

**RISK FACTORS ASSOCIATED WITH SUBCLINICAL MASTITIS AND BACTERIAL
CONTAMINATION OF COW MILK ALONG THE MARKET CHAIN, AND
ANTIMICROBIAL SUSCEPTIBILITY PATTERNS OF THE ISOLATES IN RWANDA.**

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DEDICATION

In Memoriam: my father. We have not been together for a long time, because God loved you more than us and took you very early. Know that I have become a man and I will honor you forever. RIP Daddy! I love you.

In Memoriam: my mother. When I started this journey, I remember the joy that was on your face, both of us were hoping to celebrate this achievement together, but Your Creator who loved you more than me took you on His good time. I promise you to be the one you wished me to be; a strong man! RIP Mum. I miss you!

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LIST OF ABBREVIATIONS

%	: Percentage
ABR	: Antibiotic Resistance
ADD	: Agar Disk Diffusion
AGDP	: Agricultural Gross Domestic Product
AMR	: Antimicrobial Resistance
ANOVA:	Analysis of Variance
CAMP	: Christie, Atkins, and Munch-Peterson
CEMO	: Coopérative des Eleveurs Modernes
CEZONYI	: Coopérative des Eleveurs de la Zone Nyiragikokora
cfu	: Colony forming unit
CGIS	: Centre for Geographic Information Systems
CLSI	: The Clinical and Laboratory Standards Institute
CM	: Clinical Mastitis
CMT	: California Mastitis Test
CNS	: Coagulase Negative Staphylococci
CODERU	: Coopérative des Eleveurs de Rubavu
COMESA	: Common Market for Eastern and Southern Africa
DDM	: Dairy Dynamic Management
DF	: Degrees of freedom
<i>E. coli</i>	: <i>Escherichia coli</i>
EC	: Electrical Conductivity
g	: Gram
GDP	: Gross Domestic Product
HACCP	: Hazard Analysis and Critical Control Point
IMViC	: Indole, Methyl-Red, VogasProskaur and Citrate
l	: Litre

MCC	: Milk Collection Centre
MCMT	: Modified California Mastitis Test
mg	: Milligram
MHA	: Mueller Hinton Agar
MINAGRI	: Ministry of Agriculture and Animal Resources
MINALOC	: Ministry of Local Government
ml	: Millilitre
mm	: Millimetre
MSCC	: Microscopic Somatic Cell Count
n	: Number
°C	: Celsius degrees
OIE	: Office International des Epizooties
<i>p</i>	: <i>p</i> -value
SCC	: Somatic Cell Count
SCM	: Subclinical Mastitis
SEM	: Standard Error of the Mean
SFMT	: Surf Field Mastitis Test
SLST	: Sodium Lauryl Sulphate Test
SPC	: Standard Plate Count
spp.	: Species
SPSS	: Statistical Package for Social Sciences
TBC	: Total Bacterial Counts
TSI	: Triple Sugar Iron
WST	: Whiteside Test

ABSTRACT

Milk is important as a valuable diet, but due to its nutritional value it serves as an ideal medium for development of various microorganisms under suitable conditions, hence it can be a potential source of zoonotic pathogens. Microbial contamination in milk comes from various sources, including: sick animals (mastitis cases), humans, environment, water, equipment used for milking and storage of milk. Antimicrobials are routinely used for treatment of dairy cattle affected with clinical mastitis. Over time, this usage has increased the number of antimicrobial-resistant bacteria. Thus, in order to identify the effective drug to use, antimicrobial susceptibility testing needs to be carried out. This study was carried out in the north-western region of Rwanda, to determine the prevalence of subclinical mastitis and associated risk factors, evaluate bacterial contamination of cow milk along the milk market chain, and establish the antimicrobial susceptibility patterns of isolated bacteria.

For subclinical mastitis (SCM), a cross-sectional study was carried out on a total of 123 crossbred milking cows from 13 dairy farms randomly selected, their milk screened for SCM using California Mastitis Test (CMT), and milk samples collected and processed for bacterial isolation and identification following the method of Black (2011). The overall SCM prevalence at cow level was 50.4%. Sixty eight (68) bacterial isolates were identified by morphological and biochemical characteristics; the most prevalent being coagulase negative staphylococci (CNS) (51.5%) followed by *Staph. aureus* (20.6%), while *E. coli* was isolated at 1.5%. Only 5.5% and 2.7% of the farmers reported using dry cow therapy and teat dips, respectively, to control mastitis.

For determination of milk contamination along the market chain, 67 raw milk samples were collected and analysed for Total Bacterial Counts (TBC) at four dominant stages of the raw milk chain: 36 dairy farmers, 15 milk hawkers, 4 milk collection centres (MCC) and 12 milk kiosks. A designed questionnaire was adapted to gather information regarding animal health management practices and milking procedures, factors and milk handling practices that influence milk quality along the milk market chain. The study revealed a TBC mean value of 1.2×10^6 cfu/ml (dairy

farmers), 2.6×10^7 cfu/ml (milk hawkers), 1.5×10^6 cfu/ml (MCC) and 6.9×10^6 cfu/ml (kiosks/restaurants). The most commonly isolated bacteria from the milk samples, along the milk market chain were: *E. coli* (18, 26.9%) and *Salmonella* spp. (11, 16.4%). Total bacterial count was significantly associated with containers used for milk transport, cleaning time for milk containers and source of water used to clean containers. It was moderately associated with production system, milking space, while there was no association found between bacterial loads and time used to supply milk to the MCC.

Agar disk diffusion method was used to evaluate antimicrobial susceptibility patterns of 83 isolates including: coagulase negative staphylococci (n=45), *Staphylococcus aureus* (n=19) and *Escherichia coli* (n=19) isolates from SCM and bulk tank milk samples, using ten (10) antimicrobials. Diameters of zones of inhibition were recorded and interpreted as susceptible, intermediate or resistant according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. Coagulase negative staphylococci demonstrated high susceptibility to gentamicin (100%), ceftriaxone (100%) and ciprofloxacin (97.8%). All the isolates were resistant to ampicillin, with CNS and *Staph. aureus* showing resistance to penicillin at 57.8% and 73.7%, respectively and *E. coli* being resistant to amoxicillin/clavulanate (31.6%). However, all the isolates tested were susceptible to ciprofloxacin, ceftriaxone and gentamicin.

This study has revealed a high prevalence of subclinical mastitis predominantly caused by *Staphylococcus* spp. It has also revealed that milk contamination started at the farm and increased along the market chain. The results of this research also indicate that Gentamicin, Ceftriaxone and Ciprofloxacin are promising alternative agents to combat staphylococcal infections. It is, therefore, recommended that farmers in the study area implement good milking practices so as to ensure clean milk production. Other actors of the milk value chain should observe good milk handling practices to reduce contamination along the market chain. It is also recommended that regular screening of antimicrobial susceptibility for the most used drugs in the treatment of bacterial infections be encouraged.

CHAPTER 1: INTRODUCTION

In developing countries, as the human population growth increases, demand for livestock products also increases (Delgado *et al.*, 1999). From 1995 to 2005, milk consumption increased from 3.5 to 4.0% per annum in developing countries (FAO, 2010) and is likely to increase more by 2020 (Nene *et al.*, 1999). Therefore, there is need for good management of milk and milk products to enable the dairy sector serve as an important means for poverty alleviation and creation of prosperity in developing countries (FAO, 2010). According to the Ministry of Agriculture and Animal Resources (MINAGRI, 2013), Rwanda is benefiting from the dairy subsector in that it has led to poverty alleviation and boosted economic growth, with an average contribution to both gross domestic product (GDP) and agricultural gross domestic product (AGDP) of 6 percent and 15 percent, respectively. As a result, it has improved the living standards of many households keeping livestock. However, animal diseases, mainly mastitis, is one of the challenges faced by dairy producers in Rwanda (MINAGRI, 2013).

Milk is an essential source of nutrients for both humans and animals and therefore considered to be the first and the only food for the offspring of mammals as it is almost complete food (Pandey *et al.*, 2011). The biochemical constitution of milk is complex; more than 85% is water and the remaining portion is composed of carbohydrates, fat, proteins and minerals (Parekh *et al.*, 2008). The high water activity and nutritional value of milk make it a suitable medium for growth and multiplication of numerous microorganisms, mainly bacteria, provided there are appropriate growth conditions (Parekh *et al.*, 2008). It was reported that milk-borne diseases are due to consumption of milk contaminated with pathogenic microbes while other microorganisms are known to cause milk spoilage (Ngasala *et al.*, 2015). Milk meant for human consumption must be free from any pathogenic organisms (Bertu *et al.*, 2010).

Microbial contamination of milk may be primary, secondary or tertiary. Primary contamination is from infected lactating cows. Secondary microbial contamination may occur along the milk market chain and includes contamination by milkers during milking, other milk handlers, unclean milking equipment and water supplies used in cleaning procedures (Parekh *et al.*, 2008). Other

secondary sources of microbial contamination include transportation and storage temperatures to cite a few. On the other hand, tertiary microbial contamination occurs mainly due to re-contamination of milk after processing due to unhygienic conditions and improper handling and poor storage of milk during consumption (Parekh *et al.*, 2008). The milk composition and overall hygiene are, therefore, key determinants of milk quality (Parekh *et al.*, 2008).

Human health is greatly threatened by the pathogenic microorganisms commonly isolated from milk and milk products (Shirima *et al.*, 2003). Some of these pathogens include *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* serotypes, *Listeria monocytogenes*, *Brucella abortus*, *Mycobacterium* spp., *Campylobacter* spp., *Leptospira* serovars, *Clostridium* spp., *Pseudomonas aeruginosa* and *Proteus* spp. (Shirima *et al.*, 2003; Al-tahiri, 2005). Raw milk is known to be a major vehicle for the transmission of these milk-borne pathogens to humans. On the other hand, raw milk, apart from being potential carrier of pathogens, can also cause serious health risk to consumers due to antimicrobial residues (Omore *et al.*, 2005; Kivaria *et al.*, 2006).

Factors associated with subclinical mastitis and bacterial contamination of raw milk, and antimicrobial susceptibility profiles of bacteria isolated from milk have not been extensively investigated in Rwanda. Therefore, this study aimed to establish bacterial contaminations that occur along milk market chain as well as antimicrobial resistance patterns of the isolated bacteria in Rwanda.

1.1. Objectives

1.1.1. Overall objective

To assess risk factors associated with subclinical mastitis and bacterial contamination of cow milk along the market chain, and determine antimicrobial susceptibility patterns of isolates in Rwanda.

1.1.2. Specific objectives

- 1) To assess the prevalence of subclinical mastitis and associated risk factors in the study area
- 2) To assess bacterial contamination of cow milk along the market chain and associated risk factors
- 3) To determine antimicrobial susceptibility profiles of selected isolates from the milk samples

1.2. Hypotheses

- 1) There is a high prevalence of subclinical mastitis caused by bacteria
- 2) Bacterial contamination of cow milk is associated with many factors at all the market nodes
- 3) Isolates from milk samples along the market chain are resistant to various antimicrobials

1.3. Justification of the study

According to Chatikobo (2010), mastitis is the major disease that affects the dairy subsector. Different researches have shown mastitis to be one of the most costly diseases of the dairy industry worldwide (Kossaibati and Esslemont, 1997; Biffa *et al.*, 2005). Antimicrobials are important for its therapy, though not the ultimate solution for poor udder health. Economic losses result due to mastitis such as reduction of milk yields, milk discards due to bacterial or antimicrobial contamination, veterinary intervention costs and occasionally deaths (Vaarst *et al.*, 1997). Systematic and early detection of subclinical mastitis is, therefore, essential for epidemiological investigations to establish its prevalence and recommend corrective measures (Kurjogi *et al.*, 2014). This study was conducted to determine the prevalence of subclinical mastitis as well as isolate and identify the bacterial agents associated with subclinical mastitis in lactating cows in the study area.

Poor or improper handling of milk may cause public health and economic constraints, hence it requires hygienic handling throughout the milk value chain (Swai *et al.*, 2011). When not processed or stored correctly, fresh cow milk is a good medium for bacterial growth although it contains temporary germicidal or bacteriostatic properties including lactoferrin, lactoperoxidase, lysozyme, and possibly N-acetyl- β -D-glucosaminidase (Swai *et al.*, 2011). The major health problems related to milk consumption include tuberculosis caused by *Mycobacterium bovis* and

M. tuberculosis and brucellosis caused by *Brucella* spp. (Al-tahiri, 2005). There is need to evaluate the bacterial load and identify pathogenic microorganisms present in cow milk as well as to assess associated risk factors.

On the other hand, acquired antimicrobial resistance is becoming an increasing threat in both human and veterinary medicine. World Organization for Animal Health [Office International des Epizooties (OIE)] recommends evaluation of antimicrobial susceptibility patterns in both pathogenic and commensal bacteria in animals (Acar *et al.*, 2001). This monitoring gives important data for treatment decisions thereby providing information on resistance trends for appropriate antimicrobial use (Bengtsson *et al.*, 2009). There is limited information on antimicrobial susceptibility of bacterial isolates in Rwanda, thus hindering the choice of appropriate antimicrobials for veterinary use. Determining antimicrobial susceptibility patterns of selected bacterial isolates from subclinical mastitis and bulked tank milk samples, was necessary to provide data for the treatment regimens of bacterial infections in the country.

CHAPTER 2: LITERATURE REVIEW

2.1. Mastitis in dairy cattle

The term 'mastitis' is derived from Greek word '*mastos*' which means breast (mammary gland) and '*itis*' which means inflammation, that is, inflammation of the mammary gland (Radostits *et al.*, 2007). Mastitis may be classified as either clinical or subclinical, the latter being commonly found in most herds (Gruet *et al.*, 2001; Awale *et al.*, 2012). Clinical mastitis (CM) is characterized by visible changes in milk (e.g., clots, color changes or consistence, decreased production) while subclinical mastitis (SCM) is asymptomatic; therefore, produced milk appears to be normal (Fox, 2009).

2.1.1. Pathogens involved in bovine mastitis

Laboratory methods have provided precise identification of the main pathogens involved in mastitis (Radostits *et al.*, 2007). These pathogens can be classified according to their nature and origin; whether they are contagious, teat skin opportunistic or environmental (Radostits *et al.*, 2007). Bacteria involved in the pathogenesis of contagious mastitis include *Staphylococcus aureus*, *Streptococcus agalactiae*, *Corynebacterium bovis* and *Mycoplasma bovis* (Cunningham *et al.*, 2007). Environmental pathogens may include *Streptococcus uberis*, *Streptococcus dysgalactiae*, the coliforms (*Escherichia coli*, *Klebsiella* spp., *Citrobacter* spp., *Enterobacter* spp) and *Pseudomonas* spp. (Giannechini *et al.*, 2002). Coagulase negative staphylococci form major part of opportunistic pathogens. They are able to invade the udder through teat skin abrasions (Radostits *et al.*, 2007). Bovine mastitis caused by these bacteria and other opportunistic ones has been increasingly reported over many years (Pyorala *et al.*, 2009). Other uncommon bacteria that have been shown to cause sporadic cases of mastitis in a herd, affecting few cows, include: *Nocardia* spp, *Pasteurella multocida*, *Campylobacter jejuni* and *Clostridium sporogenes* (Radostits *et al.*, 2007).

