ANTIBIOTIC SUSCEPTIBILITY PATTERNS OF PATHOGENIC BACTERIA RECOVERED FROM UNPROCESSED BOVINE MILK PRODUCED IN NDIVISI WARD, BUNGOMA COUNTY

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A thesis submitted in partial fulfillment of the requirements for the award of A Master of Science Degree in Microbiology of Masinde Muliro University of Science and Technology.

November, 2019

DECLARATION

DECLARATION

This thesis is my original work prepared with no other than the indicated sources and support and has not been presented elsewhere for a degree or any other award.

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CERTIFICATION

The undersigned certify that they have read and hereby recommend for acceptance of Masinde Muliro University of Science and Technology a thesis entitled "Antibiotic susceptibility patterns of pathogenic bacteria recovered from unprocessed bovine milk produced in Ndivisi ward, Bungoma County."

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DEDICATION

This thesis is dedicated to my loving parents Joan Sasaka and Absalom Sasaka for their words of encouragement and push for tenacity. I also dedicate this thesis to my friends who have supported me throughout this process.

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First and foremost, I appreciation God for according me good health and strength through my study period. Secondly, I also appreciative my supervisors; Dr. Mario Kollenberg and Prof. Donald Siamba for their sincere encouragement and guidance to me. Their remarks made this work to be what it is. Thirdly, I thank Mr. Peter Nyongesa who assisted me through the entire research and any other person who willingly participated in this study. Lastly, my gratitude goes to members of the family for their inspiration and encouragement.

ABSTRACT

Milk is an essential and nutritive product that fulfills the increasing demand for food in the rising population in the former western province of Kenya. Milk can be easily contaminated by bacteria posing a health risk to human consumers. Similarly, antibiotic resistance is emerging as a great concern as it makes the control of diseases difficult by reducing the effectiveness of the available drugs. The antibiotics used for treatment of animals has an effect on the levels of bacterial resilient in humans, yet the exact health impacts are poorly understood. A total of 486 samples were collected from individual animal and bulk milk and outlets market places. Bacterial communities were isolated from the samples and then subjected to antibiotic susceptibility testing. The bacteriological status of milk was assessed by total plate count, isolation and identification of pathogenic bacteria and testing for antibiotic susceptibility patterns. The level resistance to antibiotic among the isolates was tested to amoxicillin, chloramphenicol, kanamycin, gentamicin, cephalexin, and tetracycline. The responses of the isolates to antibiotics were established by measuring the diameter of the zone of inhibition around the antibiotic disk. These measurements were subsequently converted into a qualitative scale using standard charts. Data on the bacteriological quality of milk were summarized using means and standard deviation. The difference in bacterial counts between sub-locations, sources of milk and the difference in response to antibiotics and levels of antibiotics between and within groups in the study was assessed using analysis of variance (ANOVA). Statistical significance was set at $p<0.05$ using a computer package, SPSS software version 20.0. Out of 486 samples collected only 235 samples (48.4%) were contaminated. *Staphylococcus aureus* was (28.1%) in abundance, pathogenic *Escherichia coli* (21.7%), *Pseudomonas aeruginosa* (19.1%), *B subtilis* (11.5%), *Citrobacter freundii* (10.2%) and *Klebsiella pnemoniae* (9.4%). Percentages of bacteria resistant to antibiotics are amoxicillin (63%), kanamycin (19%), cephalexin (41%) and tetracycline (19%). Those that are intermediate: kanamycin (33%) and cephalexin (22%). Susceptible ones: amoxicillin (37%), gentamicin (100%), kanamycin (48%), cephalexin (37%), chloramphenicol (100%) and tetracycline (81%). Generally, 62% of the bacteria are resistant, 33% are intermediate while 5% are susceptible. Lutacho sub-location had the highest bacterial counts, followed by Misemwa, Wabukhonyi, Marinda, Makuselwa, and Lowest in Sitabicha. *B. subtilis, P. aeruginosa and C. freundii* are multidrug-resistant bacteria. Cephalexin and kanamycin are intermediate; their concentrations need to be increased to be used again against *E. coli* and *B. subtilis*. *K. pnemoniae* and *S. aureus* are susceptible amoxicillin, chloramphenicol, kanamycin, gentamicin, cephalexin, and tetracycline. The information generated from this study has shown antibiotic susceptibility patterns among pathogenic bacteria in unprocessed bovine milk. The information can be used to improve antimicrobial surveillance systems like Atlas which creates awareness. This information provides evidence of antibiotic resistance two of which are key objectives of the FAO action plan on AMR, similarly, it's of great importance to veterinary officers, public health officers, dairy technologists, dairy farmers, and consumers.

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LIST OF ABBREVIATION AND ACRONYMS

AMR	Antimicrobial resistance
AMX	Amoxicillin
C	Chloramphenicol
C	Collection points (Bulk Milk)
CDC	Centers for Disease Control and Prevention
cfu	Colony forming units
CLSI	Clinical and Laboratory Standards Institute
CN	Cephalexin
E test	Epsilometer
E. coli	*Escherichia coli*
FSANZ	Food Standards Australia New Zealand
HGT	horizontal gene transfer
K	Kanamycin
KEBS	Kenya Bureau of standards
KNBS	Kenya National Bureau of Statistics
MIC	Minimal inhibitory concentration
O	Outlets
P	Production (Individual Animal)
PCA	Plate Count Agar
PCR	Polymerase chain reaction
RCG	Resistance conferring genes
TE	Tetracycline
TPC	Total plate count
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Mammary glands of secret milk which is used as food for infants and supplies them with all necessary nutrients for their development. Milk and its products have rich nutrient contents that include minerals, proteins, and carbohydrates, which support the growth of microorganisms including some food-borne pathogens (Remenant *et. al*., 2015). Consuming contaminated products may cause illnesses oscillating from stomach upset to worst symptoms (Ahmed *et al.,* 2014). Milk contamination affect the product's nutritive and sensory quality properties hence leads to economic losses (Janštova *et. al*., 2006).

Previously, milk was taught to be sterile secreted into the alveoli of the udder (Tolle, 1980) but the current studies suggest that milk contain commensal (Rainard *et al.,* 2017).

Since milk allows growth of numerous bacterial species, preferably, mastitic pathogens multiply *in vivo* between 20–30 minutes after a few hours of udder penetration (Rainard *et al.,* 2003). Inside the lumen numerous bacterial species multiply during lactation period unless immune reaction hinders their growth. The consequence of such a high concentration of bacteria is mastitis (Hou *et. al*., 2015). Away from the udder contamination occurs through use of additives such as antibiotics, unsterilized water and hydrogen peroxide or environment contamination, milk handlers, equipment and milking practices (Rainard *et al.,* 2017).

Contamination is mostly as a result of excretion from infected animal and environment (Oliver *et al*., 2005). Similarly, the detection of coliform bacteria and pathogens in milk also shows likely contamination of bacteria from utensils used for milking, the udder or from the used water supply (Bonfoh *et al*., 2003).

The bacteria in milk are risky to persons with the compromised immune system, pregnant women, aged individuals and children. More danger is on pregnant women since *Listeria* causes miscarriage, death fetuses or newborn (CDC between 1993 and 2006). The milk harbors risky bacteria that include *Salmonella* species, *Corynebacterium diphtheria, Listeria monocytogenes*, pathogenic *Escherichia coli,* and *Campylobacter*

The bacteriological quality of milk in Harare revealed that milk and its products sold in various outlets contained a variety of bacteria that are of great health concern (Igumbor *et al*., 2000). Another research showed microbiology significance in dairy industry studying the epidemics of foodborne illnesses connected to milk consumption contaminated with pathogenic microbes or toxins.

More emphasis need to be placed on milk bacteriological analysis and evaluation of quality and regulatory compliance (Vasavada *et al*., 1993; Mubarack *et al*., 2010).

The antibiotics used in the treatment of dairy animals have got their way into the milk hence leading to the emergence of antimicrobial-resistant bacterial strains. Antimicrobial resistance emerges as a great concern as it makes the treatment of infections difficult because the available drugs become less effective. Furthermore, the transfer of bacterial resistance to antibiotics from animals to humans has become a global threat (Asperger *et al.,* 1997).

This study, therefore, proposes to investigate the pathogenic bacteria in unprocessed milk. Antimicrobial susceptibility patterns of the bacterial isolates will be determined to obtain information on the levels of milk contamination with microbes, pathogens and antimicrobial resistance patterns prevalent in Ndivisi ward in former Western Province of Kenya. This data will be useful to veterinary officers, public health officers, dairy technologists, dairy farming and consumers. The information can also be used to update and strengthen training material by County Veterinary and public health officials. The consumers can use the information to avoid the health risks associated with milk products.

1.2 Statement of the problem

The safety of dairy products concerning foodborne disease and other additives is of great concern around the world. It's evidenced in third world countries where the production of milk and various milk products occurs under unhygienic conditions and poor production practices (Mogessie *et al.,* 1990). The consumption of animal products contaminated with pathogenic organisms causes illnesses oscillating from stomach upset to more solemn symptoms (Ahmed *et al.,* 2014). These are rampant in developing countries such as Kenya. Both processed and raw are well-known vehicle of several human pathogens. Milk contamination is risky since make milk unsuitable for human consumption due to food poisoning cases and spread of diseases to humans (Asperger *et al.,* 1997). Mastitic milk transmits bacteria which causes illness in humans (Zoonotic diseases), even though, pasteurization destroys pathogens in humans, it's of concern when unprocessed milk is consumed or when pasteurization is faulty, and some strains of *S. aureus* produce heat resistance toxins, causing food poisoning (Thirapaskun *et al.,* 1999). Similarly, concerning mastitis are residues of antibiotics in milk, which can initiate allergic reactions in people to antibiotics and at

a low level causes sensitization of individuals and the development of antibiotic-resistant strains of bacteria (Faull *et al.,* 1985). It's evidenced that the amount of antibiotics used in animals influences the levels of human-resistant bacteria (Elliot *et al.,* 2015), however, exact health impacts are poorly understood. There is a need to investigate pathogenic bacteria in unprocessed bovine milk in Ndivisi ward because they pose serious risks, not only to the economy, but also to human lives.

1.3 Objectives

1.3.1 General objective

To investigate bacterial contamination levels and antibiotic susceptibility patterns of pathogenic microbes recovered from unprocessed bovine milk sources from small-scale farms in Ndivisi ward.

1.3.2 Specific objectives

1. To determine bacterial levels in unprocessed bovine milk at different production points and outlets in Ndivisi ward.
2. To isolate and identify pathogenic bacteria in unprocessed milk at different production and outlets in Ndivisi ward.
3. To determine the antibiotic susceptibility patterns of the isolated bacterial pathogens.

1.4 Hypothesis

1. There is no difference in bacterial contamination levels of unprocessed bovine milk at points of production and outlets in Ndivisi ward.
2. Unprocessed milk of Ndivisi ward at points of production and outlets are not contaminated with pathogenic bacteria.

3. Bacterial pathogens contaminating unprocessed milk in Ndivisi ward are not resistant to antibiotics.

1.5 Justification

Ndivisi ward has high reported incidences of diarrhea (12%) and other enteric diseases among children of under 5 years with the highest prevalence of diarrhea of 21% between 12 -23 months (WHO 2013/2014). Current studies from Kenya showed that higher levels of the bacterial count, Salmonella and Streptococcus were found unprocessed milk, which signifies the health hazard linked to the consumption of unproceesd milk (Matofari *et al*., 2007). High quality and uncontaminated milk are necessary to reduce the incidences of these diseases. Antibiotic resistance currently health care problem in both community and hospital settings and is a serious threat to treatment of bacterial infections (Stalder *et al*., 2012). Therefore, understanding the level of contamination in milk and the level of antibiotic resistance is the initial stage of designing preventive strategies.

CHAPTER TWO

LITERATURE REVIEW

2.1 Milk production in Kenya

Dairying is an agricultural practice involving livestock farming, in which, cattle are kept for milk production. Dairy farming in Kenya is grouped into two; commercial dairy farming and domestic dairy farming (Karanja *et al.,* 2003). Commercial dairy farming is practiced on small scale and large scale. Domestic dairy farming is practiced for domestic use (Karanja *et al.,* 2003). Though, some domestic cattle keepers do sell their milk to the markets.

2.2 Microbial assessment of milk and its products

According to a study conducted in Rwanda on milk and dairy value chain, proposed that milk and dairy products vended at outlets had poor and varied bacteriological quality (Kamana *et al.,* 2014). To be precise, the bacteriological load and pathogen in cheese were very high. Equally, raw milk soft cheeses made in small dairy farms took place under unhygienic conditions and also presented poor bacteriological quality of unprocessed milk as tested in a Brazilian study (Moraes *et al*., 2009).

Human infection transmission is achieved through direct contact with contaminated tissues, vaginal discharges urine, blood, aborted foetuses or placentas. Foodborne infection happens following the intake of unprocessed milk but, hardly from consuming raw meat from infected animals. Airborne infections in laboratories have been documented (Cloeckaert *et al.,* 2001). Accidental inoculation of live vaccines rarely occurs, causing human infections. There are also case reports of venereal and congenital infection in humans.

2.3 Indicators of microbial quality in milk

Milk has a particular characteristic (colour, taste, smell, PH) (Grimaud *et al.*, 2009). Microbial load in milk can pose many types of detrimental changes in chemical composition, nutritive value, taste, flavor, and appearance. The rates under which these changes occur depend upon not only on initial microbial load but also on storage conditions and length of time under which milk is held (Marth *et al.*, 2001).