2.1.2. Diagnostic methods for mastitis

While it is easy to detect clinical mastitis (CM; seeing clotted milk), subclinical mastitis (SCM) can only be demonstrated using various tests such as California Mastitis Test (CMT), Whiteside test (WST), Surf field mastitis test (SFMT), sodium lauryl sulphate test (SLST), Microscopic Somatic Cell Count (MSCC) (Sharma *et al.*, 2010; Hoque *et al.*, 2014) and Electrical Conductivity (EC) (Hegde *et al.*, 2013). Enzymatic analysis such as colorimetric and fluorometric assays have also been developed (Viguier *et al.*, 2009). New advanced techniques such as proteomics have been recently developed and used in detection of proteins involved in mastitis (Lippolis and Reinhardt, 2005; van Leeuwen *et al.*, 2005; Smolenski *et al.*, 2007). Most of these tests are preferred as screening tests indicating SCM since they are easy to use and yield rapid as well as satisfactory results. However, CMT has been recognized as a highly sensitive test to detect bovine subclinical mastitis (Joshi and Gokhale, 2006; Madut *et al.*, 2009). It has been reported by Sharma *et al.* (2010) that the sensitivity of the CMT was 86.1% while specificity was 59.7%, with percentage accuracy of 75.5. In a similar study, Joshi and Gokhale (2006) found that sensitivity for the Modified California Mastitis Test (MCMT) was 95.2% while its specificity was 98.0%. In order to identify mastitis causing microorganisms, the microbiological culture procedures are still the gold standard (Viguier *et al.*, 2009). Different studies have shown that SCM is mainly caused by coagulase negative staphylococci, *S. aureus*, *Str. agalactiae*, other *Streptococcus* species and coliforms (Quinn *et al.*, 2011; Hegde *et al.*, 2013).

2.1.3. Prevalence rates of subclinical mastitis

In most countries, different studies have revealed that the prevalence of mastitis is nearly 50% at cow level, whereas at quarter level it varies between 10 and 25% (Radostits *et al.*, 2007). For example, Plozza *et al.* (2011), from the United Kingdom, documented a mastitis prevalence rate of 29% while Fadlelmoula *et al.* (2015), from Germany, reported a prevalence rate of 27.6%. Elbers *et al.* (1998), from Netherlands, reported the lowest prevalence rate of 12.7%. The situation is different for East-African countries: in Uganda, Abrahmsén *et al.* (2014) reported a prevalence of 86.2%; in Kenya Mureithi and Njuguna (2016) reported a prevalence of 64%;

while in Tanzania, Mdegela *et al.* (2009) reported a prevalence of 51.6%. Other findings are prevalence of 88.6% for Vietnam (Östensson *et al.*, 2013) and 51.8% for Rwanda (Iraguha *et al.*, 2015). These differences could be due to different screening methods used.

2.2. Milk production systems and sales in Rwanda

In Rwanda, there are five geographically demarcated zones that produce and collect milk or milk products that may be supplied to the area of demand within the country; these are referred as milk sheds (MINAGRI, 2013). Every milk shed has a unique and characteristic production system which can be extensive (Nyagatare), intensive (zero grazing in Gicumbi and Kigali) or semi-intensive in Gishwati (MINAGRI, 2013). Extensive system is defined as production system where livestock are left to wander and graze during the day and are enclosed during the night (OIE, 2017). Intensive system is defined as a production system in which animals are continuously kept under housing with no access to land or otherwise called zero grazing system in which they are stall fed (OIE, 2017). Semi-intensive system is defined as a system where cattle are reared in any combination of both intensive and extensive systems, either concurrently, or mixed according to variations in climatic conditions or physiological state of the cattle (OIE, 2017).

Recently, the Ministry of Agriculture and Animal Resources (MINAGRI) field surveys have shown that, overall, the return on capital invested in dairy production ranged from 16 % to more than 30 % in both Gishwati and Nyagatare, respectively (MINAGRI, 2013). The supply of milk in wet and dry seasons vacillates the total income of milk producers; there is more milk produced during the wet season, hence forcing the farmers to sell the milk cheaply (at a loss) (MINAGRI, 2013). Dairy production provides a means for poverty reduction for many rural households in Rwanda. For its sustainability, there is a need for dairy stakeholders to be market-driven, while noting that the trading decisions at all the market points (nodes) are determined by different parameters such as market demand and its related forces (population, revenue, prices, and cultural factors) (MINAGRI, 2013).

The profits from milk production, processing and selling, which attract investments in the dairy subsector are influenced by the markets (MINAGRI, 2013). In 2013, the annual milk production was estimated at 445,000,000 litres with a related estimated farm value of US\$115.3 million and an average milk consumption of 40 litres/person/year (MINAGRI, 2013). Through its strategic plan, the National Dairy Strategy predicts that milk consumption will double to 80 litres/person/year in 2020, in regards to one of the goals of Rwanda to become a middle-income country. In 2020, it is expected that 1,161 million litres of milk will be required based on population growth estimates; meaning that the annual milk production has to increase by 13 % (MINAGRI, 2013). The use of agricultural inputs and artificial insemination has helped dairy farmers to produce more milk.

The major milk consumer outlets in urban markets are milk kiosks, milk hawkers and restaurants. Local milk processors are challenged by importers of processed milk from more competitive producers in the region (MINAGRI, 2013). A regional markets study revealed an unexploited potential for dairy products from Rwanda (MINAGRI, 2013). According to the National Dairy Strategy (MINAGRI, 2013), the two main markets presenting immediate opportunities for Rwanda are Burundi and the Democratic Republic of Congo. There is opportunity for promotion of branded Rwandan dairy products in the Ugandan market as they are cost competitive and also seem to be of high quality (MINAGRI, 2013).

2.3. Milk market/value chain in Rwanda

Market chains conventionally refer to the chain of activities that products pass through before reaching the consumer (McCormick *et al.*, 2002). It is not different when the dairy value chain in Rwanda is considered, following the supply chain and the expense of value addition at each step. The milk market chain is mainly composed of four actors: milk producers (farmers), milk collection centres, milk vendors (kiosks/restaurants/hotels) and milk processors. Surplus milk is sold to a retailer, a transporter, a processor or a chiller/bulker. The latter collects fresh milk and chills it, thereby slowing down the rate of spoilage, prior to re-selling the milk to a retailer or processor (MINAGRI, 2009).

Approximately 25 processing factories are spread throughout the country with a daily processing capacity of 160,000 litres. However, only 24,000 to 32,000 litres are currently processed (MINAGRI, 2013). In order to benefit from this surplus, dairy processors should carry out major changes in terms of quality, pricing and product diversification. In order to meet the 2020 target of 850,000,000 litres, industries that process milk must use their established capacities and new milk plants need to be opened. The Mukamira milk plant in the Western province will be an important addition for processing milk from the Gishwati zone (MINAGRI, 2013). However, like in many other African countries, more than 80% of produced milk is sold in informal market (Omoro *et al.*, 2002; Björk, 2013; Kamana *et al.*, 2014). This informal market is driven by various forces such as high prices, low demand for processed milk, easy availability of raw milk, to cite a few (RDPC, 2014). Moreover, informal market poses serious safety hazards to the consumer leading to milk-borne diseases (Omoro *et al.*, 2005) because of quality control challenges (Mbugua *et al.*, 2012). On the other hand, informal market presents an advantage over the formal one because it is a cash-based market for farmers who need immediate cash (Ndungu *et al.*, 2016).

2.4. Microbial quality of raw milk

2.4.1. Source of microbial contamination in milk

Milk is sterile when it is still contained in the udder of a healthy animal. However, it gets contaminated with microorganisms mostly during the milking process and/or after milking (Karimuribo *et al.*, 2005). There exists a difference in milk from both subclinical mastitic cows and non mastitic cows; the former containing pathogens and the latter often getting contamination from the environmental dust or poor quality of water (Kivaria *et al.*, 2006).

Bacterial contamination of milk may come from milk itself as it can be naturally contaminated or come from sick animal, human, environment, water, equipment used for milking and storage of milk. These sources of contamination include infected udder and/or teats, skin of the animal, muddying of the udder by faeces, contaminated milking and storage equipment and water used through cleaning process (Karimuribo *et al.*, 2005). The other sources of bacteria include air,

milkers, handlers, drugs (chemicals) when treating the sick animals and water used for adulteration purposes by corrupt and unscrupulous sellers (Karimuribo *et al.*, 2005). This contaminated water may result in additional health problems (Swai *et al.*, 2011).

Exposure of milk to these sources may lead to increased microbial contamination and affect its quality. Occasionally, re-contamination may occur during and after processing; this is mostly because of unhygienic conditions and inappropriate handling of milk during consumption (Parekh *et al.*, 2008). Milk contamination is considered to be of public health concern and one among the major causes of economic losses in the dairy sector worldwide, including Rwanda (Karimuribo *et al.*, 2005; Syrit, 2008; Mdegela *et al.*, 2009).

2.4.2. Hygiene, handling and microbial quality of raw milk

Milk is a suitable medium for the growth of many bacteria (Parekh *et al.*, 2008). When produced from the udder of a healthy cow, its bacterial count is very low ranging from 500 to 1,000 bacteria per millilitre (Omore *et al.*, 2005; Pandey and Voskuil, 2011). However, the environmental contamination that happens after milking subsequently increases the total bacterial counts up to 50,000 or several millions of bacteria per millilitre (Pandey *et al.*, 2011). This level is a great indicator of a very poor quality of milk either during milking and handling or milk from an unhealthy cow. Coliform bacteria, especially *E. coli*, when present in raw milk, indicate faecal contamination, therefore signifying unhygienic conditions and unclean environment since these bacteria are of faecal origin (Hasan *et al.*, 2015).

In Rwanda, like in other developing countries, milk is mainly produced by the small holder farmers (Hasan *et al.*, 2015). These farmers are spread across the rural areas which have poor infrastructure, while most markets and customers are located in the urban areas. There is a need therefore for good hygienic practices, an efficient collection together with handling and transport system, all of which are important but face a challenge of implementation towards milk safety during transportation (Hasan *et al.*, 2015).

It is, however, important to note that milk has a natural inhibitory system which prevents rapid bacterial growth up to 3 hours after milking. Hence, if the milk is chilled at 4°C immediately after

milking, it keeps close to its original quality and remains safe for processing and consumption (Pandey and Voskuil, 2011; Swai and Schoonman, 2011). The temperature at which milk is stored and time after milking are critical factors in determining the quality of milk as they both influence the multiplication rate of bacteria (Omore *et al.*, 2005). With respect to bacterial multiplication and growth curve, at ambient temperature, after every twenty minutes a bacterium, like *E. coli*, produces another (Harding, 1995). So, in order to prevent exponential bacterial growth, the milk has to be produced as hygienically as possible and should be chilled or pasteurised immediately (Pandey *et al.*, 2011).

Acceptable raw milk quality levels in East African countries are defined and good milking and storage procedures have been set and standardised (Bingi *et al.*, 2015). It is very critical that all players of the milk value chain implement these standards at their level of operation to protect the consumer from any health related issues, with respect to milk as recommended by the Common Market for Eastern and Southern Africa (COMESA, 2006). These milk standards provide a tool for ensuring milk quality at every value chain. COMESA has categorised milk quality into three grades, defined as: Grade I or A: $<2 \times 10^5$ cfu/ml; Grade II or B: 2×10^5 - 1×10^6 cfu/ml and Grade III or C: 1 - 2×10^6 cfu/ml (COMESA, 2006).

2.4.3. Total Bacterial Counting

Bacterial counting in milk offers important information on the microbiological quality of milk and the conditions under which it was produced (Kaiza, 2011). Total plate count indicates only the mesophilic aerobic organisms as incubation is done under normal atmospheric conditions at 35°C for 48 hours (Richardson, 1985). The counts also indicate whether the product was mishandled during milking, transportation, storage and processing (Cogan, 1996). The increased microbial counts in raw milk reduce its quality leading to spoilage (Cogan, 1996). For a raw milk sample the total bacterial count must be less than or equal to 100,000/ml; for a retail product the total count must be less than or equal to 20,000/ml or gram and for frozen desserts the total bacterial count must be 50,000/gram or less (Pandey *et al.*, 2011). The test is based on a hypothesis that each viable cell will develop into a visible colony when cultured on agar

containing the appropriate nutrients. However, it does not provide a measure of the entire bacterial population; thus it is a generic test for organisms that grow aerobically at mesophilic temperatures (25 to 40°C) (Richardson, 1985).

2.4.4. Prevention and control of microbial contamination in milk

Elimination of microorganisms from human carriers helps in the prevention and control of microorganisms in milk. This can be through provision of adequate water supplies, education in public health and both personal and environmental sanitation (Cogan, 1996). Prevention can also be achieved through proper pasteurization of raw milk before consumption and processing (Kaiza, 2011). Control of pathogenic microorganisms from the lactating cows can be done by improvement in animal husbandry practices whereas those from the environment and equipment can be prevented by observing good milking practices (Kaiza, 2011).

In general, microbial contamination of milk could be reduced by adhering to effective hygienic practices at the farm level. Many developing countries are ignorant about the existence of milk-borne infections and consuming raw milk predisposes small-scale livestock farmers, consumers and the general public at risk of contracting these infections (Mosalagae *et al.*, 2011).

It is recommended that milk be boiled so that most pathogenic bacteria are killed and it becomes safe for human consumption (Adams *et al.*, 2008). However, Kilango *et al.* (2012) observed that while boiling generally makes milk safer by eliminating most microorganisms, there are still other possible risks of re-contamination through other factors like utensils used for storage, storing temperature and hygienic status of milk handlers.

2.4.5. Bacterial contamination of cow milk and zoonoses

Food safety is an area of great concern in relation to public health management and particularly from an economic perspective (Kaiza, 2011). Microbial contamination of milk is a risk to the public health through transmission of food borne diseases (Pires *et al.*, 2009). Raw milk is and continues to be a major distress in the epidemiological data of campylobacteriosis, salmonellosis, tuberculosis, brucellosis, hemorrhagic colitis, Brainerd diarrhoea, Q fever, listeriosis, among others (Alvarez, 2009).

These infections inflict a significant burden to the health care systems lowering economic productivity (Kaiza, 2011). Seventy percent (70%) of deaths in children below five years are connected to microbial contaminated food and water (Motarjemi *et al.*, 1993). The sources of contamination are varied and may occur at any level of milk production and supply chain. The main milk pathogens of interest are zoonoses and environmental coliforms of faecal origin, the latter being mainly introduced in milk from unhygienic practices at farm and along the value chain (Kaiza, 2011). Contamination of faecal bacteria in milk commonly occurs through the use of contaminated water and poor handling (Kilango *et al.*, 2012).

2.4.6. Role of contaminating bacteria in milk spoilage

The breakdown of lactose, proteinaceous compounds, unsaturated fatty acids and the catabolism of triglycerides potentially leads to microbial spoilage of raw milk. The microorganisms that are principally involved in milk spoilage are psychrotrophic organisms, most of which are destroyed by pasteurization temperatures (Goff *et al.*, 1989). However, some other bacteria which are also capable of causing spoilage, like *Pseudomonas fluorescens*, *Pseudomonas fragi*, can produce proteolytic and lipolytic extracellular enzymes which are heat stable (Goff *et al.*, 1989). In addition, some species and strains of *Clostridium*, *Micrococcus*, *Mycobacterium*, *Bacillus*, *Arthrobacter*, *Cornebacterium*, *Lactobacillus*, and *Streptococcus* can resist pasteurization temperatures leading to spoilage of well-preserved milk (Banwart, 1989).

Being lactose non-fermenters, *Pseudomonas* and related species metabolize the proteinaceous compounds altering the ordinary taste of milk to bitter or fruity. While growing, lactose-positive coliforms tend to produce lactic, acetic and formic acids, carbon dioxide and hydrogen gases which cause souring and curdling of milk (Dilbaghi *et al.*, 2007). When raw milk is not refrigerated directly after milking, the mesophiles predominate e.g, *Lactococcus*, *Lactobacillus*, *Enterococcus*, *Bacillus*, *Pseudomonas*, *Proteus*, coliforms and others producing different changes in milk such as souring and curdling (Dilbaghi *et al.*, 2007).

2.4.7. Importance of establishing critical contamination points

Nutritional values of dairy products make them to be considered amongst the most complete foods. However, this feature attributes them to being highly susceptible to bacterial contamination which may result in outbreaks of food borne diseases (FDA, 2014).

Both food and agricultural products may become contaminated at any stage of food supply chain, from production to distribution, thereby becoming hazardous (APACEM, 2008). People of the same risk groups are likely to be exposed to the same health risks and hazards, therefore, these hazards must be efficiently traced in the food chains; consequently food processors and experts have elaborated the Hazard Analysis and Critical Control Point (HACCP) system (APACEM, 2008). Establishing critical contamination points will have a great impact on preventing bacterial contamination and stop their spread along the value chain. The HACCP system is a more efficient tool to safeguard food safety in critical contamination points of food processing industries, such as dairy products, meat, fisheries, and packing houses (Nikolic *et al.*, 2013).

2.5. Antimicrobial resistance

2.5.1. Use of antimicrobials and antimicrobial resistance

Antimicrobials are extensively used in the control of bacterial infections not only in humans, but also in animals and plants. Most antimicrobials/antibiotics are only active against bacteria and do not have an effect on other cells (Sköld, 2011). However, even with the use of the best possible antimicrobial treatments, bacteriological cure rates (e.g., of *Staphylococcus aureus* mastitis) rarely go beyond 50% (Saini *et al.*, 2011). It was reported that the main reason for treatment failures is the antimicrobial resistance (AMR) (Barkema *et al.*, 2006). Resistance to antimicrobials among pathogenic bacteria has developed within a short time and in many ways faster than could have been expected because their multiplication is very fast and short (Sköld, 2011). This has led to the rapid spread of antimicrobial resistance.

Almost all microorganisms have developed resistance to some antimicrobials and these resistant strains pose a serious health problem (Mainous *et al.*, 2001). Every use of an antimicrobial agent can create new resistant bacteria for many decades, resulting in a global health problem (Thanner

et al., 2016). Furthermore, following the use of antimicrobials, bacteria have established the mechanism to manipulate their own genetic makeup, leading to the development of resistance (ARAE, 2016). The use of antimicrobials in veterinary medicine should ideally rely on consultations and prescriptions by veterinarians (McEwen *et al.*, 2002). However, antimicrobials are used in food animal production with little or no veterinary consultation (McEwen *et al.*, 2002). Moreover, it has been reported that the misuse of antimicrobials in food-animal production is one of the most important factors contributing to the global surge and spread in antimicrobial resistance (Carlet *et al.*, 2012).

The management of antimicrobial resistance resides in good therapeutic regimen dosing as recommended (Parry, 2010); however, most of the times, this is not the case. Emphasis is therefore, currently, being placed on taking appropriate measures to preserve the efficacy of the existing drugs so that common and life-threatening infections can be cured (WHO, 2014). Unfortunately, the impact of the burden of AMR on the population is not well documented and it is not easy to be assessed (WHO, 2014), while resistance among animal pathogens decreases the efficacy of some antimicrobials (McEwen *et al.*, 2002).

2.5.2. Antibiotic susceptibility profiles

Different methods of screening antimicrobial resistance have been developed and may include disk diffusion, agar dilution, E-test (CLSI, 2014), broth microdilution (Burrows *et al.*, 1993) assays and molecular techniques (Tan, 2003). Although molecular advances have revolutionized AMR screening, they also present some challenges. The most important of them are characterization of bacterial mutations with variations in DNA sequences and clinical specificity (Ledeboer *et al.*, 2011). Phenotypic testing of AMR has been widely used in clinical and diagnostic microbiology laboratories with accurate standards (Qi *et al.*, 2006). They are of low cost, easily automated and are essential for new resistance discovery because their interpretation criteria is readily available for commonly encountered organisms (Qi *et al.*, 2006). Dilution and disk diffusion tests have been standardized and recommended as basic methodologies for AMR screening techniques (CLSI, 2014). Furthermore, agar disk diffusion method is recommended as

a reliable screening method for AMR in veterinary laboratories (Saini *et al.*, 2011). The Clinical and Laboratory Standards Institute (CLSI) guidelines offer standardized methods and interpretative standards for antimicrobial susceptibility testing for organisms commonly encountered in clinical microbiology laboratories (CLSI, 2014).

When treatment of diseases is based on the results from antimicrobial susceptibility tests, this leads to effective and appropriate treatment of diseases (Idriss *et al.*, 2014). Different researches were conducted to evaluate the susceptibility of many pathogenic bacteria in veterinary medicine (Malinowski *et al.*, 2008; Onono *et al.*, 2010; Ikiz *et al.*, 2013; Idriss *et al.*, 2014). Reports from these studies vary largely depending on tested isolates and applied antimicrobials. For example, *Staphylococcus aureus* was susceptible to cotrimoxazole (100%), oxytetracycline (95.65%), amoxicillin (86.95%), gentamicin (86.95%), ampicillin (82.60%), ciprofloxacin (82.60%), chloramphenicol (82.60%), enrofloxacin (69.56%) and novobiocin (60.86%) in a study carried out in Pakistan (Hussain *et al.*, 2007). The highest recorded susceptibility was to gentamicin (96.6%) while high resistance was found to penicillin (40.3%) and erythromycin (11.6%) (Gentilini *et al.*, 2000). In another study carried out in Kenya, Onono *et al.* (2010) determined the antimicrobial sensitivity of the non-sorbitol fermenting *Escherichia coli* isolated from milk samples and found that all the isolates were resistant to sulphamethoxazole.

CHAPTER 3: PREVALENCE OF SUBCLINICAL MASTITIS AND ASSOCIATED RISK FACTORS IN DAIRY FARMS OF MUSANZE, RUBAVU AND NYABIHU DISTRICTS, RWANDA.

3.1. Introduction

Mastitis is defined as inflammation of mammary gland. It is divided into two types: clinical and subclinical. Clinical mastitis (CM) is characterized by visible changes in milk (e.g., clots, color changes or consistence, decreased production) that may be associated with inflammation signs of the udder (e.g., redness, swelling, heat or pain) or the cow (e.g. dehydration, hyperthermia, lethargy) (Fox, 2009). Subclinical mastitis (SCM) is asymptomatic; therefore, produced milk appears to be normal. According to Chatikobo (2010), mastitis is the major disease that affects the dairy subsector. Several economic losses result due to mastitis such as reduction of milk yields, milk discards due to bacterial or antimicrobial contamination, veterinary intervention costs and occasionally deaths (Vaarst *et al.*, 1997).

The prevalence of SCM and causative bacteria in lactating cows of Musanze, Rubavu and Nyabihu district in Rwanda is not known. Therefore, this study was conducted to determine the prevalence of subclinical mastitis as well as isolate and identify the bacterial agents associated with SCM in lactating cows in these two districts and to assess possible association with SCM within the two production systems (extensive and intensive). Milking procedures and management practices that influence the prevalence of mastitis in the study area were also evaluated.

3.2. Materials and methods

3.2.1. Study area

This study was carried out in North-Western region of Rwanda, specifically in Musanze, Nyabihu and Rubavu districts (Figure 1b). Musanze district is located in Northern Province (1°30'6.94"S; 29°37'59.75"E at 1850 m above sea level) whereas Rubavu and Nyabihu districts are located in Western Province (1°40'52.54"S; 29°19'45.55"E and 1830 m above sea level; 1°39'9.90"S; 29°30'24.62"E and 2437m above sea level, respectively).

The average temperatures for Musanze, Rubavu and Nyabihu districts are 19.4°C, 18.1°C and 15°C, respectively. The average annual rainfalls are 1,100 mm, 1,377 mm and 1,400 mm for Musanze, Rubavu and Nyabihu districts, respectively.

There are two wet seasons in North-Western region, the first being from February to May and the second from September to November. This region has numerous milk collection centres (MCCs; about 16) thus accounts for over 70% of local cheese processors in the country.

Majority (above 91%) of the human population in North-Western region engage in agriculture. The soil types in the region consist volcanic, lateritic and humus-bearing and clayey soils (MINALOC, 2011).

The study sites were conveniently selected based on their location (near the University of Rwanda, for laboratory analysis), their potentialities of hosting more dairy cattle and their accessibility.

Figures 1a and 1b describe the location of Rwanda in Africa and the study area, respectively.

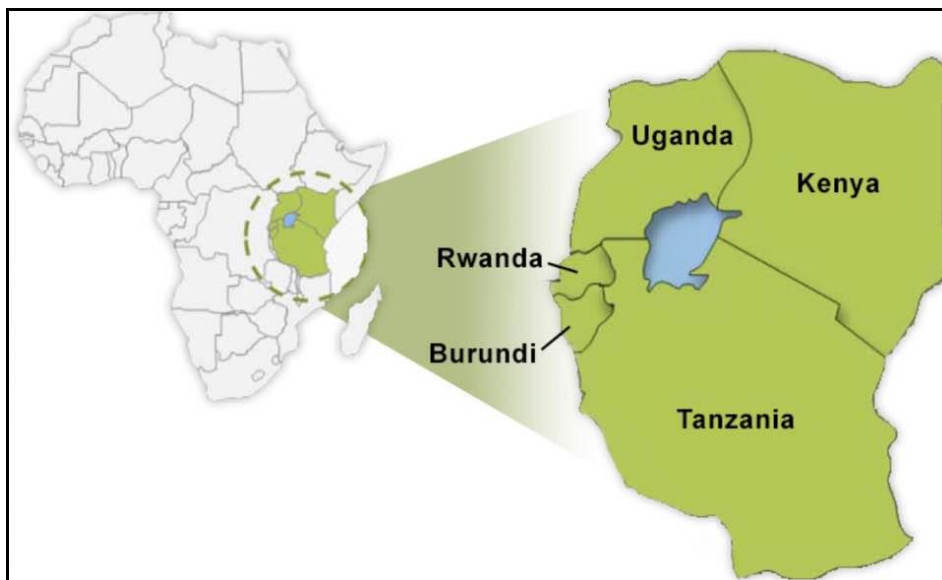


Figure 1a. Map showing the location of Rwanda in Africa

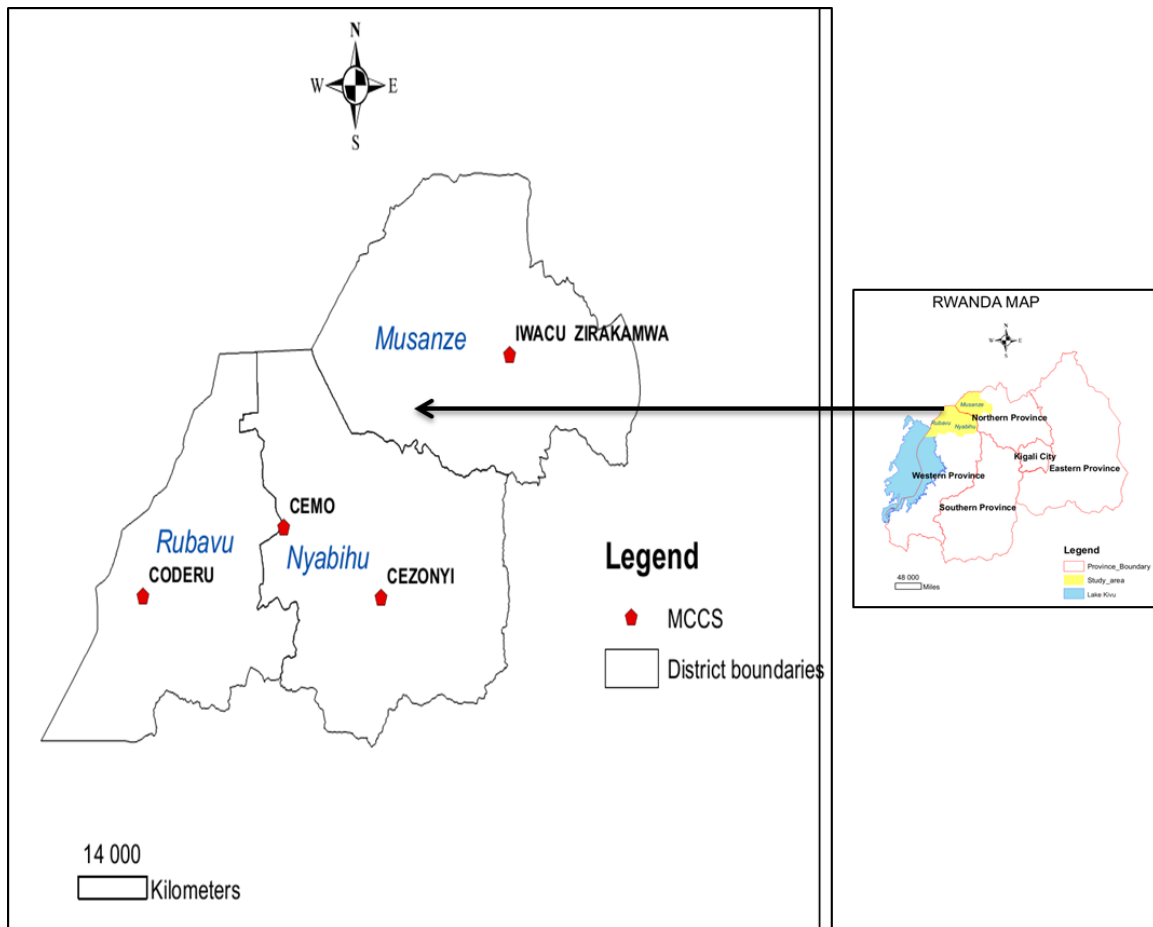


Figure 1b. Map showing the study area

Source: Derived from CGIS (2015)

3.2.2. Study design

This was a cross-sectional study where cows kept under two production systems (extensive and intensive) were screened for subclinical mastitis. Extensive system was defined as production system where livestock are left to wander and graze during the day and are enclosed during the night whereas intensive system was defined as a production system where cows are confined in kraals and zero grazed; being served with grass, supplements and water (Figure 2).



Figure 2. Typical intensive (A) and extensive (B) systems from the study areas

California mastitis test was used to test for subclinical mastitis before collection of milk samples for bacterial isolation and identification - done using standard microbiological techniques (Quinn *et al.*, 2011). As the milk collection was done, a structured questionnaire (Hyera, 2015) was administered to collect information on farmer's particulars (age, sex and level of education), herd characteristics, management practices and milking procedures. Observational assessment was also made on the hygienic status of the animals as well as cow sheds, and any other factors.

3.2.3. Sample size calculation

Sample size was calculated using the formula given by Dohoo *et al.* (2003) as follows:

$$n = \frac{t^2 \times P(1-P)}{L^2} = \frac{(1.96)^2 \times 0.15(0.85)}{(0.05)^2} = 196$$

Where:

n: sample size

t: confidence level at 95% (standard value of 1.96)

P: anticipated prevalence (15%)

L: required precision or margin of error at 5% (standard value of 0.05).

However, based on the production systems and the willingness of the farmers to be part of the research, the study was carried out on 123 lactating crossbreed (Friesian versus Ankole and

Jersey vs. Ankole) cows; 61 from intensive system and 62 from extensive system randomly selected from 13 smallholder dairy farmers; 6 and 7 in Rubavu and Nyabihu respectively.

3.2.4. Sampling method

This was based on the production systems and the willingness of the farmers to be part of the study. Deliberate efforts were made to include cows from the two management systems (extensive and intensive), for comparison sake. For small scale farmers, all lactating cows at the time of research were screened for SCM, with an average of 8 cows per farm. However, for large scale producers, cows with healthy teats were selected because some of the cows had dead teats due to clinical mammary infections.

3.2.5. Milk collection and handling

After carrying out California mastitis testing on milk from the four quarters, 15 ml of combined (composite) milk samples from multiple quarters of a cow were drawn into one sample tube (in cases of clinical mastitis, milk samples from affected quarters were aseptically collected into separate and labelled bottles), placed in a cool box and transported to the laboratory for bacteriological isolation and identification. Briefly, after carrying out CMT, the udder and teats were cleaned with water and wiped using sterile towels; the teat orifice and the skin around the teat were sprayed with 70% alcohol and dried off with sterile towels; before the milk sample was collected (Figure 3). The samples were taken shortly prior to milking and only cows expressing no clinical signs of mastitis were sampled. Milk samples from CMT positive cows were aseptically collected directly from quarters into aseptic tubes and taken to the laboratory for bacteriological analysis to identify SCM causative micro-organisms (Klastrup, 1975).