2.4 Milk spoilers and mastitis pathogens

Mastitis is a condition in which mammary glands undergo inflammation causing changes in milk quality and quantity (Amir *et al.*, 2014). Mastitis is caused by *Staphylococcus spp*. *Streptococcus spp*. *E. coli* and *K. pneumoniae*) and *Actinomyces pyogenes* (Sharma *et al.*, 2010; Zadoks *et al.*, 2011),). *Pseudomonas* and *Sarratia* produce spoilage enzymes which spoils milk (Machado *et al.*, 2017).

2.5 The microbiological contaminants of unprocessed milk

A current study has shown that unprocessed milk in Ethiopia were contaminated with pathogenic bacteria, *Listeria monocytogenes* (Oliver *et al*., 2005). The detection of pathogens in unprocessed milk requires fast regulatory mechanisms to be put in place. Milk consists of commensal organisms that are which are lactic acid bacteria namely *Lactococcus*, *Lactobacillus*, *Streptococcus* and *Leuconostoc Spp*

2.6 Consequences of pathogenic bacteria in milk and milk products

A report by World Health Organization (WHO) shows that 50 million children under 5 years in the world get diarrheal diseases each year due to contaminated water and foodstuff (Tavakoli *et al.*, 2008). *Salmonellae* is known to affect both human and animal (Van Kessel *et al*., 2007) and causes human typhoid

2.7 Antimicrobial resistance

2.7.1 The discovery of antimicrobial drugs

Louis Pasteur and Robert Koch stated that microorganisms cause several diseases (Madigan *et al*., 2006). After which target therapy was introduced with Paul Ehrlich initiating chemicals to kill infectious microorganisms without harming humans since human cell and microbe cells have different cellular structures (Strebhardt and Ullrich 2008). The arsenic compounds were introduced first after their discovery to control antimicrobial activities (Strebhardt *et al.,* 2008) and later sulphonamides were also discovered (Madigan *et al.,* 2006). In 1929, Alexander Fleming discovered penicillin which has been majorly used in treatment of malaria and emphasized that microbes produce toxin (antibacterial substances) to kill each other (Fleming *et al.,* 1929). This therefor led to an era of antibiotic discovery (Wright *et al.,* 2007) with emphasis on Waksman's antibacterial-activity screening platform (Kresge *et al.,* 2004).

Later synthetic and semisynthetic derivatives antibiotics developed especially of natural origin and were used in clinical setup after some structural modification to reduce toxic effects and improve antimicrobial activity (Pietsch *et al.,* 2015). The order of discovery is in the figure 2.1 below.

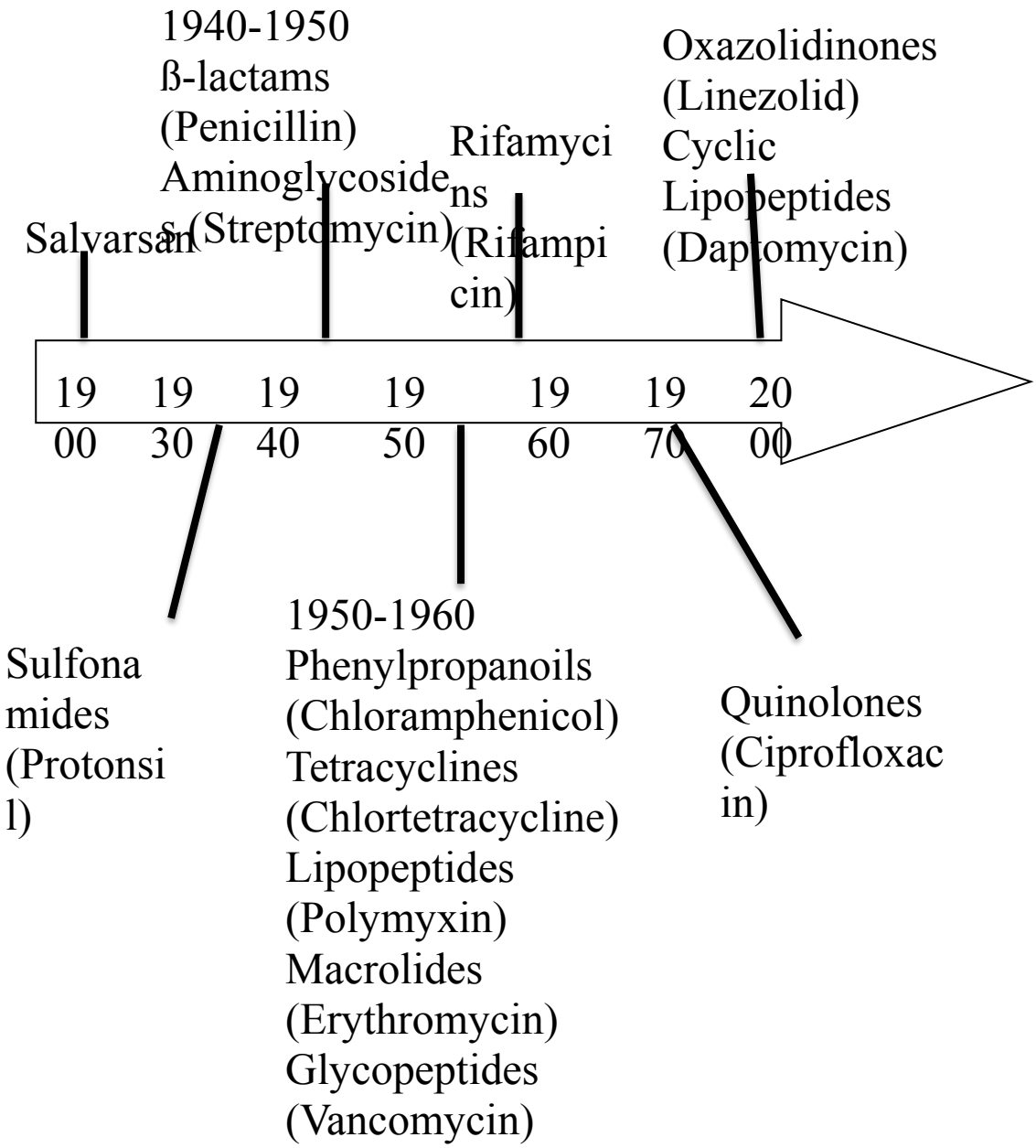

Figure 2.1: Period of antibiotic drug discovery (Pietsch *et al.*, 2015)

2.7.2 Classification of antibacterial drugs

The antimicrobial agents (Figure 2.2) have varied ways of action that include disruption of processes within the bacteria, inhibits target structures or pathways different or absent in mammalian cells (Pietsch *et al.,* 2015). As a result, antibiotics can be classified according to their modes of action namely their inhibitory effect, the

spectrum of activity or molecular target (Auerbach *et al.*, 2002; Bhattacharjee *et al.*, 2016; Hooper *et al.*, 1999). Some of these antibiotics are bacteriostatic while others are bacterial suicidal (Cioffi *et al.*, 2005; Friedman et al., 2002).

They can also be classified as broad spectrum (wide) or narrow spectrum (narrow) depending on range of activity (Bockstael *et al.*, 2009). Antibacterial drugs are also different in their bacterial targets and mechanisms of action which involve cell wall biosynthesis and membrane integrity for example ß-lactams (Lee *at el.*, 2001), protein synthesis for example tetracyclines (Auerbach *et al.*, 2002), folic acid metabolism for example Sulfonamides (Bhattacharjee *et al.*, 2016), and DNA replication and transcription for example quinolones (Hooper *et al.*, 1999; Bockstael *et al.*, 2009).

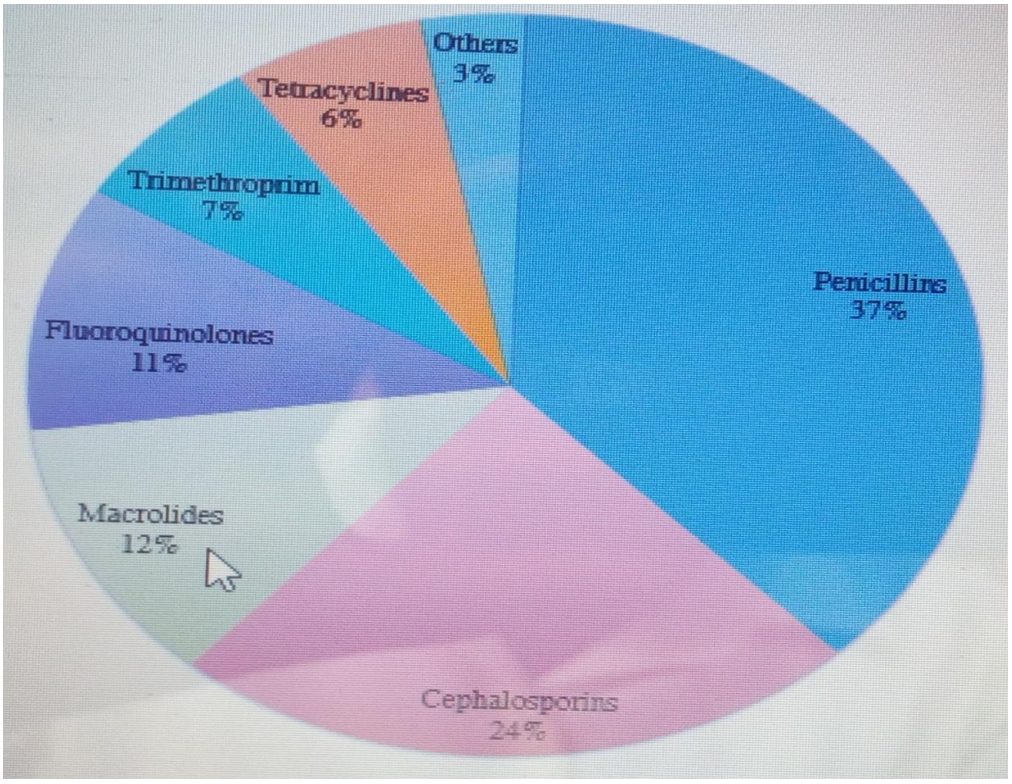

Figure 2.2: Worldwide antibiotic consumption (Boeckel *et al.*, 2014)

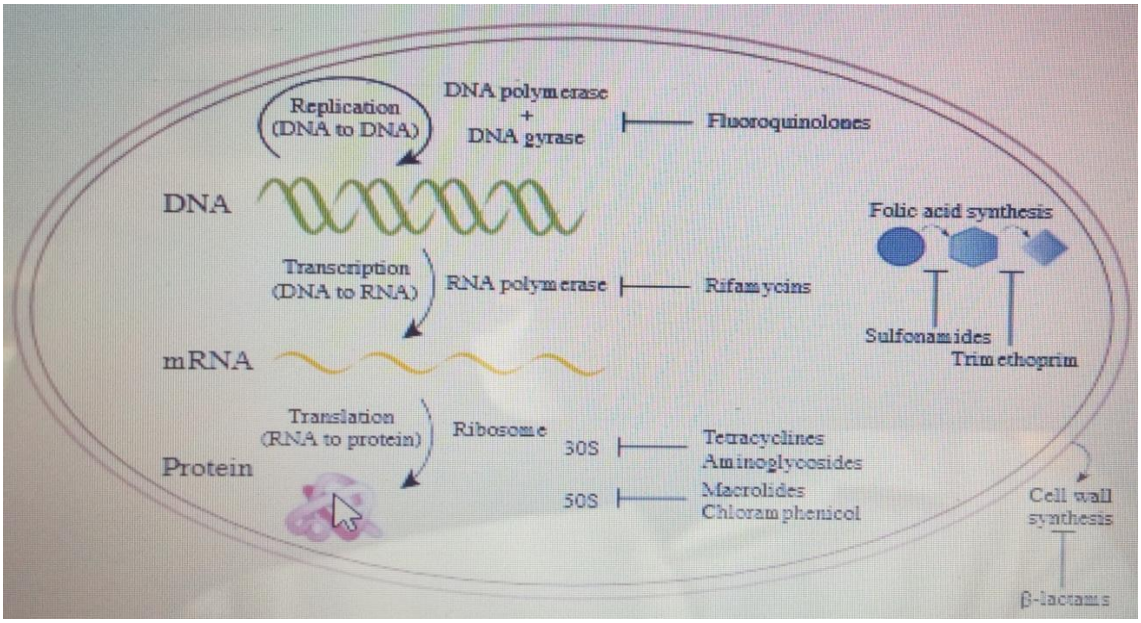

Figure 2.3: Major antibiotics and their targets (Pietsch *et al.,* 2015)

Unfortunately, some antimicrobial drugs have some limited use due to their toxicity effects, difficulty in usage, the spectrum of activity, or reserved for particular uses (Reidy *et al.,* 2013). For example, rifampicin (Campbell *et al.,* 2001).

2.7.3 Origin of antibiotic resistance

Antibiotic resistant began long before human started using them in clinical set up (Gillings *et al.,* 2013; wright *et al.,* 2007; Wright and Poinar 2012). However, application of under low concentrations contributes to quorum sensing and microbial communication (Aminov *et al.,* 2009, Davies *et al.,* 2006; Yim *et al.,* 2006; Goh *et al.,* 2002; Sengupta *et al.,* 2013). High concentrations on other hand enables antibiotic-producing organisms to harbor resistance genes used for self-protection and exchange of those genes between other bacteria (Nikaido *et al.,* 2009).

Antibiotic producers and antibiotic-resistant organisms evolved together harboring resistance to antibiotics (Cox and Wright 2013). Gram-negative antibiotics since many molecules can't penetrate their double-membrane cell wall structure (Mayrand *et al.,*

1989). They also possess efflux pumps to lower antibiotic concentrations in their cells (Cox and Wright 2013).

2.7.4 Evolution antibiotic resistance

The selection for resistant strains began more than 70 years ago leading to emergence of resistant human pathogens (Swartz *et al.,* 2002; Alanis *et al.,* 2005; Sengupta *et al.,* 2013). This led to selective pressure, from acquired resistance elements from the environmental by either horizontal gene transfer or evolved through mutations (Martinez *et al.,* 2009). Initially, susceptible pathogens to antibiotic led reduction in human mortality (Martinez *et al.,* 2009). Antimicrobial resistance led to the introduction of new drugs (Lobanovska *et al.,* 2017; Spellberg *et al.,* 2005). Since antimicrobial resistance is still in its young stages new drugs are being produced and used in therapeutics (Tacconelli *et al.,* 2018; Wright *et al.,* 2005 Aminov *et al.,* 2009; Fischbach *et al.,* 2009). Antimicrobial resistance led to increase in multidrug-resistant pathogens since available antibiotics started losing efficacy (Levy *et al.,* 2013; Bush *et al.,* 2011). Currently, resistance was noticed in most pathogens and all classes of antibiotics (Ventola *et al.,* 2015). Increased antimicrobial resistance is attributed to misuse and overuse of antibiotics (Roca et al., 2015; Boeckel *et al.,* 2014). Similarly, antibiotics use in agriculture affects the treatment in human infections (Martinez *et al.,* 2009; Cohen *et al.,* 2000; Akande *et al.,* 2009; Stalder *et al*., 2012).

In Kenya, there is emergence of antibiotic-resistant bacterial strains in food animals (Sifuna *et al.,* 2013). These studies show the spread of antibiotic resistance as a growing problem and global health issue thus, giving a broad picture of the range of spread of antibiotic resistance among the bacterial populations. These studies provide an understanding of the diversity among the natural population of enteric bacteria based and their antibiotic resistance patterns (Aarestrup *et al*., 2005; Sifuna *et al.,*

2013). Over the years it was reported that there is misuse and overuse of antibiotics. This has been proved to be a major practice that promotes antibiotic resistance. Several human practices are now contributing to the spread of bacterial strains which resistant to antibiotics (Kummerer *et al*., 2004; Williams *et al.,* 2000).

In conclusion, antibiotic resistance is one of the serious problems in community and hospital setup threatens the ability to treat bacterial infections.

2.7.5 Antibiotics and antimicrobials resistance

Minimal inhibitory concentration (MIC) value was introduced to detect and solved drug resistance (Strebhardt *et al.,* 2008). MIC is the minimal concentration of drug which inhibits observable bacterial growth under controlled conditions. Mathematical models and pharmacokinetic properties were put into account to emphasis on empirical data and medical status to enhance drug therapy (Paterson *et al.,* 2007; Murray *et al.,* 2005; Wright 2007; Turnidge *et al.*, 2007)

2.7.6 Mechanism of Resistances

The mechanisms for drug resistance can categorized into 3 (Rattan *et al.*, 1998):

(i) Alteration of the drug target, leading to reduced target susceptibility

(ii) Modification of the drug, lowering drug-target affinity

(iii) Reduction in drug concentration hence no target reached.

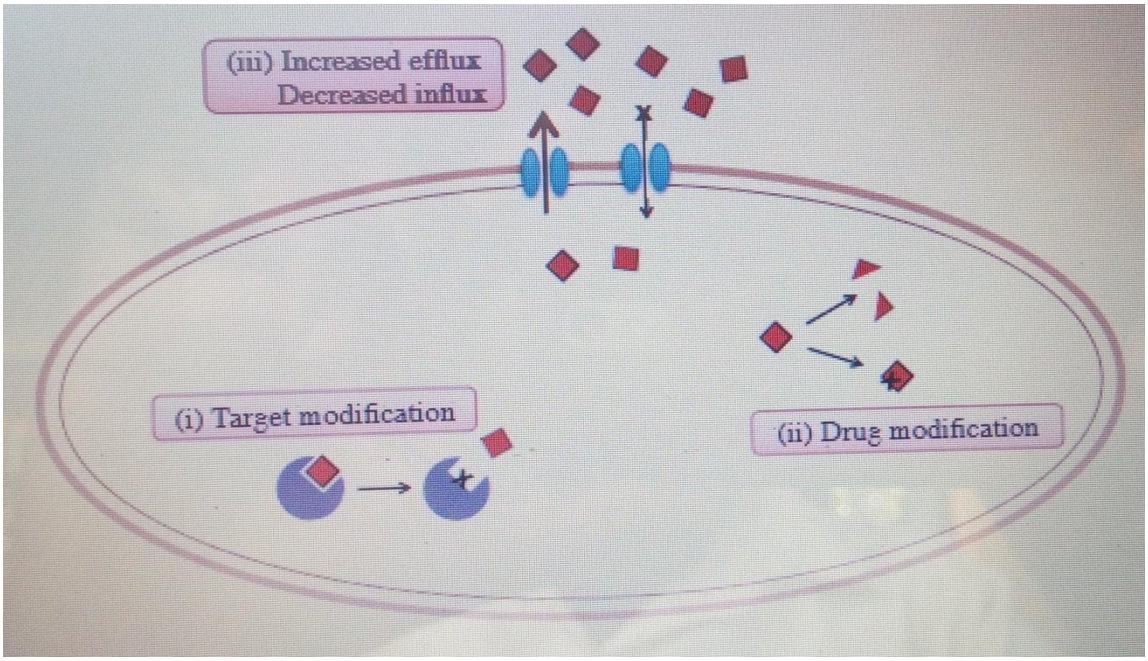

Figure 2.4: Mechanisms of resistance (Pietsch *et al.*, 2015)

Antimicrobial susceptibility has reduced heavy due to mutations (Lindgren *et al.*, 2005; Sandegren and Andersson 2009; Hawkey *et al.*, 2009; Guan *et al.*, 2013; Strahilevitz *et al.*, 2009) and genetic alterations (Jones *et al.*, 2009; Marcusson *et al.*, 2009; Poole 2004; Fernandez *et al.*, 2012; Hancock *et al.*, 2012).

2.8 Antimicrobial resistant (AMR)

2.8.1 Levels of AMR

Use of antibiotics in livestock feeds causes them to grow bigger and faster (Coates *et al.*, 1951; Elliott *et al.*, 2015; Moore *et al.*, 1946; Sneeringer *et al.*, 2015; Stokstad *et al.*, 1950). The surveillance studies have established that fluoroquinolones use in livestock accelerated rise in fluoroquinolone-resistant bacteria and diseases in humans (Silbergeld *et al.*, 2008).

2.8.2 Drivers of antimicrobial resistance (AMR)

Antibiotic resistance occurs due to selection pressure placed on susceptible microbes by use antimicrobial agents (Dione *et al.,* 2009, Glynn *et al.,* 2004, Grace *et al.,* 2008, Koningstein *et al.,* 2010), similarly, antibiotics excreted or metabolites, residue in tissues, and direct zoonotic transmission (Marshall *et al.,* 2011, Padungtod *et al.,* 2006, Aarestrup *et al.,* 2006, O'Neill *et al.,* 2016).

2.8.3 Techniques for detecting AMR among microbes

These techniques include

1. Dilution method
2. Disk-diffusion method
3. E-test method
4. PCR and DNA hybridization methods

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area

Ndivisi ward is a rural setting located in Bungoma County (Coordinates; DD, 0.5666644 34.5666644; DMS, 0°33'59.99" N 34°33'59.99" E; Geohash, sb0e4x4tj9vhg; UTM, 36N 674343.9228408 62657.040869891, in Western Kenya. It has a large and rapidly increasing population, with a current estimated total population of 39,800 people, distributed evenly within the ward with an area of about 68 sq km which is about 585 people per sq km (Kenyan census, 2009). Two rainfall pattern exist; the long rains between March and July and the short rains between August-October. The mean yearly range of rainfall is 1,200–1,800 mm. (Temperature ranges between 21 °C and 31 °C). The altitude (1200 and 2000 meters) above the Sea Level (Backes *et al.,* 2001). Farming is the main economic activity that is small scale crop and livestock production. Commonly grown crops are; maize, beans, and sugarcane. Livestock production includes; cattle ducks, chicken sheep and goats (George *et al*., 2013). Low literacy levels, high poverty levels, and one dispensary per sub-location. The area is relevant to the study because it's a child rich (0-14-year-olds) and highly dependent on milk (KNBS, 2017).

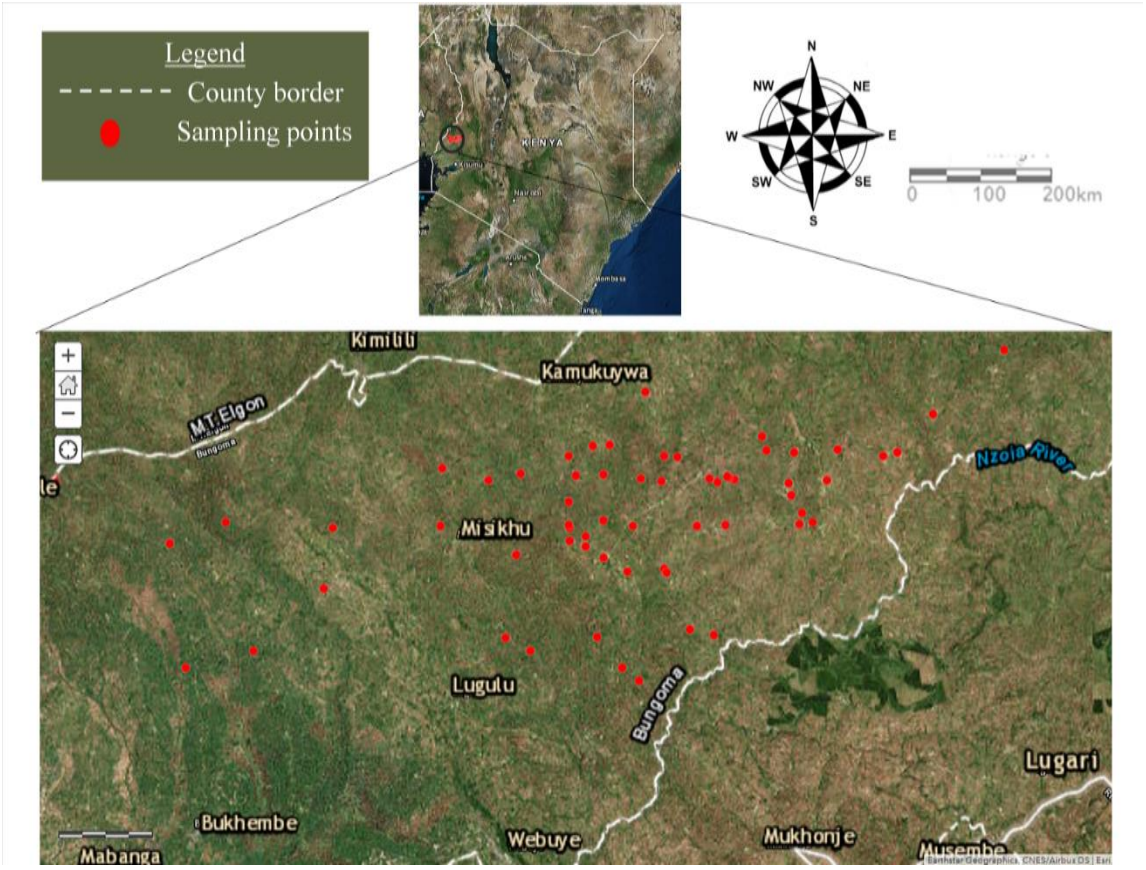

Figure 3.1: Satellite Map of Kenya showing exert location of the sampling areas (GPS readings)

3.2 Collection of samples and processing

The study was carried out in Ndivisi ward for 3 months (October to December 2016). Sampling was done once every month at each of the sampling points. Samples were taken between 6.00 am to 8.00 am. Cross-sectional study design was employed whereby milk samples from randomly selected farms and markets were collected. The sampling frame, a total of (n=486) unprocessed milk samples were randomly collected and grouped into three categories i.e. at the individual animal level, bulk milk of the herd and outlets.

Similarly, was grouped per sub-location. On the farm, milk was taken from individual cows and the bulk milk of the hard while on the market was from individual sellers. The samples were placed in a sterile universal bottle and immediately preserved on the

ice at 4 °C. They were labeled with a non-permanent pen marker as P1-P9, C1 –C9 and O1-O9, and then a follow up sampling was repeated monthly at each of the previous sampling points. The possible practices that may have led to contamination at different milk sources were also observed and recorded in the notebook. All the samples were transported on ice in insulated containers to Masinde Muliro University of Science and Technology, Microbiology laboratory for analysis.

3. 3 Study design

Nine (9) samples were sampled from each sampling points that are at production (Individual animal and bulk milk) and outlets. From each of the six sub-locations in three replicates monthly (from September to December).

9 samples ×3 collection points×6 sub-locations ×3 replicates= 486 samples

To minimize bias; an equal number of samples were taken and also follow up sampling (Replicates) was repeated monthly at each of the previous sampling points in all sub-locations.

3.4 Total plate count (TPC)

0.1ml of each sample was placed onto culture plates with plate count agar (PCA) using the pour plate method hence incubated at 37 ^{0}C for 48 hours (Monica *et al*., 2006). 0.1ml was used to give countable colonies (High concentration leads to overcrowding hence hinders proper counting of colonies). Colony-forming units (CFUs) were counted and stated as; cells per 1ml (APHA *et al.,* 2005). Bacterial colonies in 0.1ml were multiplied by 10 to give colonies in 1ml.

3.5 Isolation of bacteria

The presence or absence of bacteria was investigated by direct plating of milk on Blood agar (allows the growth of fastidious bacteria) and MacConkey agar (identifies lactose from non-lactose fermenter). They were then incubated at 37°C for 24 hours (optimum temperature and hours for mesophilic growth). The colonies formed were purified in nutrient agar, enriched in nutrient broth and were later subjected to antibiotic susceptibility patterns.