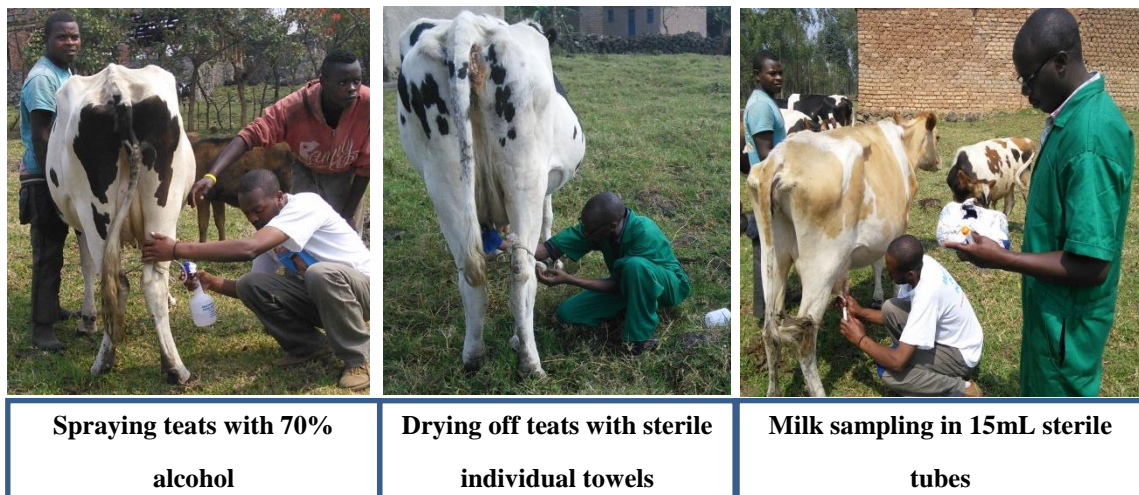


Figure 3. On farm milk sampling procedures

3.2.6. California Mastitis Test

California Mastitis Test (CMT) which is a cow-side test to estimate the somatic cell count of milk was carried out following the method described by Markey *et al.* (2013). Briefly, a squirt of milk (approximately 2 ml) from each quarter of the udder was placed in each of four shallow cups in the CMT paddle. An equal volume of commercial CMT reagent or 14% sodium lauryl sulphate (Teepol®, Shell) was added to each cup. A gentle circular motion was applied to the mixtures, in a horizontal plane and in positive cases gel formation occurred after a few seconds. Presence of gelling denotes positive reaction, as a result of disrupting the cell membrane of any cells present in the milk sample by the reagent, allowing the DNA in those cells to be released and appear as a gel; colouring done for ease of viewing (Figure 4). The extent of gelling was scored from none to 3 (Table 1) using the modified Scandinavian scoring system, based on the extent of gelling, where 0 is negative result (no gel formation), 1 is mild gelling and 3 is the thickest gelling (Quinn *et al.*, 2011). A sample was defined positive to SCM when one or more quarters with CMT grading of 2 or more was detected (Schukken *et al.*, 2003).

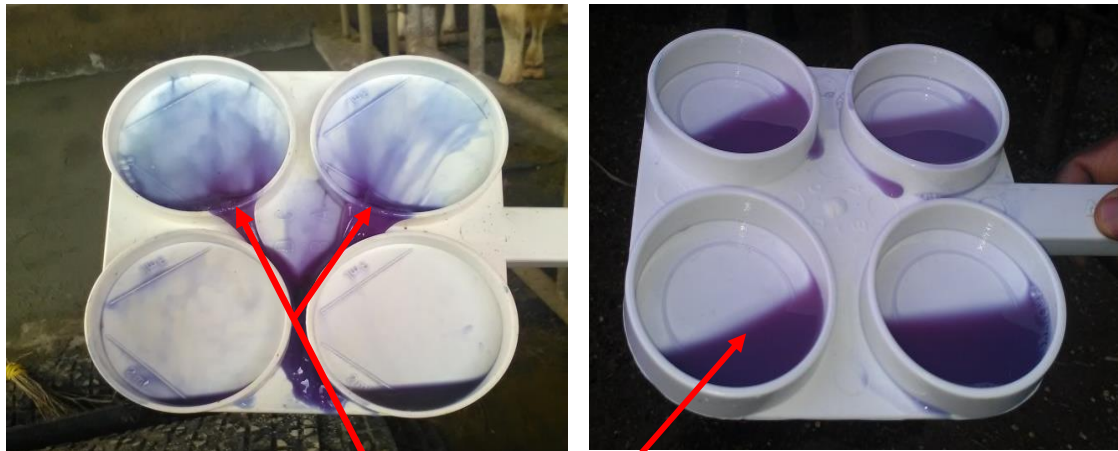


Figure 4. CMT Results (Positive and negative reaction), a DeLaval kit

Table 1. Correlation of CMT scores with somatic cell counts: Source: McFadden (2011)

CMT Score	Somatic Cell Range			Gelling
None	0	to	200,000	None
Trace	200,000	to	400,000	Very Mild
1	400,000	to	1,200,000	Mild
2	1,200,000	to	5,000,000	Moderate
3	Over 5,000,000			Heavy, almost solidifies

3.2.7. Bacterial isolation and identification

This was done using standard microbiological techniques (Black, 2011, Quinn *et al.*, 2011). The collected milk samples were inoculated separately onto MacConkey agar and blood agar plates by streaking method. Inoculated plates were then incubated aerobically at 37°C for 24-48 hours. After 24 hours, primary bacteriological identification was made based on colony morphology, colour and haemolytic characteristics; after which pure cultures were prepared through subculturing and incubation. The purified isolates were then subjected to gram staining and further biochemical testing. Staphylococci were identified based on catalase and tube coagulase tests. Streptococci were identified based on catalase and Christie, Atkins, and Munch-Peterson (CAMP) tests. Gram negative isolates were identified based on growth characteristics on

MacConkey agar and reactions to oxidase test, catalase test, Triple sugar Iron (TSI) agar and the “IMViC” tests (Indole, Methyl-Red, VogasProskaur and Citrate utilisation) (figure 5 and 6).

3.2.8. Questionnaire and observational data collection

The questionnaire, as given in Appendix 1, was administered at the time of sample collection. The data collected included: Farmer’s age, sex and level of education; herd size, milk production, record keeping, milking practices, mastitis screening and control measures; milk handling practices and factors that influence milk quality in regards to bacterial contamination. Observational assessment was made on the state of the environment under which the animals were kept (the cow sheds) or where the collected milk is kept; the milking method used plus the hygienic state of the milking area; as well as the cleanliness of the milker(s)/milk handler(s).

3.2.9. Data management and statistical analysis

Information regarding farmers’/respondents’ particulars (age, sex and level of education), herd characteristics, management practices and milking procedures were encoded into excel spreadsheet for descriptive analysis. Correlation between production systems and prevalence of SCM was computed by Statistical Package for Social Sciences (SPSS) IBM SPSS® Statistics 23 version. Statistical significance was established at 95% confidence and critical p value of 0.05.

3.3. Results

3.3.1. Results from questionnaire and observational data

Respondent’s particulars and herds’ characteristics, with respect to the two production systems (intensive and extensive), were as presented in Table 2. Of the total respondents who participated in the study, 95.9% were males and 4.1% were females. The majority of the respondents were aged above 40 years (86.3%). Regarding their level of education, 69.9% attended primary school while 15.1% did not get a formal education. All (100%) of the studied animals were crossbreed of Friesian and Ankole (local cattle) while milk production per day doubled in intensive system (110 litres/day) compared to extensive system (53 litres/day) .

Table 2. Respondents' particulars and herds' characteristics per production system in the study area

Parameter	Intensive (n=10)		Extensive (n=63)		Total percentage (n=73)
	Number of respondents	Percentage	Number of respondents	Percentage	
Sex					
Male	10	100.0%	60	95.2%	95.9%
Female	0	0.0%	3	4.8%	4.1%
Age				0.0%	
[21-30]	0	0.0%	1	1.6%	1.4%
[31-40]	3	30.0%	6	9.5%	12.3%
[41-50]	2	20.0%	23	36.5%	34.2%
>50	5	50.0%	33	52.4%	52.1%
Education level					
Informal	1	10.0%	10	15.9%	15.1%
Primary	5	50.0%	46	73.0%	69.9%
Secondary	1	10.0%	4	6.3%	6.8%
University	3	30.0%	3	4.8%	8.2%
Cattle breed				0.0%	
Cross breeds	10	100.0%	63	100.0%	100.0%
Herd size (mean)	30	-	21		-
Lactating cows (mean)	14	-	10		-
Milk production (mean l/day)	110	-	53		-
Milking frequency/day					
Once	0	0.0%	0	0.0%	0.0%
Twice	10	100.0%	63	100.0%	100.0%

Key: l means litres

Management practices employed by farmers in the study area were as given in Table 3. Overall, it was noticed that milking practices and procedures were inadequate. Among 67.1% dairy farmers who screened for mastitis only 6.8% used CMT while 58.9% observed appearance of clinical signs. Out of 84.9% who controlled mastitis, 2.7% used teat dips, 65.8% treated clinical mastitis cases while 5.5% applied dry cow therapy. All (100%) farmers in the study area milked their cows by hand while 89% dairy farmers milked their cows from open space. It was noticed that

cows in extensive production system were milked outside while 80% of cows in intensive system were milked from stanchions.

Table 3. Management practices employed by dairy farmers in the study area

Management practice	Intensive (n=10)		Extensive (n=63)		Total (n=73)
	Number of respondents	%	Number of respondents	%	
Mastitis detection					
Yes	6	60.0%	43	68.3%	67.1%
No	4	40.0%	20	31.7%	32.9%
If yes how					
CMT	3	30.0%	2	3.2%	6.8%
Strip cup	0	0.0%	1	1.6%	1.4%
Milk checks for abnormal appearance	3	30.0%	40	63.5%	58.9%
If no, why					
Lack of knowledge	3	30.0%	17	27.0%	27.4%
Lack of screening materials	1	10.0%	1	1.6%	2.7%
No mastitis cases	0	0.0%	2	3.2%	2.7%
Mastitis control					
Yes	10	100.0%	52	82.5%	84.9%
No	0	0.0%	11	17.5%	15.1%
If yes how					
Cow hygiene	1	10.0%	7	11.1%	11.0%
Dry cow therapy	0	0.0%	4	6.3%	5.5%
Use of teat dips	2	20.0%	0	0.0%	2.7%
Treatment of clinical cases	7	70.0%	41	65.1%	65.8%
If no, why					
Lack of knowledge	0	0.0%	11	17.5%	15.1%
Milking technique					
Hand milking	10	100.0%	63	100.0%	100.0%
Milking machine	0	0.0%	0	0.0%	0.0%
Milking place					
Open space	2	20.0%	63	100.0%	89.0%
Milking from stanchion/tie stalls	8	80.0%	0	0.0%	11.0%

3.3.2. Prevalence of subclinical mastitis and isolated bacteria

Tables 4 and 5 give the prevalences of subclinical mastitis (SCM) and isolated bacteria. The overall SCM prevalence at cow level was 50.4% (62/123) (Table 4); prevalence being higher in Rubavu district (intensive system) 62.3% (38/61) than in Nyabihu district (extensive system) 38.7% (24/62). However, the differences between the two farming systems were not statistically significant, ($p=0.087$, CI=95%). In the current study systems, there was variation in hygienic standards of dairy environment and milking conditions as the cows were maintained in dirty and wet areas (in intensive system) which favors proliferation and transmission of mastitis-causing organisms.

Table 4. Subclinical mastitis prevalence in relation to production system

Area (District)	Production system	Number of tested cows	Number of CMT Mastitis Positive	Number of CMT Mastitis Negative	% Mastitis positive	P-value
Rubavu	Intensive	61	38	23	62.3	0.087
Nyabihu	Extensive	62	24	38	38.7	
Total		123	62	61	50.4	
Overall Prevalence					50.4%	

CMT: California Mastitis Test

Table 5. Prevalence of bacterial agents isolated from CMT positive (subclinical mastitis) milk samples

Bacterial isolates	Number of isolates (n=68)	Prevalence
<i>Staph. aureus</i>	14	20.6%
CNS	35	51.5%
<i>Bacillus</i> spp	7	10.3%
<i>Str. agalactiae</i>	4	5.8%
Other streptococci	7	10.3%
<i>E. coli</i>	1	1.5%

Staph. - *Staphylococcus*

CNS: coagulase negative staphylococci

Str. - *Streptococcus*

E. - *Escherichia*

From a total of 62 composite SCM positive samples cultured, 68 bacterial isolates were recovered (Table 5); 6 samples containing a mixture of *Staph. aureus* and CNS; the other 56 samples yielded single bacterial types. In this study, the most predominant bacteria were CNS at 51.5% (35/68) followed by *Staph. aureus* at 20.6% (14/68). *Streptococcus* species other than *Strep. agalactiae* 10.3% (7/68), *Bacillus* spp 10.3% (7/68), *Strep. agalactiae* 5.8% (4/68) and the least was *E. coli* 1.5% (1/68). Figures 5 and 6 demonstrate bacterial isolation from milk samples.



Figure 5. Bacterial isolation from milk samples

A: Culturing milk samples on blood agar media

B: Primary growth after 24h incubation

C: Pure cultures from primary culture, after 24 h incubation

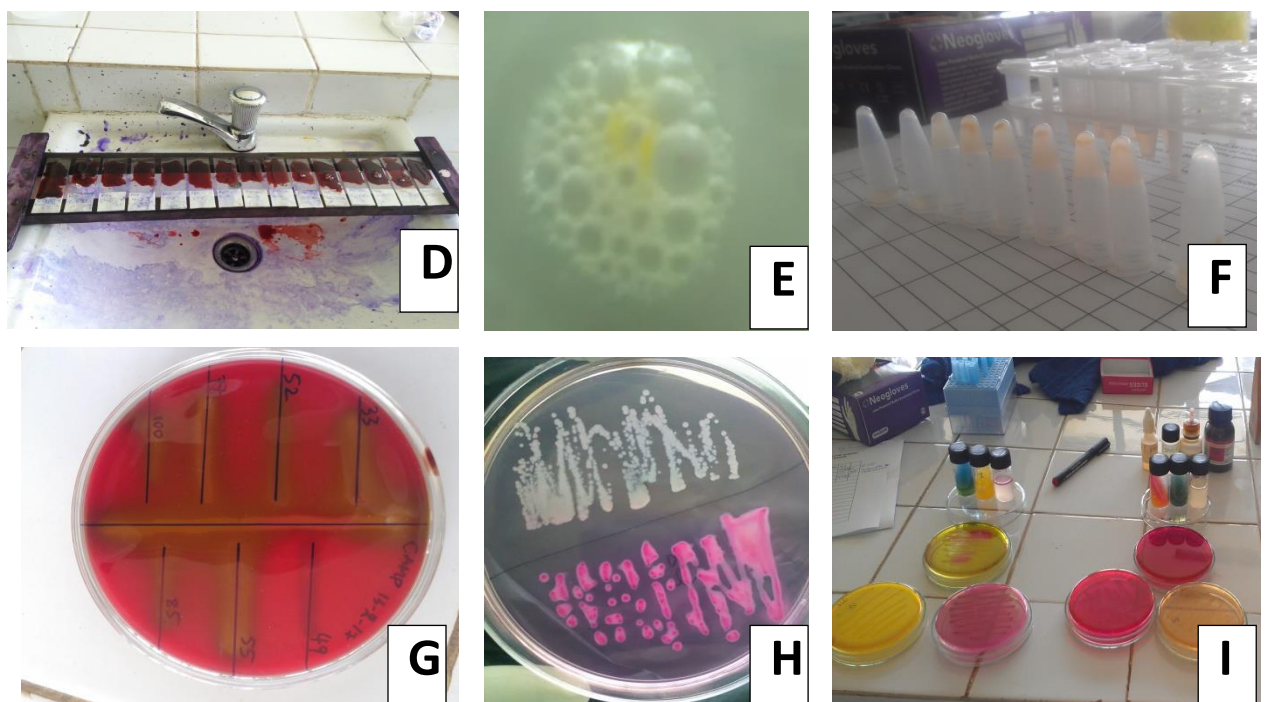


Figure 6. Bacterial identification

D: Gram staining procedure

E: Catalase test (catalase positive isolate)

F: Coagulase test for the confirmation of *Staphylococcus aureus*

G: CAMP Test for *Streptococcus* spp.

H: MacConkey Agar Plates showing a Lactose fermenter (Pink) and a Non-lactose fermenter (Pale) isolate

I: IMViC Tests and some selective media for gram negative bacteria identification

3.4. Discussion

The results from this study show a high prevalence of SCM. A possible explanation for this finding could be that most farmers in the study area were not practicing proper farming management and screening for mastitis at earlier stage. Results from the survey have revealed that only 6.8% of the farmers screened for SCM using CMT, while 32.9% did not screen for mastitis. Others (58.9%) only observed appearance of clinical signs which is difficult in SCM. This could also be supported by the fact that 97.3% of farmers in the study area did not practice teat dipping.

The current prevalence rate corroborates with those reported in recent studies: 51.8% in Rwanda (Iraguha *et al.*, 2015), using electrical conductivity; 51.6 %, in Tanzania (Mdegela *et al.*, 2009) and Ethiopia (Abebe *et al.*, 2016), both using CMT to screen for SCM at cow level. It was also similar to those reported from other countries: 49.5%, 51.8% and 52.4% in South Wales in Australia (Plozza *et al.*, 2011), in Bangladesh (Tripura *et al.*, 2014) and in Uruguay (Giannechini *et al.*, 2002), respectively, all using CMT. However, this reported SCM prevalence was lower than those reported in recent studies in East Africa; 86.2%, 64% and 59.2%, in Uganda (Abrahmsén *et al.*, 2014), in Kenya (Mureithi *et al.*, 2016), and in Ethiopia (Abebe *et al.*, 2016), respectively who also used CMT to screen for SCM at cow level and 88.6%, by Östensson *et al.* (2013) in Vietnam who used SCC. These differences seem to be due to different screening methods used as in the case of Östensson *et al.* (2013) and the breed of animal used; as in the case of Mureithi and Njuguna (2016) – 62.8% of their study animals were Friesian and Jersey; while,

in the current study, all cows were crossbreeds which are less prone to mastitis than exotic breeds (Kurjogi *et al.*, 2014).

In contrast, the prevalence of SCM reported in the current study was 16.3% higher than the prevalence reported by Ondiek *et al.* (2013) in Njoro District of Kenya, 41.0% by Ayano *et al.* (2013) in Ethiopia, 42.5% by Hashemi *et al.* (2011) in Iran, 28.5% by Kayesh *et al.* (2014) in Bangladesh, all of which used CMT test. These differences could be due to farming systems, management practices and cow breeds; for instance, Ondiek *et al.* (2013) in Kenya screened for SCM on cows reared in paddocks where animals were grazed on green pastures. This is also supported by Barrett *et al.* (2005) in UK who found that grass-based herds were less exposed to environmental bacteria, hence less prevalence of subclinical mastitis. On the other hand, Kayesh *et al.* (2014), using CMT, found a low SCM prevalence because 74% of their study animals were local breeds (zebu) which are less prone to mastitis (Kurjogi *et al.*, 2014).

According to Lévesque (2004), herd mastitis prevalence of 40% or over must sound an alarm to the producer; hence, this study reveals how serious mastitis is the problem in the dairy industry sector of Rwanda; it requires attention. Pre- and post-milking teat disinfection has been recommended as an important procedure to prevent prevalence and incidence of mastitis (Sampimon *et al.*, 2008). However it is not practised in any of the farms in the current study.

The distribution of CNS as the most predominant bacteria isolated from the CMT positive samples, followed by *S. aureus* and *Streptococcus* species in this study, is supported by Thorberg (2008) who found that the majority of the positive subclinical cases were associated with CNS organisms. In a similar way, Hogan and Smith (1997) found the CNS, coagulase positive staphylococci (CPS; *Staph. aureus*), the environmental streptococci and coliforms as the prevalent mastitis pathogens associated with SCM in lactating cows.

The predominance of CNS in SCM in this study is also in line with the findings of Cervinkova *et al.* (2013) in Czech Republic, Abrahmsén *et al.* (2014) and Björk *et al.* (2014) in Uganda and Lim *et al.* (2007) in Canada. The high predominance of CNS in the current study areas can be explained by poor milking hygienic practices in the farms and lack of routine mastitis screening

tests. These provide an opportunity for the CNS to invade the udder and develop into an intramammary infection. It is also stated that staphylococcal mastitis is the most common form of contagious mastitis and these organisms are spread from infected to clean cows on hands or equipment from one udder to another (Bagley, 1997).

Coagulase negative staphylococci (CNS) are considered to be teat skin opportunists that normally reside on the teat skin and cause mastitis via ascending infection through the teat canal (Radostits *et al.*, 2007). However, recent reports suggest that CNS have become the most common bovine mastitis isolates in many countries and could therefore be described as emerging mastitis pathogens (Wilson *et al.*, 1997; Taponen, 2008; Pyorala and Taponen, 2009).

Being a contagious pathogen (Jones *et al.*, 1998), *Staph. aureus* prevalence rate could be associated with poor milking hygiene and lack of teat dipping in the current study. It has been reported that *Staph. aureus* has adaptive mechanisms that allow it to survive on the udder and cause intra-mammary infections during milking processes (Radostits *et al.*, 1994). In some studies, *Staph. aureus* are the second most prevalent pathogens, while in other studies the environmental mastitis pathogens are more prevalent (Fox, 2009). As reported by Iraguha *et al.* (2015) in Eastern Rwanda, coliform bacteria were mostly isolated from SCM positive milk samples. It should, however, be noted that Iraguha's study was carried out during dry season (where there was contamination by soil and fecal matter) whereas the current study was conducted during the short rainy season.

Although environmental streptococci (10.3%) were ranked third followed by *Strept. agalactiae* (5.8%) in the current study, Östensson *et al.* (2013) in Vietnam reported *Strept. agalactiae* as the most predominantly (21%) isolated bacteria. Similar findings have been reported by Hegde *et al.* (2013) who found *Streptococcus* spp. ranked the second among all isolates from subclinical mastitis at a rate of 26.3%. These findings are also in line with those reported by Björk, (2013) in Uganda, who found *Strept. agalactiae* at 8.4% in subclinical mastitis. *Strept. agalactiae* has been associated with subclinical mastitis; it can also cause clinical mastitis (Hegde *et al.*, 2013).

Although *Bacillus* organisms have been reported to be an uncommon cause of mastitis in cattle (Parkinson *et al.*, 1999), and affected animals express acute to gangrenous form of mastitis (Logan, 1988), this species has been reported in the current study at a slightly high rate. This could be explained by the poor hygienic conditions of milkers in the study area. It has been found that *Bacillus* organisms are widely distributed in nature and most species exist in soil, water, dust, air, faeces and on vegetation (Gonzalez, 1996). Therefore, *Bacillus* organisms should be considered as a cause of intra-mammary infection in a cow with high SCC or clinical signs of udder disease, otherwise the presence of few *Bacillus* colonies on blood agar would be expressed as contamination (Gonzalez, 1996).

Findings of the current study have shown a low prevalence of *E. coli*, though the study was conducted during the rainy season; case which should depict the opposite findings as coliforms are environmental bacteria associated with wet and muddy conditions (Hogan *et al.*, 2003). However, the same authors have reported coliform bacteria to be of more importance in clinical mastitis (CM) than in subclinical mastitis (SCM), despite the environmental factors. This confirms that the prevalence of coliform bacteria in this study would be low, as the selection criteria identified cows with SCM as opposed to CM. On the other hand, coliforms have been confirmed to be commonly involved in CM characterized by a rapid onset associated with acute and peracute forms (Quinn *et al.*, 2011, NMC, 2010) and rarely cause SCM (Björk, 2013). These findings corroborate with those reported in Tanzania, whereby Kivaria *et al.* (2007) found that SCM was associated with coliforms at 4.1%. However, it has been found that chronic and subclinical infections occur and recurring infections with *E. coli* may be more common than previously thought (Bradley *et al.*, 2001) and could also be associated with immune-depressed animals (Kremer *et al.*, 1990).

This study also revealed that subclinical mastitis prevalence was high in intensive system (62.3%) and lower in extensive system (38.7%). These findings corroborate with those of Biressaw and Tesfaye, (2015), Kebebew and Jorga, (2016) and Sanotharan *et al.* (2016), who reported that mastitis prevalence was higher in intensive systems. This could be attributed to the fact that, in

intensive systems, cows are crowded together, hence the environmental bacteria tend to be concentrated; requiring high hygienic practices to be adhered to in order to lower their concentration. In the current study systems, there was variation in hygienic standards of dairy environment and milking conditions as the cows were maintained in dirty and wet areas (in intensive system) which favors the proliferation and transmission of mastitis-causing organisms. However, Iraguha *et al.* (2015) in the same country found a high prevalence in extensive system; this can only be explained by the observation that their study was conducted in a Savannah vegetation which may expose cows to teat damage and hence further predispose the teats to mastitis infections.

Although we did not perform linear regression on the risk factors associated with subclinical mastitis, the high prevalence recorded in the intensive system can be explained by the fact that in that area, cows were kept in dirty conditions. Combined with lack of mastitis control strategies, it is clear that mastitis causing bacteria were more comfortable in these conditions. These findings are in agreement with those by Iraguha *et al.* (2015) who found that mastitis was more associated with animal cleanliness, production system as well as other management practices.

CHAPTER 4: ASSESSMENT OF BACTERIAL CONTAMINATIONS AND ASSOCIATED RISK FACTORS ALONG THE MILK MARKET CHAIN IN NORTH-WESTERN REGION OF RWANDA

4.1. Introduction

Microbial contamination in milk may result in milk-borne diseases to humans while others are known to cause milk spoilage (Ngasala *et al.*, 2015). Microbial contamination of milk may be primary (from infected lactating cows), secondary (from milkers during milking, milk handlers during transportation and storage, milk handling procedures, water used in cleaning, unclean utensils and/or milking equipment, or tertiary (occurring mainly due to re-contamination of milk after processing due to unhygienic conditions, improper handling and poor storage of milk before serving) (Parekh *et al.*, 2008). Rwanda adheres to the Common Market for Eastern and Southern Africa (COMESA) milk grading system, which is based on bacterial count as follows: Grade I or A: $<2 \times 10^5$ cfu/ml; Grade II or B: $2 \times 10^5 - 1 \times 10^6$ cfu/ml and Grade III or C: $1-2 \times 10^6$ cfu/ml (COMESA, 2006). While most of microorganisms that are associated with milk spoilage are killed by pasteurization process (Goff *et al.*, 1989), some other bacteria which are capable of causing spoilage, like *Pseudomonas spp.* can produce proteolytic and lipolytic extracellular enzymes which are heat stable (Kaiza, 2011). This study was conducted to establish microbial contamination and assess factors and management practices associated with bacterial contamination along raw milk value chain in three districts (Musanze, Nyabihu and Rubavu) of Rwanda.

4.2. Materials and methods

4.2.1. Study area

This was as given in Section 3.2.1

4.2.2. Study design

This was a cross-sectional study where milk samples from various collection points (dairy farmers, milk kiosks/restaurants, milk collection centres and milk hawkers) were aseptically

collected and tested for bacterial load and type. Bacterial load (total viable bacterial count) was determined using dilution method (Black, 2011); bacterial type (isolation and identification) was determined using standard microbiological techniques (Quinn *et al.*, 2011). A structured questionnaire (Hyera, 2015) was used to collect information on age, sex and level of education of the interviewees, storage and handling of the milk at the different points. Observational assessment was also made on the hygienic status of the premises and the milk handling equipment (containers, type of the floors of the MCC and milk kiosks) and handler(s). These factors were statistically assessed to identify their possible association with bacterial contamination of raw milk along the market. Sampling was also based on convenience and willingness of the involved parties.

4.2.3. Sample size calculation

A formula by Kothari, (2004) for unknown population was used to calculate the sample size for this study (this is applicable because the population is infinite), where Z, is the estimated standard variation at 95% confidence interval (CI) which was considered the point of the normal distribution corresponding to the level of significance (Z=1.96). Standard deviation (SD) estimated at 0.25 or 25% and e, the estimated error, considered at 0.05 or 5%.

Therefore, the sample size ‘n’ calculated as:

$$n = \frac{Z^2 \times SD^2}{e^2} = \frac{(1.96)^2 \times (0.25)^2}{(0.05)^2} = 96$$

4.2.4. Sampling method

Depending on the structure of the study area, a multistage sampling method was used to select sixty-seven (67) stakeholders of the milk value chain as applied in Rwanda. These involved thirty-six (36) dairy farmers, fifteen (15) milk hawkers, twelve (12) milk kiosks and four (4) MCCs. The sampling procedure was as given in the schematic presentation of raw milk commodity in the value chain; Figure 7 .

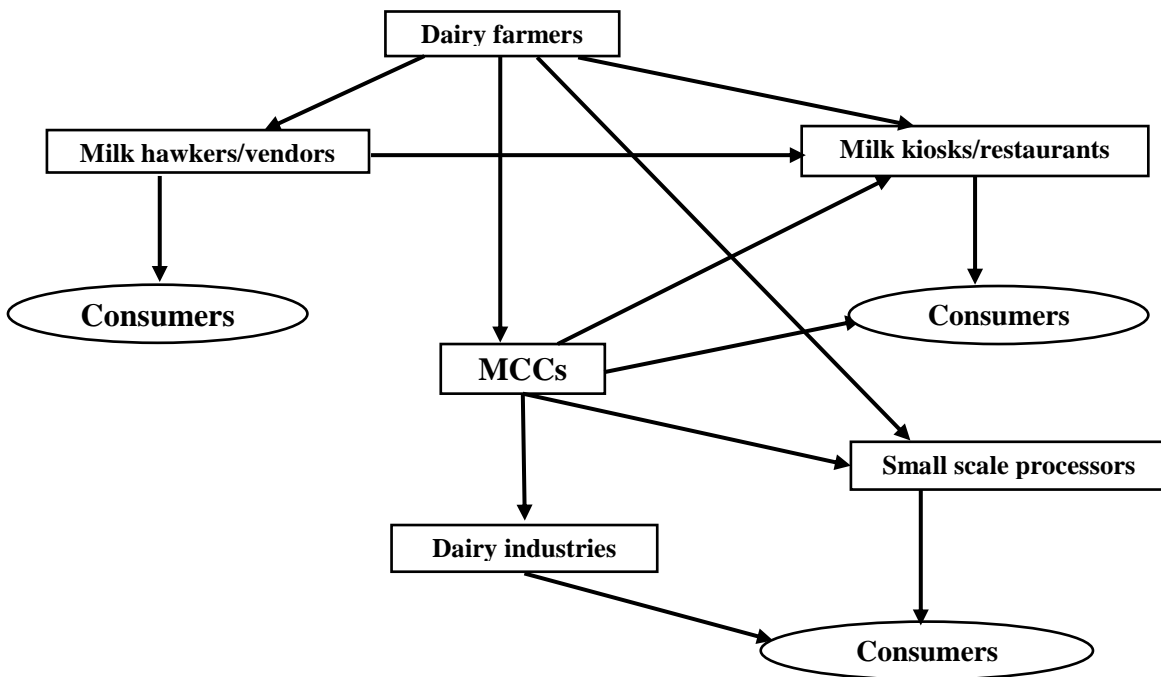


Figure 7. Raw milk value chain in the study area

Farmers were conveniently sampled, with the following inclusion criteria: a smallholder farmer - man and woman, with lactating cows during the study period, willing to participate in the study and whose milk was supplied to any milk collection centre (MCC). Four (4) MCCs were chosen based on their accessibility, being the ones collecting milk from the selected farmers. Vendors, milk kiosks were chosen based on their ability to get milk from the selected MCCs. Milk hawkers were chosen based on their willingness and availability to participate in the study.