3.6 Identification of bacterial isolates

Identification and confirmation of bacterial isolates were performed using standard techniques as described by Ewing (1986). Characteristic colonies resembling bacteria were randomly picked from selective and differential media plates (Blood agar and MacConkey agar) and identified based on biochemical tests, namely triple sugar iron Simmon's Citrate Agar Motility lysine indole (Kovacks reagent is added to confirm), oxidase test and coagulase test

Gram staining was used to distinguish between the gram positives and gram negatives. The standard reference strain of *E. coli* 25922 and *S. aureus* 25923 were used as negative controls. Purification was done on nutrient agar while enrichment on nutrient broth, then confirmed isolates were then stored at -80 ^{o}C in 10 % glycerol broth until used in other experiments.

3.7 Antimicrobial Response Tests (AST)

Bacterial isolates obtained were inspected for antibiotic resistance using the standard Kirby-Bauer disk diffusion method. The antibiotics tested were; tetracycline, chloramphenicol, cephalexin, gentamicin, kanamycin, and amoxicillin. Mueller – Hinton medium plates were swabbed (cotton swabs of 0.1ml as per manufacturer's

recommendation) with the inoculums and the six commercially prepared antimicrobial agent disks placed on each of the inoculated plates. The plates were incubated at 37 °C for 24hours. The diameters of clear zones of growth inhibition around the antibiotics disks were measured as well as the 6 mm disk diameter by use of the precision calipers and compared to the Standard reference organisms. The break-points used to group isolates as resistant to each antimicrobial agent were those recommended by CLSI (2016).

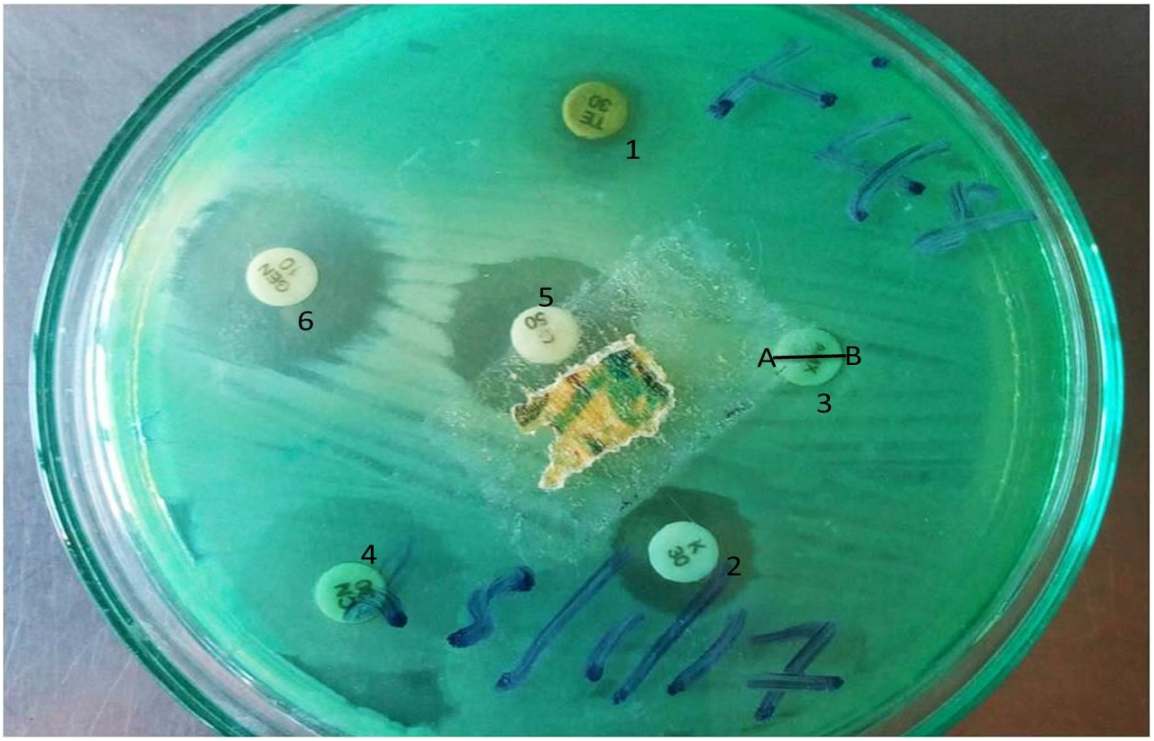

Figure 3.2: *pseudomonas aeruginosa* sensitivity patterns

3.8 Data Analysis

Data on the bacteriological quality of milk were summarized using means and standard deviations. Frequency and percentages described the occurrence of antimicrobial resistance. The difference in bacterial counts between sub-locations, sources of milk and the difference in response to antibiotics and levels of antibiotics between and

within groups in the study was assessed using analysis of variance (ANOVA). The p-value was set at $p<0.05$ using a computer package, SPSS software version 20.0.

CHAPTER FOUR

RESULTS

4.1 Bacterial Counts

The figure below shows, the results of bacterial counts in 1ml of milk samples from Sitabicha, Marinda, Wabukhonyi, Misemwa, Lutacho, and Makuselwa. A summary of all 6 sub-locations shows that the bacterial counts were highest at outlets and lowest at production from the individual dairy animal.

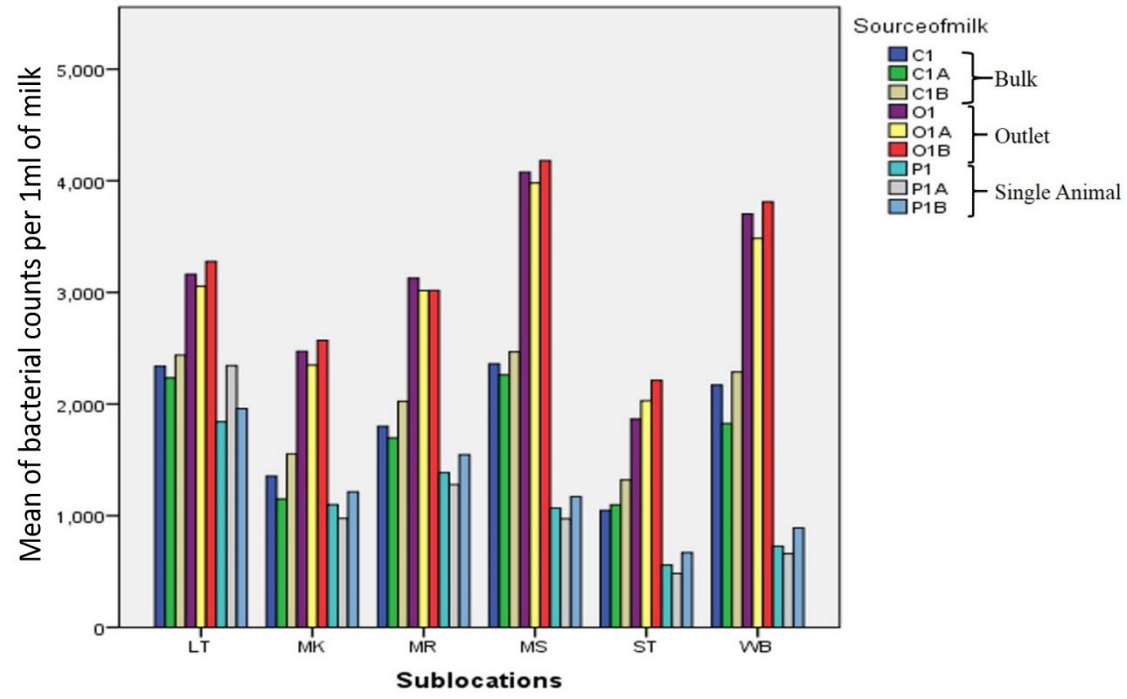

Figure 4.1: Bacterial counts per 1ml of milk.

KEY:

Lutacho(LT), Marinda(MR), Makuselwa(MK), Misemwa(MS), Wabukhonyi(WB) Sitabicha(ST) P-Individual Animal, C-Bulk Milk of the herd, O-Outlets, 1-First Collection,1A-Second Collection, 1B-Third Collection

Bacterial counts in milk from sub-locations in Ndivisi ward between production (individual cow P and bulk milk of the herd C) and market outlet (many herds O) over a period time of 3 months.

There were high bacterial counts in outlets followed by Bulk milk and the lowest milk from an individual animal. There were also highest bacterial counts during the third collection (in December) then the first collection (in October) and lowest bacteria counts were recorded in the second collection (in November). See table 2 below has the statistics (Appendix 2).

On individual animals, there were high bacterial counts in Lutacho sub-location, followed by Marinda, Makuselwa, Misemwa, Wabukhonyi, and Lowest in Sitabicha. On bulk milk of the herd, there were high bacterial counts in Lutacho sub-location, followed by Misemwa, Wabukhonyi, Marinda, Makuselwa, and Lowest in Sitabicha. At outlets, there were high bacterial counts in Misemwa, sub-location, followed by Wabukhonyi, Lutacho, Marinda, Makuselwa, and Lowest in Sitabicha. These results show the standards of hygiene within Ndivisi ward. There was significant difference in the bacterial counts of unprocessed bovine milk at production (S- individual animal and M-bulk milk of the herd) and outlets in Ndivisi ward (Appendix 12).

Combining bacterial counts at production points and outlets within the ward, Lutacho sub-location had the highest bacterial counts, followed by Misemwa, Wabukhonyi, Marinda, Makuselwa, and Lowest in Sitabicha (Table 1, Appendix 1). Milk in Ndivisi ward is of very good quality hence safe for human consumption since bacterial counts are lower than the recommended standards by the Kenya Bureau of standards (KeBS). According to KeBS bacterial counts of 0-1,000,000 cfu/ml means very good quality, 1,000,000-2,000,000 cfu/ml means good quality, >2,000,000 cfu/ml denotes bad quality for milk to be drunk raw.

Table 4.1: Bacterial counts per sub-location

Sub locations	Mean	Number of samples	Std. Deviation
Lutacho	2516.91	81	±1303.809
Makuselwa	1637.78	81	±1022.662
Marinda	2099.01	81	±1284.255
Misemwa	2504.07	81	±1621.936
Sitabicha	1246.18	81	±1223.391
Wabukhonyi	2173.21	81	±1287.460
Total	2031.14	486	1374.178

n- Number of samples

Table 4.2: Bacterial density per collection point

Source of milk	Mean	Number of samples	Std. Deviation
C1	1845.81	54	±1062.424
C1A	1710.74	54	±1054.820
C1B	2015.56	54	±1066.638
O1	3090.57	54	±1280.459
O1A	2985.74	54	±1284.778
O1B	3178.15	54	±1308.179
P1	1112.96	54	±861.647
P1A	1118.89	54	±1244.315
P1B	1241.48	54	±854.918
Total	2031.14	486	1374.178

P-Individual Animal, C-Bulk Milk, O-Outlets, 1-First Collection, 1A-Second Collection, 1B-Third Collection, N-Number of samples

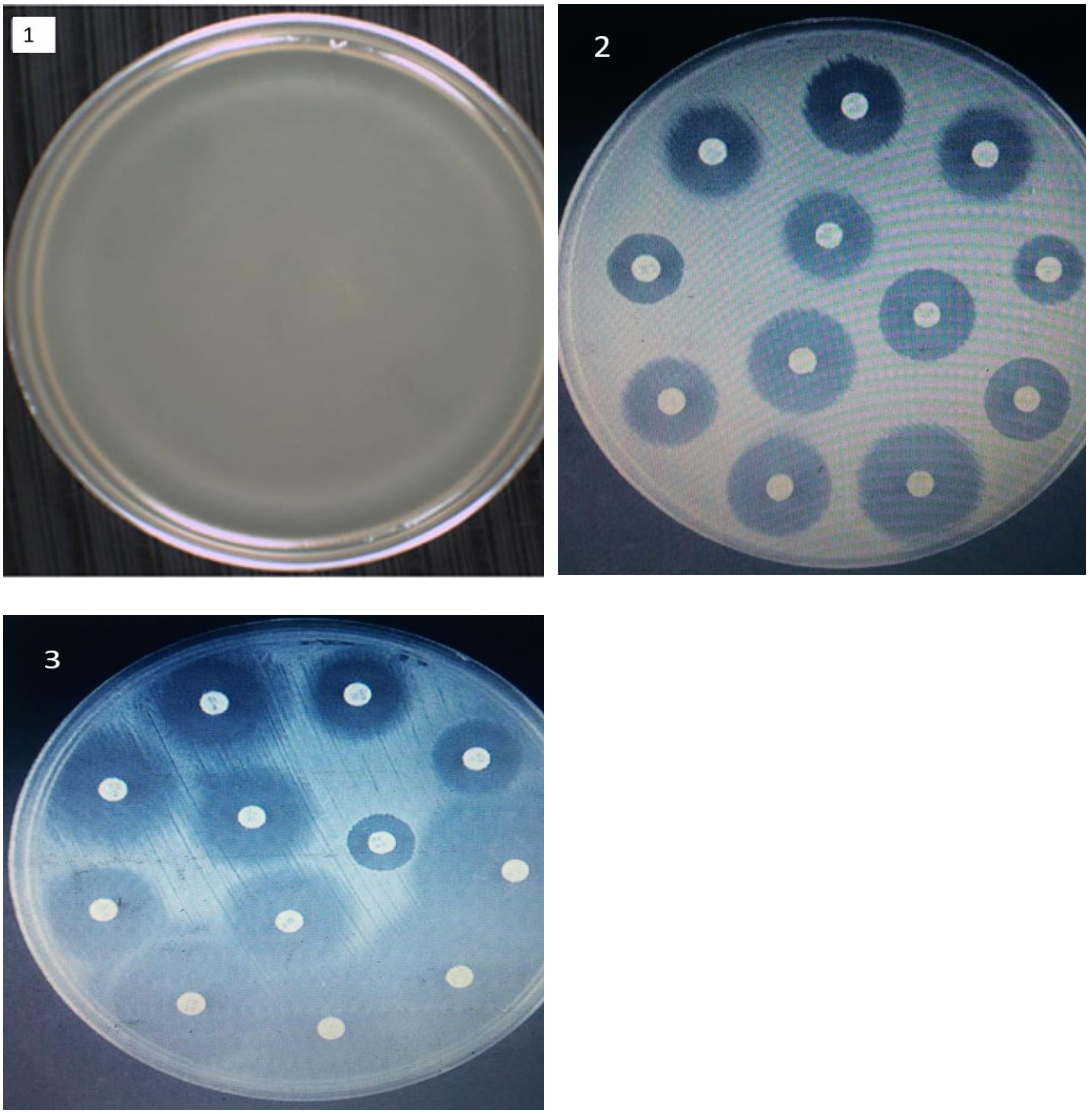

Figure 4.2: Negative controls
Bacterial counts on PCA (1), *E. coli* 25922 and *S. aureus* 25923 are susceptible to all antibiotics (2 and 3) respectively.