Figure 8. Milk testing (A) and cooling (B) at MCC

Dairy farmers were defined as individual cow owners who, immediately after milking, took the milk to the MCC for quality testing and cooling (Figure 8). Milk hawkers/vendors were defined as those who collected milk from different farms and sold it directly in public places or carried it over long distances, distributing it to customers (individual consumers, kiosks/restaurants). A milk collection centre was defined as a place with milk quality testing equipment and cooling facilities, where milk from different dairy farmers and milk transporters within the same location was gathered and cooled before sale. Milk kiosk/restaurant was defined as a safe/certified commercial place where milk was sold either as fresh-raw, skimmed or mixed with tea (African tea) (MINAGRI, 2016).

4.2.5. Milk sample collection and handling

A total of sixty-seven samples were collected from four different levels of the value chain. Of these, 36 were from dairy farmers (26 in Nyabihu and 10 in Rubavu district); 15 from milk hawkers; 7 from milk vendors in Byangabo city (3 in Musanze town and 5 in Rubavu town); twelve (12) from milk kiosks (4 in Nyabihu, 5 in Musanze town and 3 in Rubavu town); four (4) from MCCs (CEMO and CEZONYI in Nyabihu, CODERU in Rubavu and IWACU ZIRAKAMWA in Musanze).

Figure 9 describes the main points where milk samples were collected from. Briefly, milk samples from dairy farmers and MCCs were collected in the morning. This was because local farmers normally milk and bring the milk to the MCC in the morning hours. However, milk samples from milk hawkers and restaurants were collected in the afternoon hours because hawkers normally collect milk from different farmers during the morning hours and supply to the restaurants in the afternoon hours. At the dairy farmer's level, milk was sampled from the bulked milk containers prior to carrying it to the MCC. At the MCC level, milk was sampled from the bulk tank during cooling. At the hawker's level, milk was sampled from milk containers at the selling point (public road, market, etc.) whereas at the kiosks/restaurants level, milk samples were collected from previously boiled and cooled milk, ready to be served.

At each stage, 15 ml of milk was aseptically collected into sterile labelled tubes, kept in a cool box and then taken to the University of Rwanda, Busogo campus Microbiology Laboratory within 1-2 hours, for further analysis.



Figure 9. Milk sample collection at different points of the raw milk value chain in the study area

A & B: Milker with dirty clothes milking with hand

C: Milk hawker selling milk on the street

D: Milk sample collection from milk hawkers on the street

E: Farmers delivering their milk at MCC in plastic Jerry cans

4.2.6. Total bacterial counting

Total bacterial count was performed as described by Richardson (1985). The standard plate count (SPC) agar (Oxoid-CM0325, UK) was cooled to 50°C before 15-20 ml volumes were poured onto sterile petri dishes and left to solidify. Ten-fold dilutions of the milk samples were prepared,

up to a dilution of 10^{-7} , using sterile peptone water (Oxoid-CM0509, UK) and sterile test tubes; mixing done using electronic vortex. A standard volume (1 ml) of milk sample was poured in duplicate onto the solid agar, prepared earlier; this was then incubated at 37°C for 48 hours. Colony count was determined using electronic colony counter (Bioblock Colony Counter 5097, Taiwan), focusing mainly on plates containing 30-300 colony forming units. The bacterial concentration (colony forming units) in the respective original milk sample was then calculated using the formula given by Richardson (1985).

$$\text{cfu/ml} = \frac{\text{Average count (number of colonies)}}{(\text{Dilution plated}) \times (\text{Volume plated})}$$

4.2.7. Bacterial isolation and identification

This was as given in Section 3.2.7

4.2.8. Data management and statistical analysis

All data collected through questionnaire and/or observation were encoded into excel spreadsheet, Microsoft Excel, 2016. Means of bacterial counts at different levels of the value chain were compared using ANOVA. The Least Significant Difference (LSD), Levene's test (Page *et al.*, 2003), Welch test (Welch, 1951), and Games-Howell post-hoc test (Games *et al.*, 1976) were performed to determine homogeneity of variance and overall statistical significance. Association between farming/milk handling practices with milk quality was determined using Chi-square, while strength of association was determined using Cramer's V test. The interpretation of Cramer's V test was described as follows: a value of less than 0.5 is considered to be a weak association, range of 0.5-0.69 is a moderate strength and 0.7 or larger is a strong association (Cramér, 1946). Distribution of microorganisms invading milk at different levels of the raw milk chain was performed using descriptive statistics.

4.3. Results

4.3.1. Total bacterial counts

Total bacterial count (TBC) was interpreted according to COMESA milk grading standards (COMESA, 2006). The current findings revealed that the mean TBC at dairy farmers' level was 1.2×10^6 cfu/ml ($SE \pm 2 \times 10^5$); at milk hawkers' level, it was 2.6×10^7 cfu/ml ($SE \pm 8.5 \times 10^6$); while at MCC's and kiosks/restaurants' levels, it was 1.5×10^6 cfu/ml ($SE \pm 2.6 \times 10^5$) and 6.9×10^6 cfu/ml ($SE \pm 1.8 \times 10^6$) (Table 6).

Table 6. Summary of bacterial count (in cfu/ml) and mean correlation between milk hawkers and other levels of the raw milk chain

Source of samples	No. of samples	Mean counts	D.F	SE	<i>P-value</i>
Dairy farmers	36	1.2×10^6	35	2×10^5	<0.001
MCCs	4	1.5×10^6	3	2.6×10^5	0.008
Kiosks	12	6.9×10^6	11	1.8×10^6	0.003
Milk hawkers	15	2.6×10^7	14	8.5×10^6	-

MCC: Milk Collection Centre

D.F: Degrees of freedom

SE: Standard Error of the Mean

Statistically, there was a significant difference ($p < 0.001$) in the mean values among the four levels of the value chain considered. There was also significant difference in TBC mean values between milk hawkers, dairy farmers ($p < 0.001$), MCC ($p = 0.008$) and kiosks ($p = 0.003$), and between dairy farmers and kiosks ($p = 0.044$). There was no significant difference observed between dairy farmers and MCC ($p = 0.975$) and between MCC and kiosks ($p = 0.551$).

Based on COMESA milk grades, it was found that all tested hawker's milk ($n = 15$) and 10/12 (83.3%) of the milk samples collected from kiosks were above COMESA standard ($> 2 \times 10^6$ cfu/ml) (Table 7). This study also showed that 3/4 (75%) of the milk samples collected from MCC were within COMESA Grade III/C ($1-2 \times 10^6$ cfu/ml) whereas 15/36 of the milk samples from dairy farmers were within COMESA Grade I/A ($< 2 \times 10^5$ cfu/ml).

Table 7. Quality grade of milk samples tested on the basis of bacterial load (TBC)

Source of samples	No. of samples	Milk quality grade			Above COMESA standards
		Grade I or A	Grade II or B	Grade III or C	
Dairy farmers	36	15 (41.7%)	6 (16.9%)	8 (22.2%)	7 (19.4%)
MCCs	4	0	0	3 (75%)	1 (25%)
Kiosks	12	2 (16.7%)	0	0	10 (83.3%)
Milk hawkers	15	0	0	0	15 (100%)

Grade I or A: $<2 \times 10^3$ cfu/ml
 Grade II or B: $2 \times 10^5 - 1 \times 10^6$ cfu/ml
 Grade III or C: $1 - 2 \times 10^6$ cfu/ml
 TBC – Total bacterial count
 MCC – Milk collection centre

Figure 10 demonstrates bacterial counting using electronic counter and record keeping.

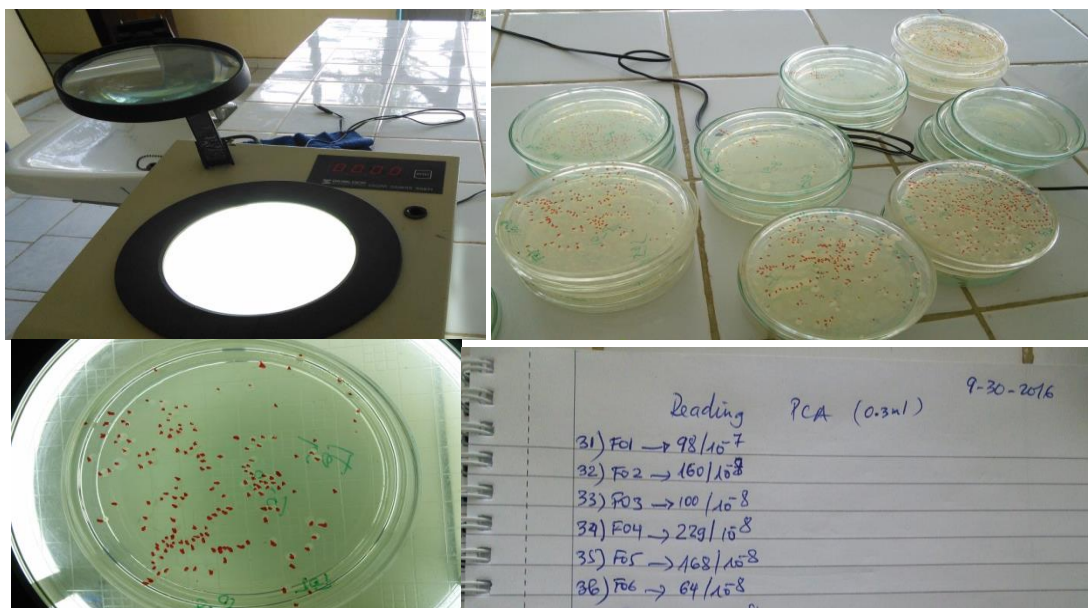


Figure 10. Bacterial counting using electronic counter and record keeping

4.3.2. Isolated bacteria

Of the microorganisms which contaminated milk at different levels of the raw milk chain, coliforms were the most predominant at 34.4%. With respect to individual bacteria, *E. coli* (18; 26.9%) was the most predominant isolate followed by *Salmonella* serotype (11; 16.4%), *Streptococcus* spp (11; 16.4%), coagulase negative staphylococci (10; 14.9%), *Enterobacter* spp.

(5; 7.5%), *Bacillus* spp. (5; 7.5%), *Staphylococcus aureus* (5; 7.5%) and *Yersinia enterocolitica* (2; 3%) (Table 8).

Table 8. Prevalence by bacterial isolates

Bacterial isolates	(n=67)	Isolation per collection points				Prevalence
		Farms	MCCs	Kiosks	Milk hawkers	
<i>Escherichia coli</i>	18	4 (22.2%)	1 (5.6%)	4 (22.2%)	9 (50.0%)	26.9%
<i>Streptococcus</i> spp.	11	5 (45.5%)	2 (18.2%)	0	4 (36.4%)	16.4%
<i>Salmonella</i> spp.	11	2 (18.2%)	1 (9.1%)	18.2%	6 (54.5%)	16.4%
CNS	10	4 (40.0%)	3 (30.0%)	0	3 (30.0%)	14.9%
<i>Bacillus</i> spp.	5	3 (60.0%)	2 (40.0%)	0	0	7.5%
<i>Enterobacter</i> spp.	5	0	0	20.0%	4 (80.0%)	7.5%
<i>Staphylococcus aureus</i>	5	3 (60.0%)	0	0	2 (40.0%)	7.5%
<i>Yersinia enterocolitica</i>	2	0	0	0	2 (100.0%)	3.0%

CNS – coagulase negative staphylococci

MCCs – milk collection centres

Bacterial isolates were distributed across the market chain in such a way that high prevalence rates were recorded at milk hawkers for gram negative bacteria (Table 8). This predominance can be partially explained by the fact that milk hawkers store their milk at ambient temperatures for a long time before they reach their customers.

4.3.3. Factors associated with bacterial contamination at farm level

This study also investigated factors and milk handling practices associated with increased TBC mean values at dairy farmer's level (Table 9). Results indicated the following: (1) there were lower TBC values in milk from cows kept under intensive system than from those kept in extensive system, (2) milk from cows milked from cow sheds showed lower TBC values than from those milked in open space, (3) milk supplied to MCC immediately after milking had lower bacterial counts than that which was kept for more than one hour after milking, (4) milk which was transported from farms to MCC in aluminum containers had lower TBC mean values than milk transported in plastic jericans/containers, (5) farmers who cleaned their utensils (milking and milk transport equipment) five to eight hours before milking delivered milk that had lower bacterial counts than those who cleaned one to two hours before milking, (6) farmers who used

tap water to clean utensils had reduced bacterial contamination of their milk than those who used stream water, and (7) farmers who used warm water with disinfectant (soap) to clean utensils had milk with lower bacterial contamination (TBC mean values) than those who used cold water with disinfectant (soap) (Table 9).

Table 9. Illustration of factors influencing milk quality on dairy farms: number and percentage of milk samples showing contamination at stated grades

Selected factors		Milk grades			
		Grade I	Grade II	Grade III	Above COMESA Standards
Production system	Intensive (n=10)	10 (100%)	0	0	0
	Extensive (n=26)	6 (23.1%)	6 (23.1%)	8 (30.8%)	6 (23.1%)
Milking space	Open (n=26)	6 (23.1%)	6 (23.1%)	8 (30.8%)	6 (23.1%)
	Kraal (n=10)	10 (100%)	0	0	0
Time to supply to MCC	Immediately after milking (n=34)	16 (47.1%)	6 (17.6%)	7 (20.6%)	5 (14.7%)
	One (1) hour after milking (n=2)	0	0	1 (50%)	1 (50%)
Containers for milk transport	Aluminum (n=13)	13 (100%)	0	0	0
	Plastic (n=23)	3 (13%)	6 (26.1%)	8 (34.8%)	6 (26.1%)
Milk containers cleaning time	Five to eight hours before milking (n=24)	16 (66.7%)	5 (20.8%)	3 (12.5%)	0
	One to two hours before milking (n=12)	0	1 (8.3%)	5 (41.7%)	6 (50%)
Source of water for cleaning containers	Tap water (n=19)	15 (78.9%)	2 (10.5%)	1 (5.3%)	1 (5.3%)
	Stream water (n=17)	1 (5.9%)	4 (23.5%)	7 (41.2%)	5 (29.4%)
Type of water used in cleaning	Warm water only (n=17)	1 (5.9%)	5 (29.4%)	6 (35.3%)	5 (29.4%)
	Warm water with disinfectant (soap) (n=17)	15 (88.2%)	2 (11.8%)	0	0
	Cold water with disinfectant (Soap) (n=3)	0	0	2 (66.7%)	1 (33.3%)

Grade I or A: $<2 \times 10^3$ cfu/ml

Grade II or B: 2×10^5 - 1×10^6 cfu/ml

Grade III or C: 1 - 2×10^6 cfu/ml

MCC: Milk collection centre

When statistically evaluated, it was revealed that bacterial contamination of milk was significantly and strongly associated with containers used for milking ($p < 0.0001$), milk containers cleaning time ($p < 0.0001$) and source of water used to clean containers ($p < 0.0001$) (Table 10). There was a correlation and moderate strength of association between bacterial contamination of milk and production system ($p < 0.001$), milking space ($p < 0.001$) and type of water used to clean utensils ($p < 0.001$) whereas the study revealed that there was no association between raw milk bacterial contamination and time taken to supply milk to the MCC ($p > 0.328$) (Table 10).

Table 10. Association between milk grade and bacterial contamination risk factors at farm level

Selected factors	Chi-square value	D.F	Significance level (P-Value)	Cramer's V
Production system	17.308 ^a	1	0.001	0.693
Milking space	17.308 ^a	1	0.001	0.693
Time to supply to MCC	3.441 ^a	1	0.328	0.309
Containers for Transport	25.435 ^a	1	0.000	0.841
Milk containers cleaning time	23.813 ^a	1	0.000	0.813
Source of water for cleaning containers	20.034 ^a	1	0.000	0.746
Type of water used in cleaning	28.832 ^a	2	0.000	0.633

4.4. Discussion

The TBC mean values for all the hawkers' milk samples (15/15; 100%) and kiosks/restaurant's samples (10/12; 83.3%) were above COMESA's acceptable levels. Multiple factors affected contamination at hawkers' level including: storage and transport in unclean milk containers, prolonged time for milk storage and uncontrolled temperature along transportation. In the study area, some farmers milked their cows in the morning hours and stored the milk (for about 5 hours) at ambient temperature before milk hawkers collected it for distribution to consumers, milk kiosks and restaurants. Additionally, hawkers often sold milk in the afternoon on public roads or in milk "markets". Indeed, 80% of Rwanda's milk market is designated as "informal"

due to the fact that the milk coming from a majority of small-holder farmers does not enter the regulatory food chain (MINAGRI, 2013).

According to bacterial multiplication and growth curve, at ambient temperature, for example *Escherichia coli* divides into two after every twenty minutes (Harding, 1995); this seems to be the most likely scenario at hawker's level, where milk undergoes prolonged storage time at ambient temperature and *E. coli* isolates were found to be many. Furthermore, bacterial contamination was exacerbated when the milk was stored in unhygienic plastic containers, and when it was subjected to poor handling practises during distribution to different customers. This observation is in agreement with the results of Grimaud *et al.* (2007), when they analysed raw milk marketed through the informal subsector in Uganda. They reported that unhygienic conditions from the production source to the consumer combined with an improper milk storage were associated with increased bacterial contamination.

The findings of the current study corroborate with those obtained by Doyle *et al.* (2015), who recorded TBC mean values at kiosks of 9.8×10^6 CFU/ml; they were slightly higher than 6.9×10^6 CFU/mL obtained in the current study but also above COMESA's acceptable level) in Kigali, Rwanda.

Normally, in the study area, milk sold in kiosks/restaurants is obtained either directly from dairy farmers, or from MCC and/or milk hawkers. It is normally boiled before consumption; served either hot or cold; however it needs to be noted that, even though boiled before consumption, the milk may still harbour pathogenic bacteria which it can transmit to respective consumer(s). Contamination of milk after boiling is another food safety concern highlighted by other scientists (Kilango *et al.*, 2012). This type of contamination can occur through use of storage utensils, storing temperature and hygiene of milk handlers. Kiosks/restaurants in the study area also served unpasteurized milk which was found to be of very poor quality. This could have been due to milk contamination at source, unhygienic milk handling conditions after boiling, inadequate refrigeration and recontamination during milk storage and serving (consumption). While the current study did not evaluate the impact /safety hazards of poor milk quality on human health on

consumption, in the United States, Oliver *et al.* (2009) did so and showed that several documented milk-borne disease outbreaks which occurred within the years 2000–2008 were traced back to consumption of raw unpasteurized milk.

The TBC mean values of MCC's and dairy farmer's milk (1.5×10^6 cfu/ml and 1.2×10^6 cfu/ml, respectively) laid in grade III/C according to COMESA's milk grades. Similar results were found by Doyle *et al.* (2015); they recorded MCC milk samples' TBC mean values of 1.5×10^6 cfu/ml. In this study, it was found that, once milk reached the MCC, it was directly tested via platform tests for organoleptic properties which include pH, fat contents, other solids non fats, density and then cooled in cooling tanks at 4°C (MINAGRI, 2016). The slight increase of the TBC mean values from dairy farmers to MCC, observed in this study, could have been caused by use of contaminated transportation containers and/or time the utensils were cleaned after supplying milk to a MCC. These findings are supported by Grimaud *et al.* (2007) in Uganda (with similar milk handling and transport conditions); they demonstrated increase of bacterial load in milk during transportation.

The highest TBC mean values obtained at dairy farmer's level was found to be associated with the investigated factors and milk handling practices used by farmers in the study area. These include: source of water used to clean containers, milking space, production systems, milking hygiene and cleanliness of milk containers used during milking. This is in agreement with findings of Banwart (1989); he concluded that unhygienic milking utensils are the source of many microorganisms which transform high quality milk to an unacceptable product. The current findings also corroborate with those of Grimaud *et al.* (2007), who during an evaluation of milk quality in Uganda, noted that milk contamination took place as early as at the farm level - beginning of the value chain. They concluded that raw milk contamination along the value chain is associated with storage and milk handling conditions especially during transportation from the primary production area to the urban market place.

In this study, coliforms (*E. coli* and *Enterobacter* spp) were the most isolated bacteria from different levels of the raw milk chain, representing 34.4%. The results are in agreement with

those of Garedew *et al.* (2012) who found *E. coli* (29.6%) to be the most isolated gram-negative staining bacterial pathogen. Adams and Moss (2008) concluded that *E. coli* is the most prominent faecal coliform and that its presence indicates faecal contamination of raw milk and its products; which is in agreement with the findings of the current study. Mellenberger and Roth (2009) also stated that coliform bacteria are normal inhabitants of soil and the intestines of cows. They accumulate and multiply in manure, polluted water, dirt and contaminated bedding. Iraguha *et al.* (2015), from his study in Eastern Rwanda, found that the predominance of coliform bacteria were largely of environmental origin, at farm level. The predominance of coliforms found in the current study seems to be associated with milking practices like: unclean water used during milking and cleaning of milk utensils, poor milker's hygiene, milking space and non-use of teat dips. The coliform contamination at other levels of the value chain (milk hawkers and kiosks) increased largely due to poor hygiene of milk handlers and poorly cleaned utensils used for milk transport. *Salmonella* organisms were found at 16.4% in the current study. These findings corroborate with those found by Lubote *et al.* (2014) in Arusha, Tanzania; they reported prevalence of *Salmonella* organisms at 37.3% of all the bacterial isolates along the raw milk chain. This high prevalence is explained by factors such as: poor animal husbandry and hygienic practices, inappropriate transportation and storage facilities, lack of cooling systems and use of unclean water. The presence of *E. coli* and *Salmonella* organisms is also an indication of faecal contamination by milk handlers as previously reported (Kamana *et al.*, 2014).

The current results have also revealed high prevalence of coagulase negative staphylococci (CNS), at 14.9%. These organisms are commonly considered to be teat skin opportunists that normally reside on the teat skin (Radostits *et al.*, 2007). So, they may contaminate milk during udder washing and milking, which also explains their association with poor milking hygienic practices and non-use of teat disinfectant(s) before milking. Other bacteria isolated in this study were: *Streptococcus* spp at 16.4%, *Bacillus* spp at 7.5% and *Staphylococcus aureus* at 7.5% . These bacteria could originate from mastitic milk (O'Brien *et al.*, 2009).

Although the current study did not go further to establish diseases associated with consumption of raw milk; De Buyser *et al.* (2001) did so when they explored the implication of milk and milk products in food-borne diseases in France and in different industrialized countries. They found that 37.5% of the food vehicles were from raw milk where *Salmonella* organisms were responsible for 29 outbreaks, *Listeria monocytogenes* for 10 outbreaks, pathogenic *E. coli* 11 outbreaks, and *Staphylococcus aureus* 10 outbreaks. Furthermore, Rohrbach *et al.* (1992) reported that 68 of 195 (34.9%) dairy producers in East Tennessee and Southwest Virginia consumed raw bulk-tank milk produced on their farm. Twenty-five percent (17 of 68) of the bulk-tank milk samples were shown to contain *Listeria monocytogenes*, *Campylobacter jejuni*, *Yersinia enterocolitica* and/or *Salmonella*.

CHAPTER 5: ANTIMICROBIAL SUSCEPTIBILITY TESTING OF SELECTED BACTERIA ISOLATED FROM CASES OF SUBCLINICAL MASTITIS AND BULKED MILK SAMPLES

5.1. Introduction

Antimicrobials have been extensively used in both human and veterinary medicine in recent years (WHO, 2012). In veterinary medicine, antimicrobials are used as therapeutic, prophylactic and growth promoters. Mastitis is one of the most serious diseases in dairy industry worldwide (Bradley, 2002) and its treatment is mainly based on the use of antimicrobials (Katholm, 2003). The use of antimicrobials in veterinary medicine should ideally rely on consultations and prescriptions by veterinarians (McEwen *et al.*, 2002), but in many contexts, antimicrobials are used in food animal production with little or no veterinary consultation (McEwen *et al.*, 2002). The rise of antimicrobial resistance (AMR) is becoming a major problem, with respect to treating many serious infections. It also leads to unpredicted impacts on a wide range of medical procedures (Consumers Union, 2012; Woolhouse *et al.*, 2015). Food animals are not only vehicles of transmission for AMR bacteria but are also endpoints in the dissemination, selection, and spread of respective resistance genes (Thanner *et al.*, 2016).

Different methods of screening antimicrobial susceptibility have been developed and may include disk diffusion, agar dilution, E-test (CLSI, 2014), broth microdilution (Burrows *et al.*, 1993) assays and molecular techniques (Tan, 2003). Agar disk diffusion method is considered to be a reliable screening method for AMR in veterinary laboratories (Saini *et al.*, 2011). The Clinical and Laboratory Standards Institute (CLSI) guidelines offer standardized methods and interpretative standards for antimicrobial susceptibility testing for organisms commonly encountered in clinical microbiology laboratories (CLSI, 2014).

So far, no reports exist on AMR in the study area. Therefore, this study was conducted to assess the extent of AMR in bacteria isolated from subclinical mastitis and bulked tank milk samples in Nyabihu, Rubavu and Musanze districts, Rwanda.

5.2. Materials and methods

5.2.1. Study organisms

Samples of the three most prevalent bacterial types isolated from the milk samples tested for subclinical mastitis and contamination along milk market chain were used for this study. Of the 135 isolates recovered, 83 were tested; they included: coagulase negative staphylococci (45), *Escherichia coli* (19) and *Staphylococcus aureus* (19). They were obtained from 62 dairy cows with subclinical mastitis and 67 bulked milk samples from milk value chain (dairy farms, milk collection centres (MCCs), milk hawkers and milk kiosks) (Table 11). According to the source of isolates, CNS and *S. aureus* were most prevalent in subclinical mastitis samples, while *E. coli* were more prevalent from bulk samples (Table 11). The bacterial isolates used were streaked for isolation and stored in 20% glycerol at -20°C until revived for antimicrobial susceptibility testing. Cultures were revived on 5% bovine blood agar and incubated at 37°C for 24 hours.

Table 11. Source of isolates used for antibiotic susceptibility testing

Isolates	Bulked samples	Mastitic samples	Total
CNS	10	35	45
<i>E. coli</i>	18	1	19
<i>Staph. aureus</i>	5	14	19
Total	33	50	83

CNS means coagulase negative staphylococci

E. means *Escherichia*

Staph. means *Staphylococcus*

5.2.2. Antimicrobial susceptibility testing

5.2.2.1. Preparation of Mueller Hinton Agar and bacterial suspensions

Mueller Hinton Agar (MHA) (CM-MHA135, Rapid Labs, UK) plates were prepared according to the manufacturer's instructions. Sterile saline solution (0.85%) was prepared by dissolving 0.85g of NaCl in 100 ml of distilled water and autoclaved for 15 minutes at 121°C; it was used to make

bacterial suspensions. After sterilisation, 2 ml aliquots of saline solution were cooled to room temperature prior to use. Bacterial suspensions were made by homogenising 3-5 well isolated colonies from fresh cultures into 2 ml of sterile saline solution and turbidity adjusted to correspond to 0.5 McFarland standard (CLSI, 2014).

5.2.2.2. Antimicrobial susceptibility testing procedure

Agar Disk Diffusion method as previously described by Bauer *et al.* (1966) and recommended by CLSI (2014) was used. Bacterial suspensions were plated on MHA plates by streaking method using cotton swabs. Briefly, after dipping into the bacterial suspension, the swab was firmly rotated against the side of the tube to remove excess inoculum. The swab was then repeatedly streaked on the MHA medium, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. Ten (10) antimicrobial disks (Rapid labs, UK): Nalidixic acid (NAL) (30 µg), Gentamicin (GEN) (10 µg), Amoxicillin/clavulanate (AMC) (20/10 µg), Penicillin (PEN) (10 µg), Erythromycin (ERY) (15µg), Ceftriaxone (CTR) (30 µg), Doxycycline (DOX) (30 µg), Tetracycline (TET) (30 µg), Ampicillin (AMP) (10 µg) and Ciprofloxacin (CPR) (5 µg) were used. These antibiotic disks were individually applied to the inoculated plates within 15 minutes. Maximum 3-5 disks were applied on each plate and the plate incubated for 18-24 hours. Diameters of the zones of complete inhibition (clear zones) were measured using a ruler and recorded for further analyses. Size of the zone of inhibition was used to interpret the bacterium's susceptibility to the respective antibiotic, graded as susceptible, intermediate and resistant, according to the Clinical Laboratory Standards Institute (CLSI) guidelines (CLSI, 2014). Isolates resistant to three or more antimicrobials were considered to be multi-resistant (Krumperman, 1983).

Figure 11 demonstrates how measuring and recording zones of inhibition was done.

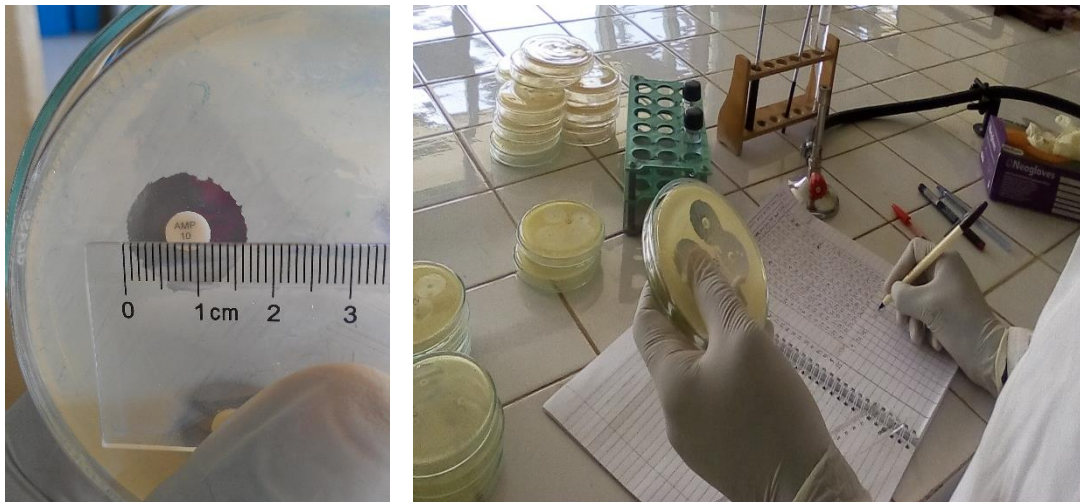


Figure 11. Measuring and recording zones of inhibition

5.2.3. Data management and statistical analysis

Inhibition zone diameters were recorded and statistical analyses were performed to compare the susceptibilities/resistances of isolates from the various market points, using the Statistical Package for Social Sciences (SPSS), IBM SPSS Statistics 23rd version.