4.2 Identification of Bacteria

Six bacterial species were identified using biochemical tests (see table below).

Table 4.3: Identification of Bacteria based on biochemical tests

SOURCE	GRAM STAIN	SHAPE	HAEMO-LYSIS	COLONY COLOUR	MOTI-LITY	LY-SINE	IN-DOLE	CIT-RATE	TSI	CATA-LASE	COAGU-LASE	OXI-DASE	IDENTITY
P,B and O	+ve	Cocci	Beta	Yellow	-ve	-ve	-ve	-ve	+ve	+ve	+ve	-ve	*S aureus*
P and C	-ve	Rods	Beta	Green	+ve	-ve	-ve	-ve	-ve	+ve	-ve	+ve	*P aeruginosa*
P,B and O	+ve	Rods Mono-Polar	Beta	White	+ve	-ve	-ve	+ve	+ve	+ve	-ve	+ve	*B subtilis*
P,B and O	-ve	Rods	Beta	Pink Slow fermenter	+ve	-ve	-ve	+ve	+ve	-ve	-ve	-ve	*C freundii*
P,B and O	-ve	Rods	Beta	Pink Fast fermenter	-ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	*K pnemoniae*
P,B and O	-ve	Rods	Beta	Pink Fast fermenter	+ve	+ve	+ve	-ve	+ve	+ve	-ve	-ve	*E coli*

P-Individual Animal, B-Bulk Milk of herd, O-Outlets, +ve-Positive, -ve-Negative

Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis, Citrobacter freundii, Escherichia coli and *Klebsiella pneumoniae* were found at points (P- Individual animal and C- Bulk milk of the herd) and outlets in all the six sub-locations in all the three replicates. *Pseudomonas aeruginosa was* found only in Wabukhonyi and Lutacho at production (S- individual animal and M-bulk milk of the herd) and outlet in all the three replicates. This may be as a result of additives to which *P. aeruginosa* is susceptible to.

4.3 Susceptibility patterns for the six isolated bacteria pathogens

The means which represent the diameter of the zone of inhibition for each bacterial species is the average of the number of isolates since the study did not identify different bacterial serotypes.

The tables below show susceptibility patterns for the six isolated bacteria (*S. aureus, P. aeruginosa, E. coli, K. pnemoniae, C. freundii, B. subtilis)* against 6 antibiotics (amoxicillin, chloramphenicol, kanamycin, gentamicin, cephalexin, and tetracycline). The concentration of antibiotic is given in µg. Means represent the diameter of inhibition zone from triplicates, n = Total Number of tests. The isolated organism was compared to Standard reference organism.

Table 4.4: Sensitivity patterns of *Staphylococcus aureus*

Staphylococcus aureus	AMX 30 µg	K 30µg	GEN 10µg	CN 5 µg	C 50µg	TE 30µg
Mean	18.27	21.68	22.32	22.95	29.50	23.36
Number of isolates	66	66	66	66	66	66
RESISTANT	≤13	≤13	≤12	≤15	≤12	≤14
INTERMEDIATE	14-17	14-17	13-14	16-20	13-17	15-18
SUSCEPTIBLE	≥18	≥18	≥15	≥21	≥18	≥19

Standard reference organism (ATCC25923)

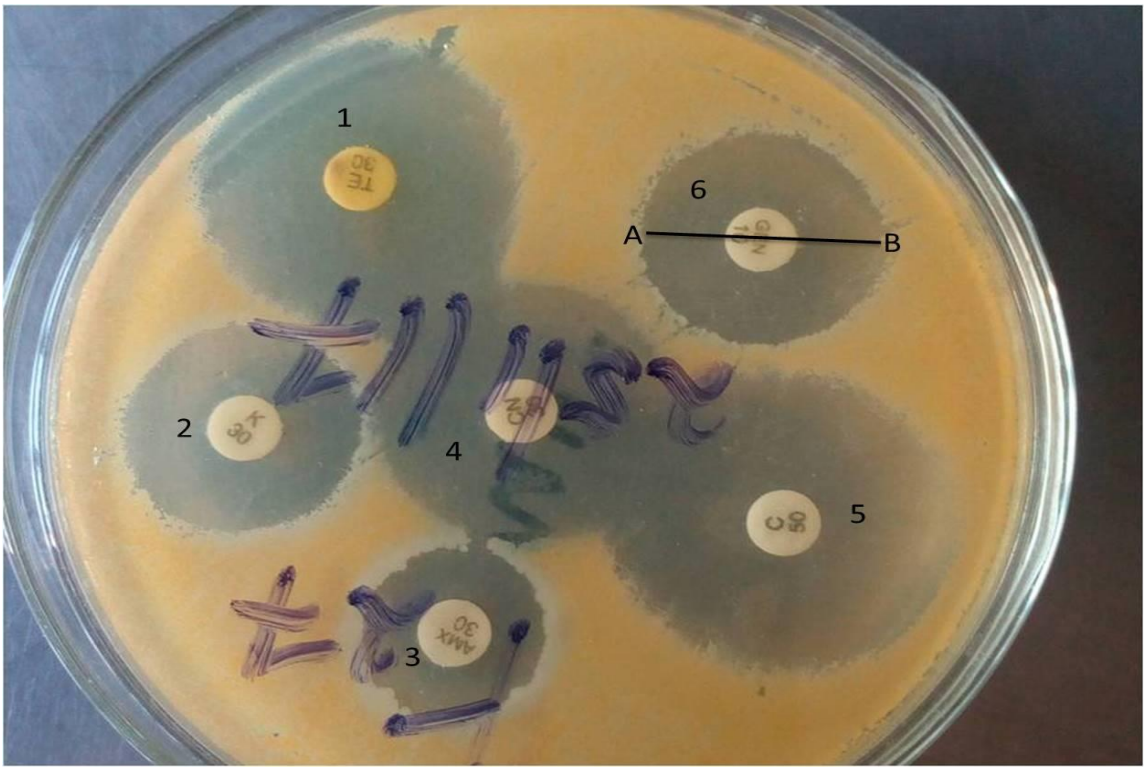

Figure 4.3: *Staphylococcus aureus* sensitivity patterns.

KEY

1. Tetracycline (TE)
2. Cephalexin (CN)
3. Kanamycin (K)
4. Chloramphenicol (C)
5. Amoxicillin (AMX)
6. Gentamycin (GEN)

Staphylococcus aureus is still susceptible to tetracycline, chloramphenicol, cephalexin, gentamicin, kanamycin, and amoxicillin.

Table 4.5: Sensitivity patterns of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa	AMX 30 µg	K 30µg	GEN 10µg	CN 5 µg	C 50µg	TE 30µg
Mean	**6.00**	**10.93**	21.30	**6.00**	21.63	**6.80**
Number of isolates	45	45	45	45	45	45
RESISTANT	≤13	≤13	≤12	≤15	≤12	≤11
INTERMEDIATE	14-17	14-17	13-14	16-20	13-17	12-14
SUSCEPTIBLE	≥18	≥18	≥15	≥21	≥18	≥15

Standard reference organism (ATCC27853)

Pseudomonas aeruginosa is resistant to tetracycline, cephalexin, kanamycin, and amoxicillin but susceptible to gentamicin and chloramphenicol (Figure 2.6).

Table 4.6: Sensitivity patterns of *Escherichia coli*

Escherichia coli	AMX 30 µg	K 30µg	GEN 10µg	CN 5 µg	C 50µg	TE 30µg
Mean	**6.00**	**17.84**	19.90	**16.59**	28.63	20.76
Number of isolates	51	51	51	51	51	51
RESISTANT	≤13	≤13	≤12	≤15	≤12	≤11
INTERMEDIATE	14-17	14-17	13-14	16-20	13-17	12-14
SUSCEPTIBLE	≥18	≥18	≥15	≥21	≥18	≥15

Standard reference organism (ATCC35218)

Escherichia coli is resistant to amoxicillin, intermediate to cephalexin and kanamycin, but susceptible to gentamicin, chloramphenicol, and tetracycline. The concentration of cephalexin and kanamycin can be increased for it to be used again against *E coli* (Appendix 11).

Table 4.7: Sensitivity patterns of *Klebsiella pnemoniae*

Klebsiella pnemoniae	AMX 30 µg	K 30µg	GEN 10µg	CN 5 µg	C 50µg	TE 30µg
Mean	22.71	22.81	23.67	21.71	27.71	23.43
Number of isolates	22	22	22	22	22	22
RESISTANT	≤13	≤13	≤12	≤15	≤12	≤11
INTERMEDIATE	14-17	14-17	13-14	16-20	13-17	12-14
SUSCEPTIBLE	≥18	≥18	≥15	≥21	≥18	≥15

Standard reference organism (ATCC700603)

Klebsiella pnemoniae is susceptible to gentamicin, chloramphenicol, tetracycline, cephalexin, amoxicillin, and kanamycin (Appendix 10).

Table 4.8: Sensitivity patterns of *Citrobacter freundii*

C freundii	AMX 30 µg	K 30µg	GEN 10µg	CN 5 µg	C 50µg	TE 30µg
Mean	**6.00**	19.37	20.79	**6.00**	23.12	19.46
Number of isolates	24	24	24	24	24	24
RESISTANT	≤13	≤13	≤12	≤15	≤12	≤11
INTERMEDIATE	14-17	14-17	13-14	16-20	13-17	12-14
SUSCEPTIBLE	≥18	≥18	≥15	≥21	≥18	≥15

Standard reference organism (ATCC8090)

Citrobacter freundii is resistant to amoxicillin and cephalexin but susceptible to gentamicin, chloramphenicol, tetracycline, and kanamycin (Appendix 9).

Table 4.9: Sensitivity patterns of *Bacillus subtilis*

Bacillus subtilis	AMX	K	GEN	CN	C	TE
Mean	**6.00**	**17.11**	19.85	**12.48**	25.52	18.56
Number of isolates	27	27	27	27	27	27
RESISTANT	≤13	≤13	≤12	≤15	≤12	≤11
INTERMEDIATE	14-17	14-17	13-14	16-20	13-17	12-14
SUSCEPTIBLE	≥18	≥18	≥15	≥21	≥18	≥15

Standard reference organism (ATCC23857)

Bacillus subtilis is resistant to amoxicillin and cephalexin, intermediate to kanamycin, but susceptible to gentamicin, chloramphenicol, and tetracycline. The concentration of kanamycin can be increased for it to be used again against *B. subtilis* (Appendix 8).

Table 4.10: The table of frequency and percentages of bacterial isolates

Bacteria species	Frequency	Percent %
Bacillus subtilis	27	11.5
Citrobacter freundii	24	10.2
Escherichia coli	51	21.7
Klebsiella pnemoniae	22	9.4
Pseudomonas aeruginosa	45	19.1
Staphylococcus aureus	66	28.1
Total	235	100.0

The percentage and frequency suggest that *S aureus* were most abundant in Ndivisi ward with (28.1%) followed by *E. coli* (21.7%), *P. aeruginosa* (19.1%), *B. subtilis* (11.5%), *C. freundii* (10.2%) and finally *K. pnemoniae* (9.4%).

Table 4.11: The table showing percentages of bacterial susceptibility patterns to antibiotics

Antibiotics	Percentage (%) of bacterial resistance to antibiotics	Percentage(%) of bacterial intermediate to antibiotics	Percentage (%) of bacterial susceptibility to antibiotics
Amoxicillin	63%	0%	37%
Cephalexin	41%	22%	37%
Kanamycin	19%	33%	48%
Tetracycline	19%	0%	81%
Chloramphenicol	0%	0%	100%
Gentamicin	0%	0%	100%

Percentages of bacteria resistant to antibiotics are amoxicillin (63%), kanamycin (19%), cephalexin (41%) and tetracycline (19%). Those that are intermediate: kanamycin (33%) and cephalexin (22%). Susceptible ones: amoxicillin (37%), gentamicin (100%), kanamycin (48%), cephalexin (37%), chloramphenicol (100%) and tetracycline (81%). In general, 62% of the bacteria are resistant, 33% are intermediate while 5% are susceptible.

The percentage of bacteria resistant to antibiotics is extremely high (62%) this trend explains the reason why mastitis infections are rampant within Ndivisi ward reducing the efficacy of the available and commonly used antibiotics.

From the table above chloramphenicol and Gentamicin are antibiotics of choice to be used since they have an efficacy of 100%.

Table 4.12: Practices leading to milk contamination

Milk Sources	Practices leading to milk contamination
Production (Individual animal)	Application of cow dung to prevent calves from suckling. Rubbing of hand on the dairy animal during milking. Poor milking techniques such as incomplete milking. Poor sanitation from milk handlers. Poor udder cleaning, dirty udders milking, maintaining an unclean Contaminated water used for udder preparation before milking.
Bulk milk	Mixing of milk from different containers Lack of cooling technology.
Outlets	Dilution of milk by adding water to increase the quantity of milk. Addition of hydrogen peroxide to prevent milk spoilage. Carrying milk in open plastic Jeri cans which difficult to clean hence harbor bacteria which cause milk spoilage. Open containers expose milk to more contaminants. Poor sanitation among transporters especially children and milkmen. Lack of cooling technology.

CHAPTER FIVE

DISCUSSION

Bacteria colonies were counted and obtained results were presented in chapter four. As was observed, there are high bacterial counts at outlets, then in bulk milk and lastly from the individual dairy animal. High bacterial counts at outlets are due to the following reasons. Western Kenya has temperatures of about 29^{o}C on average (Backes *et al.,* 2001). During the day when milk sellers are at the market (the outlet), milk is exposed to ambient temperature which provides optimum temperature for mesophilic growth. Ambient temperature experienced in western Kenya and nutrients in the milk provides optimum conditions for *E coli* and other human pathogens to multiply (Gitao *et al.,* 2017, Wayua *et al*., 2012). This supports the results of an earlier study that had high numbers and faster growth of mesophilic microbes occurs under ambient temperatures (Ashenafi *et al*., 1996). The reduction in temperature by the maintenance of a cold chain along the milk value chain reduces losses and upholds milk quality (Walstra *et al*., 2007). Low temperatures decrease physiological, biochemical and microbial activities, which are the causes of quality deterioration (Walstra *et al*., 2007). Other factors that may have contributed to an increase in bacterial counts at the outlets include (i) contamination along the way to the market from the environment since milk is carried in open plastic Jeri cans (Younan *et al*., 2002). The plastic Jerri cans are cheap and readily available but harbor bacteria responsible for milk spoilage. Used of aluminium containers is recommended (Wayua *et al*., 2012). (ii) poor sanitation among transporters especially children and milkmen (FSANZ, 2009), (iii) Adulteration of milk with water from contaminated sources increases bacterial reducing its quality (Hossain *et al*., 2011; Karimuribo *et al*., 2015).

In bulk milk, during bulking, milk from different containers was mixed, enhancing spoilage and microbial contamination (Wayua *et al*., 2012). Lack of cooling and use of plastic containers increases bacterial counts Finding from this study agree with other studies carried out by (Kivaria *et al*., 2006) and (Adesina *et al*., 2011). Findings by (Hossain *et al*., 2011; Dehinenet *et al*., 2013; (Mubarack *et al*., 2010).

High bacterial counts at the production are attributed to clinical and sub-clinical mastitis hence the main cause of mastitis among the dairy animals. In Ndivisi ward mastitis incidences results from poor milking techniques such as incomplete milking. Incomplete milking creates a favorable nutritious environment for bacterial growth and multiplication (Bradley *et al*., 2002). Another poor milking practice used by milkers is wiping their hands on the fur of the dairy animal. By so doing, they pick up bacteria, and in the process introduce the bacterial pathogens into milk and the teats. This contaminates milk and causes mastitis in case those bacterial causes mastitis infection like *Streptococcus agalactae* (Bradley *et al*., 2002). The third poor milking practice is applying cow dung on the teats to prevent calves from suckling. In cases where cow dung comes from an infected animal, this practice will introduce pathogens to the teats causing mastitis (FSANZ, 2009).

The common bacteria at the source and along the milk chain levels are *S. aureus, E. coli, K. pneumoniae, B. subtilis, C. freundii* and *P. aeruginosa.* Although we have other bacteria in milk which have been reported by other studies (Sharma *et al.*, 2010, Mubarack *et al*., 2010, CDC, 2006) like *Staph. epidermidis, Streptococcus spp*. (*Strep. agalactiae, Strep. dysgalactiae, Strep. uberis & Strep. bovis*), *Lactobacillus spp, Pseudomonas fluorescens, Salmonella spp, Corynebacterium diphtheria, Campylobacter coli* and *Listeria monocytogenes*). In this study only *S. aureus, E. coli*

and *Klebsiella pneumoniae* were found to be the main causes of mastitis within Ndivisi ward.

There were highest bacterial counts during the third collection (in December) which was a dry season, high temperatures enhanced bacterial multiplication (Gitao *et al.*, 2017). Similarly, farmers were diluting their milk by adding water to increase the quantity of milk (Hossain *et al*., 2011). Doing this enables them to keep profit levels unchanged even when milk production plummets during this season (dairy animals during this season produce little milk as a result of inadequate pasture and water). The second highest bacterial counts were recorded in the first collection (in October). This was during the wet season and water provided a medium for contamination of milk by bacteria hence contributing to slightly higher bacterial counts (Hossain *et al*., 2011) than the second collection. Finally, the second collection (in November) had the lowest bacterial counts. This was at the end of the wet season; therefore pasture and water were not yet a limiting factor. Bacterial counts, in this case, were lowest.

This study is similar to other studies where milk has bacterial counts highest at outlets and lowest at production from individual dairy animals (FSANZ, 2009) but the study has not addressed the decline of *pseudomonas aeruginosa* at outlets.

Lutacho sub-location had the highest bacterial counts, followed by Misemwa, Wabukhonyi, Marinda, Makuselwa, and Lowest in Sitabicha. Lutacho, Misemwa, Wabukhonyi are rural setups with only one cooperative society in each sub-location, milk was solely sold to consumers and farmers lack cold chains, milk is prone to bacterial multiplication hence high bacterial counts. Similarly, there is little knowledge of bacterial contamination and poor milking practices like the application of contaminated cow dung to teats to prevent calves from suckling were major causes of

mastitis and contamination. Marinda, Makuselwa, and Sitabicha are market places with four cooperative societies each hence full aware of microbial contamination, here milk was sold solely to cooperative societies that require high standards to which farmers must comply to thus milk produced had low bacterial counts. Low bacteria counts were also attributed to hybrid dairy animals which require keen monitoring and treatment.

Contamination favor the drastic increase of psychotropic bacteria, predominantly pseudomonas spp. (Perko *et al.,* 2011). Transportation of milk in refrigerated tanks because the raw milk microbiota to change. The psychotropic species of *Pseudomonas, Achromobacter, Aeromonas, Serratia, Alcaligenes, Chromobacterium, Flavobacterium* and *Enterobacter* as they grow, and these bacteria usually account for more than 90 % of the microbial population in cold raw milk (Ryser *et al.,* 1999; Martins *et al.,* 2006). These can grow at refrigeration temperatures below 7 °C, produce enzymes, toxins and other metabolites (Jay *et al.,* 1996) and contribute to high standard plate counts in raw milk as witnessed in Ndivisi ward with high bacterial counts and is also due to milking dirty udders, maintaining an unclean milking and housing environment and failing to rapidly cool milk, use of plastic jerry cans which are impossible to clean and are often used for transporting milk by most motorbike transporters (Orregård *et al.,* 2013; Gemechu *et al.,* 2015).

Low bacterial counts in milk from Ndivisi ward meet the recommended standards by the Kenya Bureau of standards (KeBS). Similarly, milk microbiological quality was still good when compared to international standards. Today, the consumers in Ndivisi ward appreciates the importance of uncontaminated milk and are willing buy and sell quality milk (Wayua *et al*., 2009).

The presence of *Escherichia coli* in milk is a common indicator of fecal contamination. There is fecal contamination in Ndivisi ward due to high bacterial counts (21.7%) of *Escherichia coli*. This proves the presence of fecal contamination (Adesina *et al*., 2011; Abeer *et al*., 2012).

Pseudomonas aeruginosa is resistant to tetracycline, cephalexin, kanamycin, and amoxicillin which makes it multi-drug resistant bacteria. These findings are similar to Baker's study. Moreover, resistance results from horizontal gene transfer and denovo mutation (Baker *et al.,* 2018). It is susceptible to gentamicin and chloramphenicol.

Bacillus subtilis is resistant to amoxicillin and cephalexin, intermediate to kanamycin, but susceptible to gentamicin, chloramphenicol, and tetracycline. From Arias' study, it's resistant to several other antibiotics, such as chloramphenicol, tetracycline, erythromycin, lincomycin, penicillin, and streptomycin. *Citrobacter freundii* is resistant to amoxicillin and cephalexin but according to CLSI guidelines, it's resistant to all aminoglycosides, sulfonamides, tetracycline, tigecycline, nitrofurantoin, and fluoroquinolones and remained susceptible to fosfomycin which makes it multi-drug resistant bacteria (Feng *et al.,* 2015). *C. freundii* is susceptible to gentamicin, chloramphenicol, tetracycline and kanamycin.

Staphylococcus aureus is susceptible to the six tested antibiotics but from the literature, it is resistant to Methicillin (Morrison et *al.,* 2007). These antibiotics are still effective in the control of *S. aureus*. Resistance is mainly witnessed in Methicillin-resistant *Staphylococcus aureus* (MRSA) which is a major threat in clinical setup (Poorabbas *et al.,* 2015)

E coli is resistant to Amoxicillin and intermediate to cephalexin but from the literature, it has increased resistance trend for ampicillin, sulfonamide, trimethoprim, and gentamicin hence studies of the farms have shown an association of multidrug-resistant *E. coli* with chronic antimicrobial drug exposure (Ribot *et al.,* 2018). Cephalexin- concentration of the drug has to be increased for it to be used again, it is still susceptible to Gentamicin, chloramphenicol, tetracycline, and kanamycin.

K. pneumoniae is susceptible to the six tested antibiotics but from the literature, it is resistant to tetracycline (Zheng *et al.,* 2018).

The bacteria are resistant to drugs as a result of the following; evolution where cell walls become impermeable to antibiotics, the mutation in chromosomes and plasmids due to exposure to antibiotics at levels below the inhibitory concentration and misuse and overuse of antibiotics for both humans and animals (Andersson *et al*., 2012). The indiscriminate use of these antibiotics in veterinary and agriculture contributes to the selection of resistant bacteria (Martinez, 2009, Chang *et al*., 2014). Further phenotypic and genotypic studies are needed to establish and clarify the genetic mechanism behind reduced susceptibilities to antibiotics.

Tetracycline, chloramphenicol, and gentamicin are used less frequently as drugs of choice, they are still effective in control several bacteria namely *S. aureus, P. aeruginosa, E.coli, K. pneumoniae, C. freundii* and *B. subtilis*. In accord to other studies, they can be used to treat brucellosis, rickettsial infections, tularemia, early Lyme disease, and typhus (Standiford *et al.,* 1990). On other hands, there are multiple resistance against amoxicillin, kanamycin and cephalexin since they have been frequently used. This is has been witnessed by *P. aeruginosa, E.coli, C. freundii* and *B. subtilis*. Multiple resistances are more common as compared to resistance to a single antibiotic (Ibekwe *et al*., 2011, Thi *et al.,* 2017, DebMandal *et al*., 2011, Nyamboya *et*

al., 2013). According to (Normark *et al*., 2002), multiple resistances are carried in the same plasmid and frequently regulated by genes that are normally associated with large conjugative plasmids. Nonetheless, further spread of their resistance could render them obsolete for the treatment of other infections.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

This study examined bacterial counts in milk with a focus on contamination by bacteria and their resistance to antibiotics. The main objective was to investigate bacterial contamination levels and antibiotic susceptibility patterns of pathogenic microbes recovered from unprocessed bovine milk sources from small-scale farms in Ndivisi ward. Analysis of 486 samples showed that milk in Ndivisi ward is contaminated. Besides, there was bacterial antibiotic resistance which makes treatment of infectious diseases difficult as it reduces the effectiveness of the available drugs. Bacterial contamination of milk and bacterial resistance to antibiotics pose serious problems that must be addressed as a matter of agency. In this regard, the following recommendations are to be made.

6.2 Recommendations

- Consumers are advised to buy milk at production (From individual animal) since has low bacterial counts.
- Milk in Ndivisi ward is contaminated by *P. aeruginosa*, *E. coli*, *B. subtilis*, *C. freundii*, *K. pnemoniae* and *P. aeruginosa*.
- Tetracycline, cephalexin, kanamycin and amoxicillin should not be used against *P. aeruginosa*, amoxicillin should not be used against *E. coli*, amoxicillin should not be used against *B. subtilis*, amoxicillin and cephalexin should not be used against *C. freundii*. The concentrations of cephalexin and kanamycin should be increased to be used against *E.coli* and *B. subtilis*

- Chloramphenicol and Gentamicin are antibiotics of choice to be used since they have the efficacy of 100%.
- Farmers should employ cold chains in transportation and storage of milk.

6.3 Further data gaps and areas for research

It is also important that further research is conducted. My research examined an issue of importance, but some gaps need to be filled by conducting further research. Such additional research has the potential of improving our understanding of milk quality and developing effective ameliorative measures.

More research aeas:

- Research should be done on the disappearance of p. aeruginosa at outlets in Ndivisi ward.
- Feacal contamination on bovine unprocessed milk.
- Milking practices and implications on milk quality.

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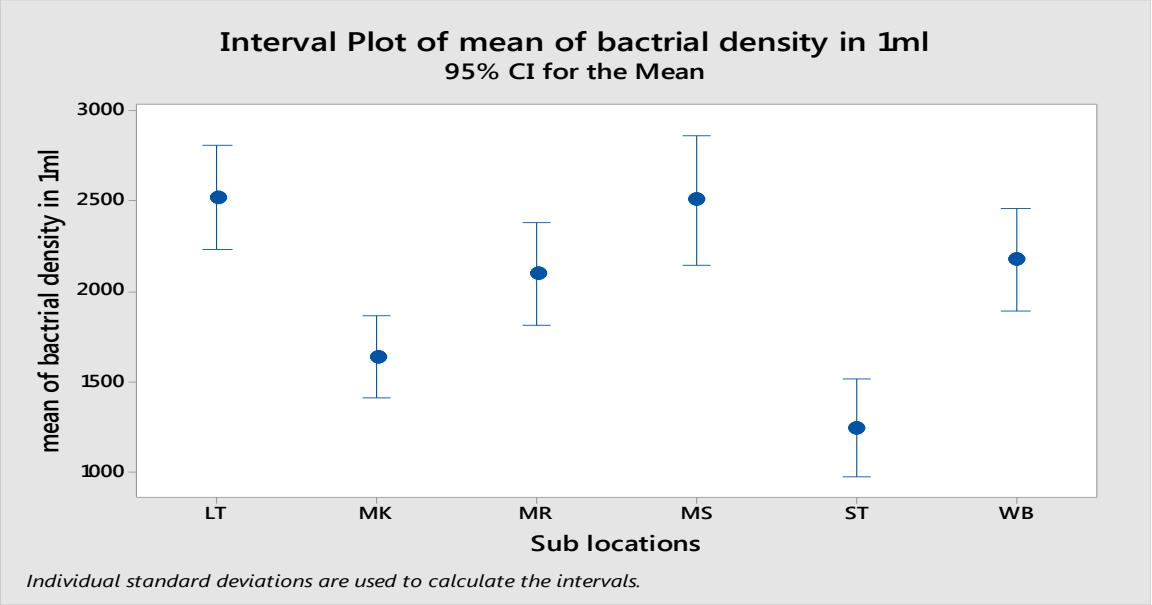
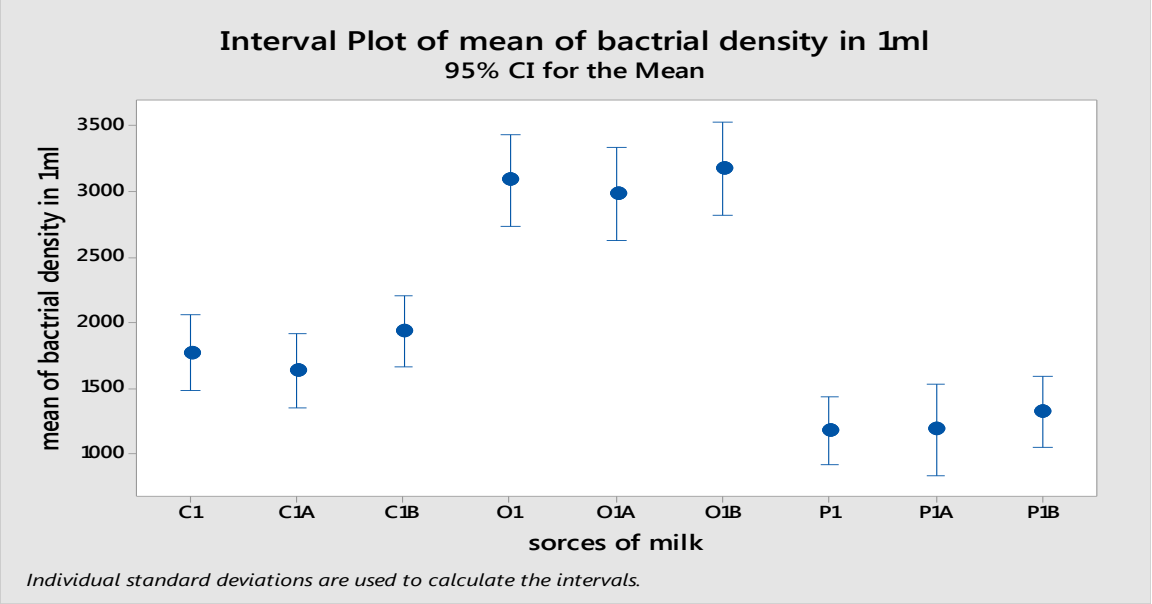
APPENDICES



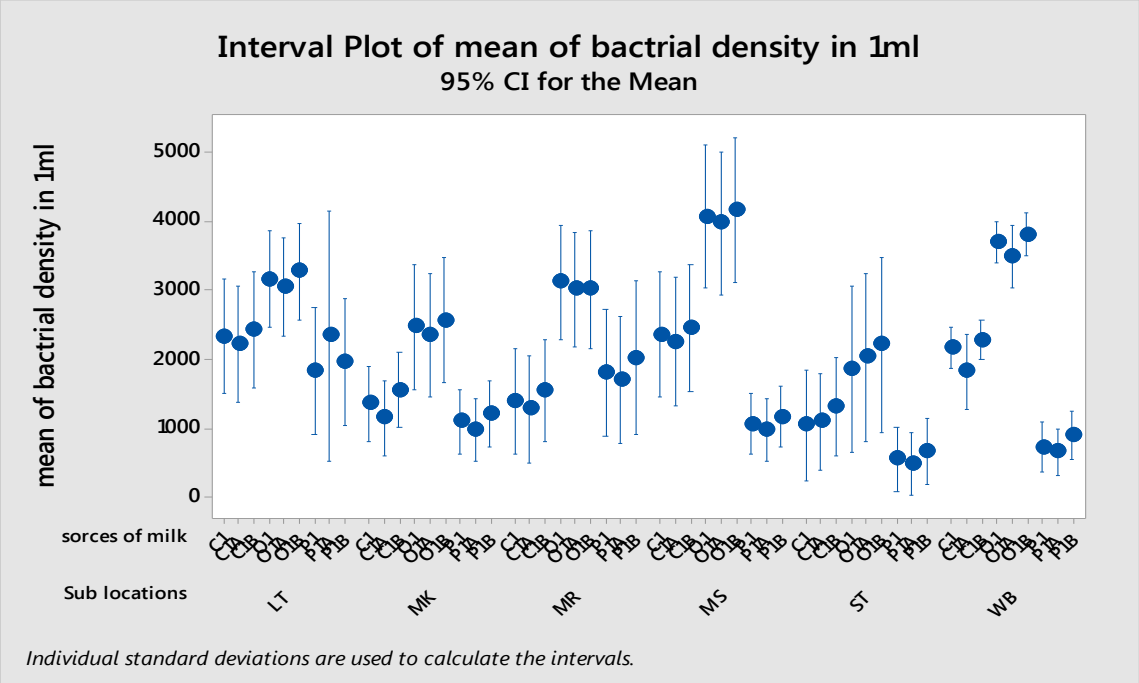
Appendix 1: Mean bacterial counts per 1 ml of milk in each sub location

Lutacho (LT), Marinda (MR), Makuselwa (MK), Misemwa (MS), Wabukhonyi (WB) Sitabicha (ST) CL -confidence interval



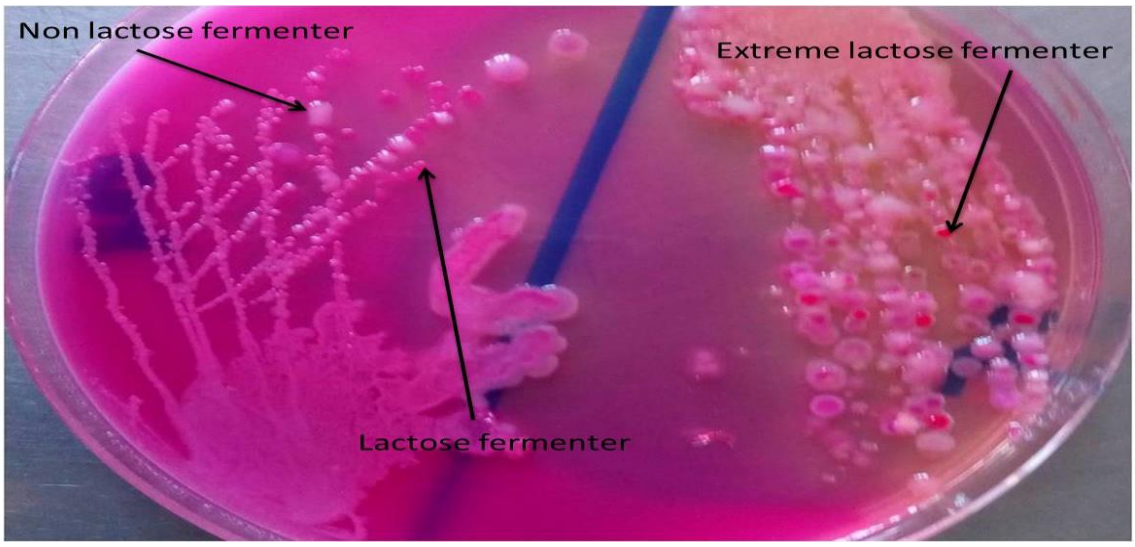
Appendix 2: Mean bacterial counts per 1 ml of milk in each sub location

P-Single Animal, C-Bulk Milk, O-Outlets, 1-First Collection,1A-Second Collection,1B-Third Collection, CL -confidence interval



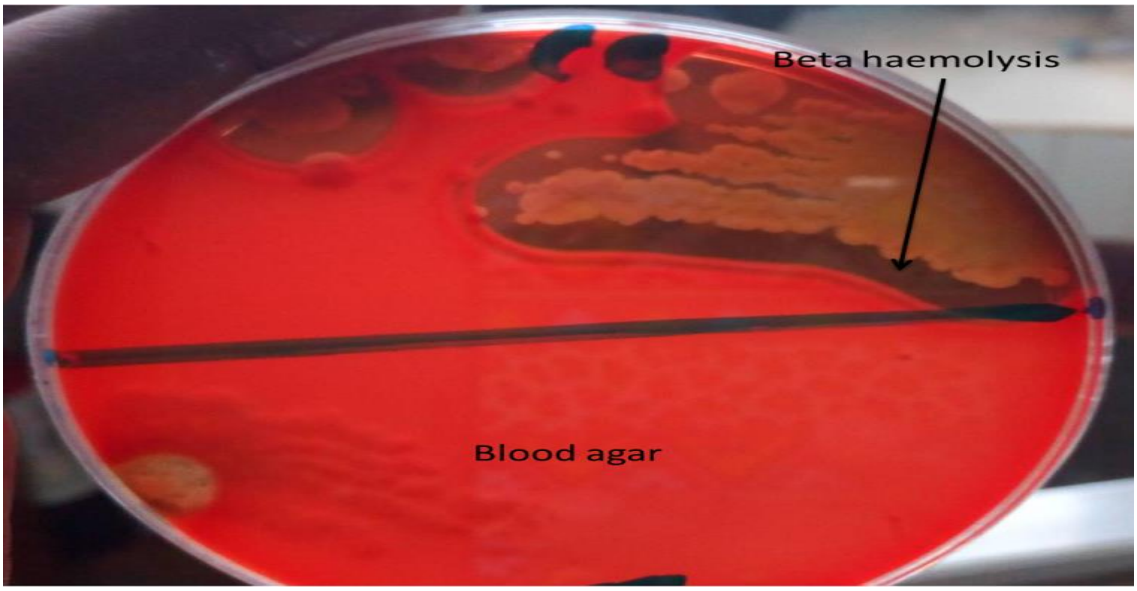
Appendix 3**:** Mean bacterial counts per 1ml milk per sub location (per collection point**)**

Lutacho (LT), Marinda (MR), Makuselwa (MK), Misemwa (MS), Wabukhonyi (WB) Sitabicha (ST) CL -confidence interval, P-Single Animal, C-Bulk Milk, O-Outlets, 1-First Collection,1A-Second Collection,1B-Third Collection, CL -confidence interval



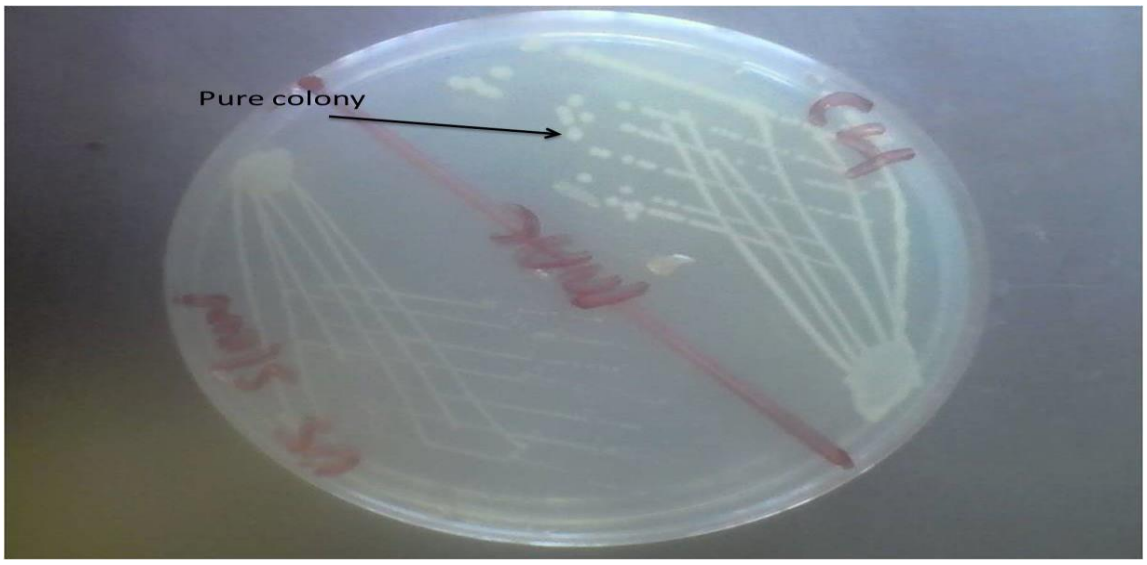
Appendix 4: Bacteria growing on MacConkey agar.

Pink and red colonies are lactose fermenters while white colonies are non-lactose fermenters. Red colony shows that the bacteria ferment lactose extremely fast.



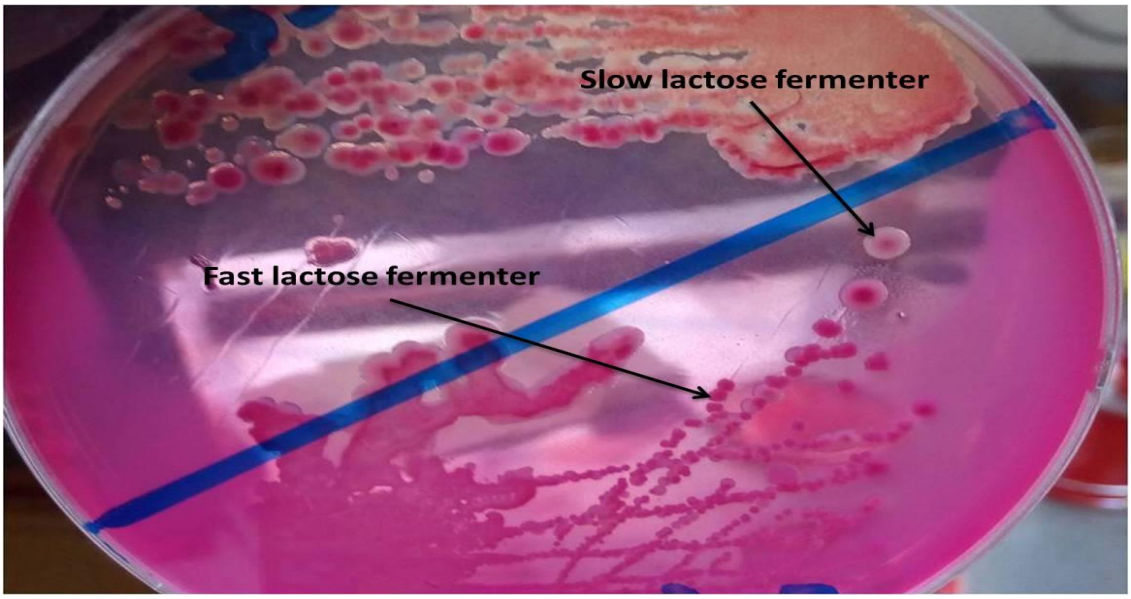
Appendix 5: Beta haemolysis by haemolytic bacteria on blood agar.

Haemolysis is the destruction of red blood cells; a clear haemolysis is called beta haemolysis while a green haemolysis is alpha haemolysis.



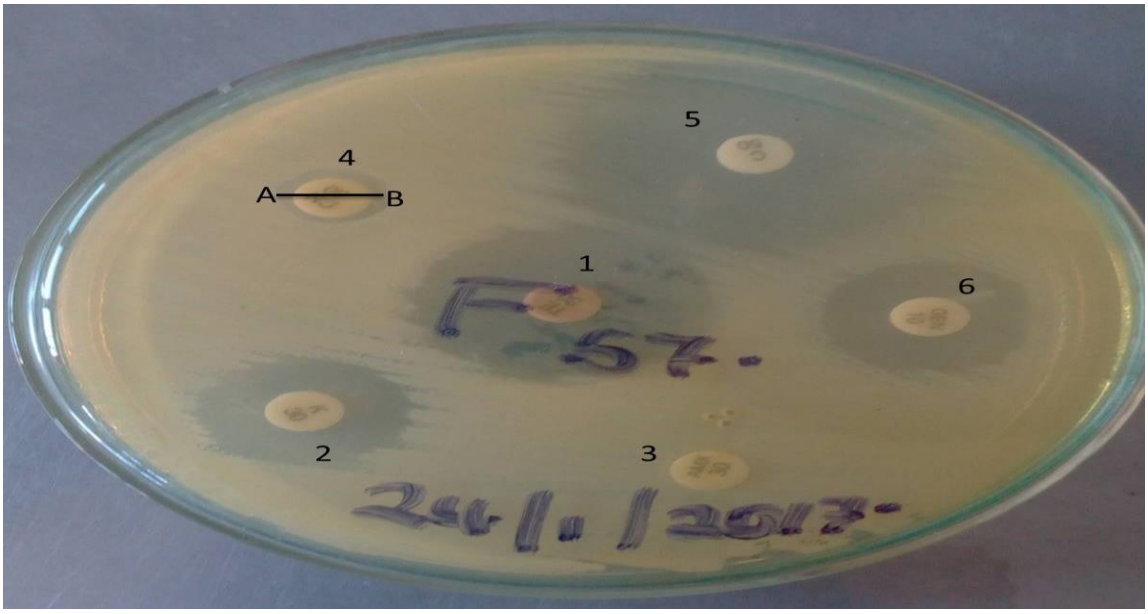
Appendix 6: Purification of bacteria on nutrient agar

Single colonies represent pure colonies. It was achieved by streak plate method.

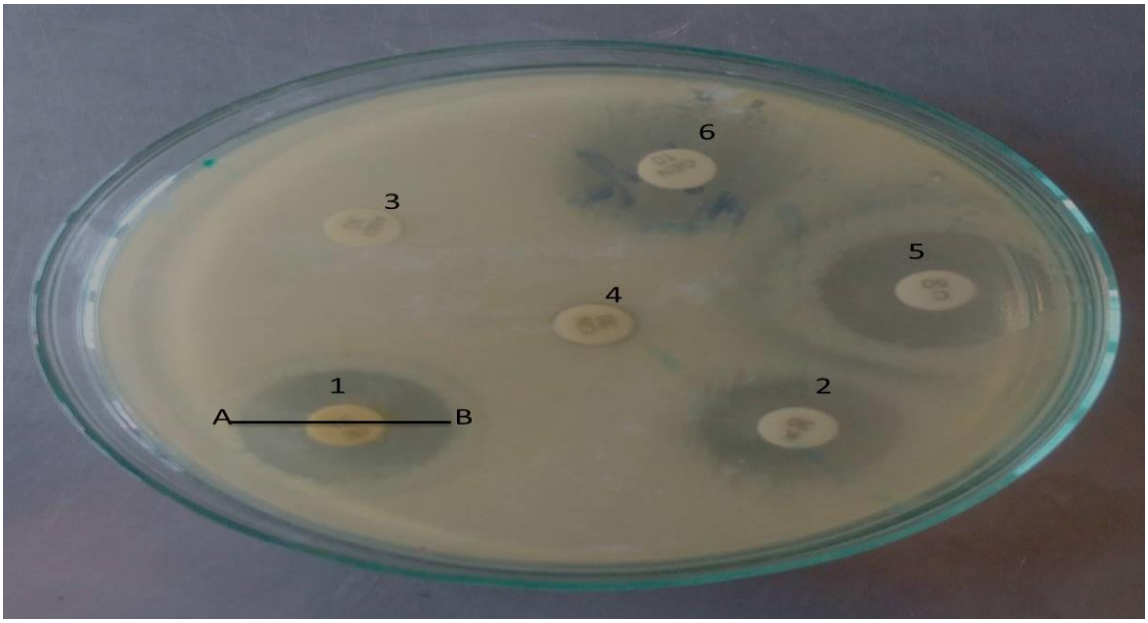


Appendix 7: Slow and fast lactose fermenter on MacConkey agar.

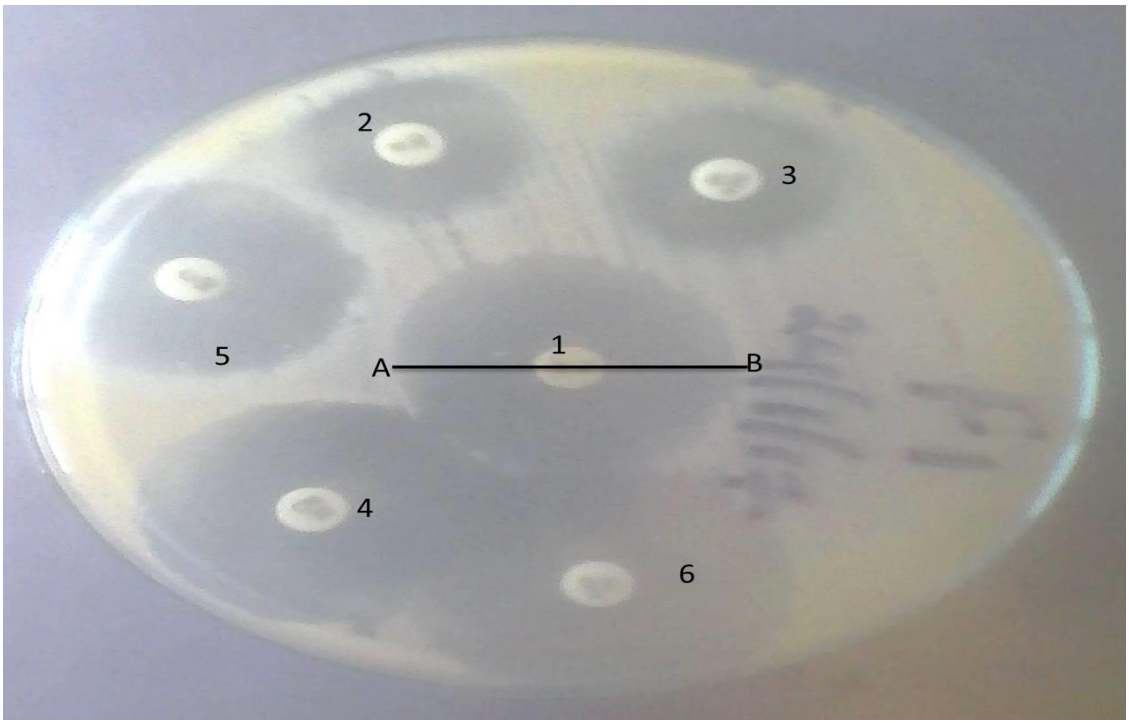
Those colonies that turn pink completely within 24 hours are fast lactose fermenters while those that take 48 hours to turn pink are slow lactose fermenters.



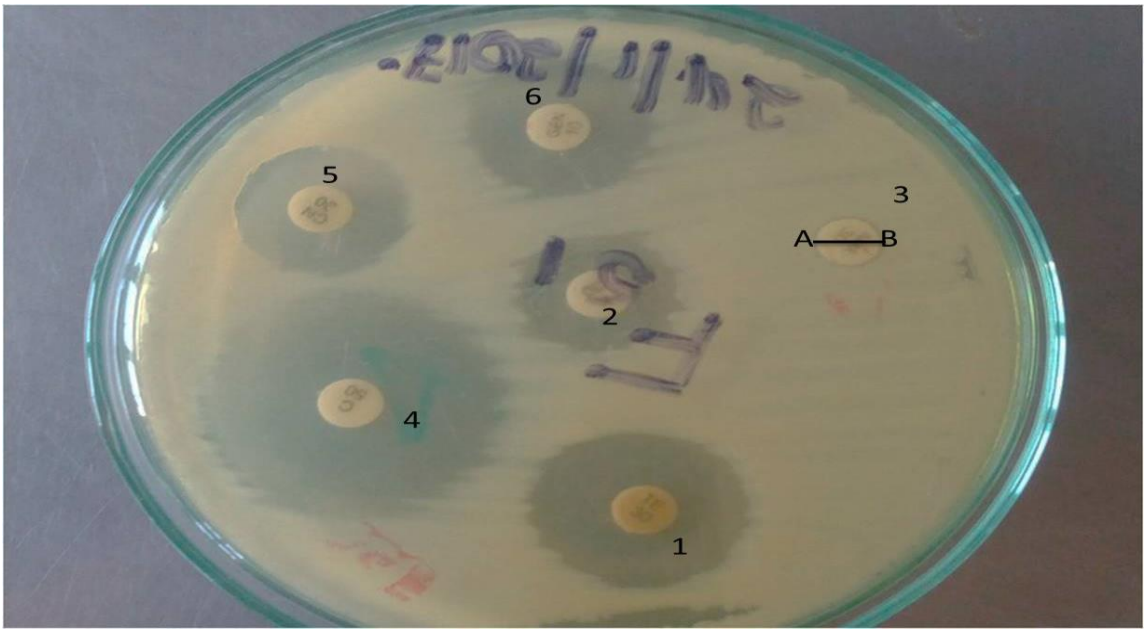
Appendix 8: Sensitivity patterns of *Bacillus subtilis*



Appendix 9: Sensitivity patterns of *Citrobacter freundii*



Appendix 10: Sensitivity patterns of *Klebsiella pnemoniae*



Appendix 11: Sensitivity patterns of *Escherichia coli*

Appendix 12: Significance test.

		Sum of Squares	Df	Mean Square	F	p-value
Sub-Locations	Between Groups	937.100	309	3.033	1.111	.220
	Within Groups	480.400	176	2.730		
	Total	1417.500	485			
Milk Sources	Between Groups	255.450	309	.827	2.123	.000
	Within Groups	68.550	176	.389		
	Total	324.000	485			
Replicates per Month	Between Groups	207.433	309	.671	1.014	.465
	Within Groups	116.567	176	.662		
	Total	324.000	485			

df- degree of freedom, p-value-probability value, f- variance among means

Appendix 13: Four Focus Areas of the FAO Action Plan on AMR (FAO, 2016)

Appendix 14: FAO Focus Areas of work as they relate to the five objectives of the Global Action Plan on AMR (FAO, 2016)