5.3. Results

The susceptibility patterns of the three most-isolated bacteria (CNS, *S. aureus* and *E. coli*) were as shown in Figure 12. All of them were resistant to Ampicillin at 48.1%, 73.7% and 73.7%, respectively; CNS and *Staph. aureus* were resistant to penicillin at 57.8% and 73.7%, respectively. All *Staphylococcus* spp tested were resistant to nalidixic acid as they have an intrinsic resistance to nalidixic acid (CLSI, 2014). All isolates tested exhibited a high degree of susceptibility to gentamycin (100%), ceftriaxone (94.7-100%), ciprofloxacin (94.7-100%), doxycycline (93.3-94.7%); however, CNS were less susceptible (60%) to tetracycline compared to *Staph. aureus* (78.9%).

The susceptibility of *E. coli* in regards to penicillin and erythromycin was not interpreted because the guidelines from CLSI did not provide the cut-off values for these antibiotics when tested using disk diffusion method.

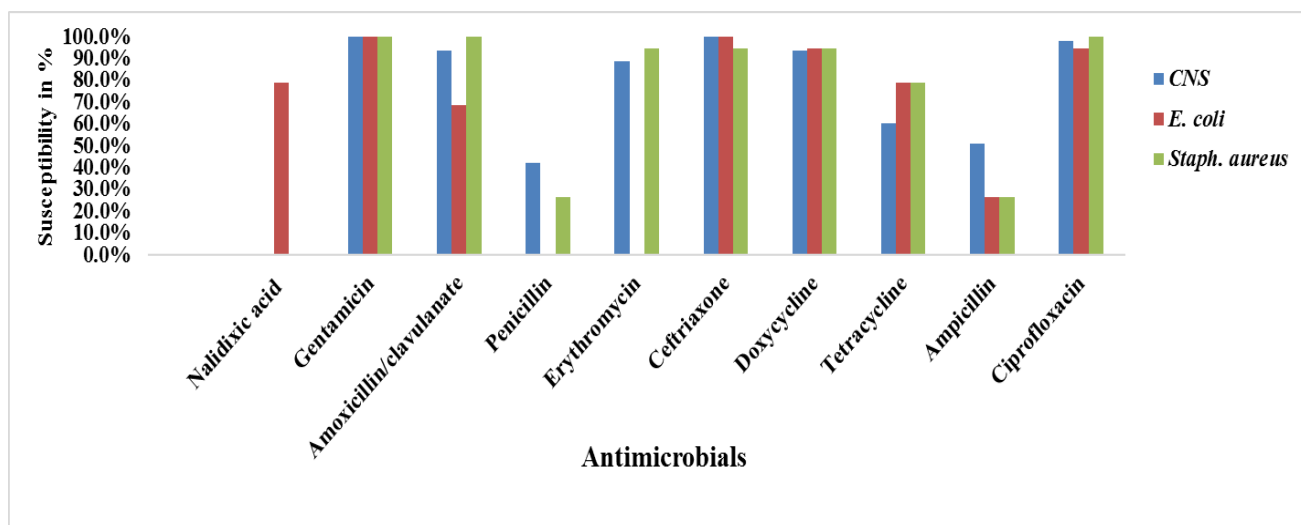


Figure 12. Susceptibility of test isolates in regards to the tested antimicrobials

Results from this study have revealed the presence of multidrug resistant bacteria present in the dairy value chain. Sixteen isolates, out of the 83, expressed multidrug resistance. Additionally, seven *E. coli* isolates showed resistance to at least two antimicrobials. All isolates expressed resistance to Ampicillin while three CNS isolates were resistant to at least 4 antimicrobials (Table 12).

Table 12. Multi-drug resistance patterns exhibited by the test isolates

Antimicrobial resistance patterns	Bacterial isolates (%)		
	CNS (n=3)	<i>Staph. aureus</i> (n=6)	<i>E. coli</i> (n=7)
AMC/AMP	0 (0.0)	0 (0.0)	4 (57.1)
TET/AMP	0 (0.0)	0 (0.0)	2 (28.8)
NAL/AMP	0 (0.0)	0 (0.0)	1 (14.3)
PEN/ERY/AMP	0 (0.0)	1 (16.7)	0 (0.0)
PEN/CTR/AMP	0 (0.0)	1 (16.7)	0 (0.0)
PEN/TET/AMP	0 (0.0)	4 (66.7)	0 (0.0)
AMC/PEN/ERY/AMP	2 (66.7)	0 (0.0)	0 (0.0)
AMC/PEN/ERY/TET/AMP	1 (33.3)	0 (0.0)	0 (0.0)

Key: NAL: Nalidixic acid, GEN: Gentamicin, AMC: Amoxicillin/Clavulanate, PEN: Penicillin, ERY: Erythromycin, CTR: Ceftriaxone, TET: Tetracycline, AMP: Ampicillin
 CNS: Coagulase negative staphylococci, *Staph*: *Staphylococcus*, *E.*- *Escherichia*

5.4. Discussion

The current study has revealed multidrug resistance in some isolates at different rates. Three CNS isolates have expressed resistance to at least four antimicrobials and six *Staph. aureus* isolates showed multidrug resistance to four antimicrobials. Similar antimicrobial resistance patterns have been reported in *Staphylococcus* organisms (Hiramatsu *et al.*, 2014; Neyra *et al.*, 2014). The current study has revealed that all tested microorganisms were susceptible to Gentamicin.

Coagulase negative staphylococci (CNS) showed high susceptibility; 100%, 100% and 97.8% to Gentamicin, Ceftriaxone and Ciprofloxacin, respectively. These findings corroborate with those reported by Reda *et al.* (2014) who reported that *Staphylococcus* organisms isolated in Ethiopia were susceptible to Gentamicin at 100%. However, in this study, CNS have shown susceptibility to tetracycline, ampicillin and penicillin at 60, 51.1 and 42.2%, respectively. The same findings have been reported by Köck *et al.* (2017) who found that CNS were resistant to tetracycline (71%) and penicillin (65%). High resistance could be explained by the fact that the most common drugs used to treat mastitis in Rwanda contain β -lactams such as Biomycin-M, which is a combination of amoxicillin and neomycin. Indiscriminate and prolonged use of these antibiotics in the study area could be contributing to increased pressure and prevalence of bacterial pathogens that are resistant (Sawant *et al.*, 2009). However, there is limited data on AMR occurrence and contributing factors available in Rwanda, compared to other East African countries (Omulo *et al.*, 2015).

Antimicrobial resistant genes can encode β -lactamase, an enzyme that inhibits penicillin and other related antimicrobial (John *et al.*, 2012). Resistance of CNS to tetracyclines is genetically mediated by *tet(K)* genes that encode for efflux of the drug and *tet(M)* genes that encode for decrease of sensitivity of the ribosome to the drug (Archer *et al.*, 1994). However, in this study, some CNS showed low susceptibility to all penicillinase-labile penicillins compared to previous findings of Cuevas *et al.* (2004) who reported a high resistance rate among *Staphylococcus* organisms to ampicillin (73.3%), penicillin (26.7%) and tetracycline (6.7%). In the current study,

CNS have expressed resistance to erythromycin (11.1%); resistance development that has been reported to occur progressively over years (Cuevas *et al.*, 2004).

The current findings have shown *Staph. aureus* to be very susceptible to gentamicin, amoxicillin/clavulanate and ciprofloxacin. However, in the same study, *Staph. aureus* were shown to be resistant to penicillin and ampicillin. These findings corroborate with those of Pillar *et al.* (2014) who reported *Staph. aureus*' resistance to penicillin at 31.2%. These findings are also similar to those reported by Szweda *et al.* (2014) and this could be explained mainly by the fact that *Staphylococcus* organisms produce β -lactamase which inactivate penicillin and ampicillin (Adams *et al.*, 2008).

Escherichia coli usually causes transient intramammary infections in dairy cows. Its presence in milk is considered to be from faecal contamination (Adams and Moss, 2008). Most of the isolates used in the current study were from bulk tank milk samples. It was found that *E. coli* showed the highest resistance rate compared to all other isolates and these findings are supported by Okeke *et al.* (1999) who found that the overall resistance of *E. coli* to the tested antimicrobials was high. The highest resistance rate was recorded for Ampicillin (73.7%) followed by amoxicillin/clavulanate (31.6%). These antibiotics are commonly misused in developing countries as reported by Omulo *et al.* (2015). The findings of this study are similar to those of Fairbrother *et al.* (2015) who reported that *E. coli* organisms isolated from mastitic milk samples were resistant to Ampicillin and Tetracycline (27.8%). Habrun *et al.* (2010) reported that *E. coli* was resistant to oxytetracycline and ampicillin at 98% and 85%, respectively in isolates from environment. The current findings also corroborate with those by Kibret and Abera (2011) who found *E. coli* to be resistant to amoxicillin/clavulanate at 29%. However, the current findings have shown all *E. coli* isolates to be susceptible to gentamicin and ceftriaxone. Clinical and Laboratory Standards Institute has reported comparable susceptibility rates (CLSI, 2014). This could be explained by the fact that these antimicrobials are not commonly used in the study area. Although CLSI guidelines do not provide cut off values of amoxicillin for CNS, *Staph. aureus* and *E. coli*, it has been reported that gram positive bacteria which are resistant to ampicillin also

express the same resistance to Amoxicillin (CLSI, 2014); the two antimicrobials belong to the same amino-penicillin subclass (Omulo *et al.*, 2015).

Additionally, multi-drug resistance (defined as lack of susceptibility to at least two antimicrobials from different classes) was also observed in several bacterial isolates. This indicates that many of the antimicrobials that are used in livestock production are no longer suitable for use in the study area. Many factors could have contributed to this multiple resistance including: antimicrobial concentration, long-term exposure, organism type, antimicrobial type and host's immune status (Shitandi, 2004). Therefore, based on the fact that majority of small-scale farmers have tendency to treat animals without consulting a veterinarian; the unsuitable use of veterinary drugs increases the risk of resistant bacteria in herds. This leads to lack of response to the antimicrobial treatment, which will eventually lead to chronic diseases as well as spread of AMR.

CHAPTER 6: GENERAL DISCUSSION

The current study revealed a high SCM prevalence rate (50.4%). This prevalence is associated with poor farming practices and management. It was noticed that all the farmers in the study do not practice pre-and post- teat dipping. Hand milking technique which is practiced by all farmers in the study area could have also contributed to this high SCM prevalence rate. Different researches have shown the correlation between farming practices, milking techniques and other sanitation procedures and the occurrence of mastitis in dairy cows (Busato *et al.*, 2000). Regular screening by CMT has been recommended as one of the measures to reduce the risk of occurrence of mastitis (Salvador *et al.*, 2014). However, in the current study, only 6.8% of the dairy farmers screened for mastitis using CMT; this has certainly contributed to the high SCM prevalence recorded in the study area. California mastitis test provides a cheap and reliable indirect method to estimate SCC of individual quarters. In a recent study, Royster and Wagner (2015) have recommended CMT as cow-side test for screening SCM over other field diagnostic tests in replacement of the PortaSCC[®] which is considered the best option in screening for SCM under field conditions (Royster *et al.*, 2015).

Based on previous studies, relating the information of the pathogen causing mastitis and SCC history has been shown to be the most important aspect in making decision for an appropriate treatment or management action at cow as well as herd level (White *et al.*, 2006). However, this requires good record-keeping practices, including all the information regarding case history of the cow as well as previous treatment(s) administered (Reksen *et al.*, 2006). In a recent study, mathematical models have been developed to predict the occurrence of mastitis in regards to treatment and have concluded that early culling would reduce the high incidence of mastitis (Grimaud *et al.*, 2009) based on history records. The consequences are linked to low income from milk production, increase in antimicrobial resistance at farm level and occurrence of clinical mastitis cases.

The prevalence and dominance of staphylococcal mastitis in the current study is alarming. *Staphylococcus* is not easy to treat and eliminate from a herd and it is linked to poor milk

handling and milker's hygiene. Even with the best treatment strategies, it will remain in the herd and has grave consequences on cow's survival and health. In an attempt to control staphylococcal mastitis, in developed countries all *Staphylococcus* positive cows were slaughtered (Grimaud *et al.*, 2009). Also, where there is no cold chain for milk delivery, *Staphylococcus* can produce toxins that cause food poisoning. The high prevalence of staphylococcal mastitis, thus threatens survival of the Rwanda dairy industry, and hence should be given priority attention.

The high TBC recorded in milk samples from milk hawkers in the current study is probably due to lack of adequate transport and storage conditions. Similar findings have been reported in Uganda (Grimaud *et al.*, 2009) whereby the researchers found that the increase in TBC was due to lack of adequate infrastructures along the value chain. Findings of this study revealed that bacterial contamination of milk was significantly and strongly associated with containers used for milk transport ($p < 0.0001$), milk containers cleaning time ($p < 0.0001$) and source of water used to clean containers ($p < 0.0001$). The reasons for this increase in TBC could be that a big number of milk producers in the study area did not practice cleaning and disinfection of the milk containers, suggesting lack of required awareness about the importance of keeping the hygienic condition of the milk. These findings are in line with those reported by Szweda *et al.* (2014) who found that in milk production area, besides udder infection and water quality, hygienic behavior with respect to hand washing, container cleaning and disinfection are the key areas that remain of relevance to milk hygiene intervention.

The current results indicated a considerable prevalence of antimicrobial resistant strains among isolated CNS and *Staph. aureus*. Similar findings have been reported worldwide and the highest rate of resistance was detected for β -lactam antimicrobial agents. For example, in a study carried out in Turkey, Szweda *et al.* (2014) found high percentages of resistance against β -lactam antimicrobials. The authors reported that all *Staph. aureus* strains from SCM cases were resistant to penicillin, ampicillin and amoxicillin at 62.1%, 56.3% and 45.6%, respectively. The most probable reason for this particular resistance is that these organisms produce β -lactamase, an enzyme which inactivates β -lactam antimicrobials (Hart *et al.*, 1998).

In the current study CNS has shown high sensitivity to gentamicin, ceftriaxone and ciprofloxacin. These findings are supported by Archer and Climo (1994) who found that *Staphylococcus* spp were susceptible to Gentamicin at 100%. The credible reason could be that these chemicals are newly developed and less used in treatment of mastitis in the study area. However, the present study has demonstrated the tested isolates' resistance to tetracycline, ampicillin and penicillin. The main reason for this could be misuse of these chemicals in the study area. It has been reported that the emergence of antimicrobial resistance in developing countries is related to misuse of antimicrobials, lack of regulation in terms of supply and purchase, self-medication (Shitandi, 2004) to cite few.

CHAPTER 7. CONCLUSIONS AND RECOMMENDATIONS

7.1. Conclusions

- ❖ This study has revealed a high prevalence of subclinical mastitis predominantly caused by *Staphylococcus* spp. It was also noticed that farmers in the study area did not control mastitis and only a few regularly screened for subclinical mastitis.
- ❖ High bacterial contamination of the milk along market chain was recorded and more than 70% of tested milk had bacterial counts of $1-2 \times 10^6$ cfu/ml (grade III according to COMESA's grading system). Milk contamination started at the farm and increased along the market chain and was associated with many risk factors.
- ❖ The overall predominance of resistance for β -lactam antimicrobials is probably the consequence of the fact that they are still one of the most widely used for treatment of bovine mastitis in Rwanda. The results of this research also indicated that gentamicin, ceftriaxone and ciprofloxacin are promising alternative agents to combat staphylococcal infections.

7.2. Recommendations

In view of the conclusions above, it is therefore recommended that:

- 1) Farmers in the study area should be educated on Dairy Dynamic Management (DDM) program which was introduced in Rwanda in 2014, animal health management as well as milk handling practices to ensure clean milk production.
- 2) The level of bacterial contamination observed from this study needs more elaborate studies at the point of production of milk to understand which factor combinations are more responsible for milk spoilage.
- 3) There is need to enforce the implementation of the Ministerial Order (N° 001/11.30 of 10/02/2016) regulating the collection, transportation and selling of milk to ensure the safety and quality of milk at all market nodes with appropriate inspection of milk production facilities with respect to milk quality.

- 4) Small scale milk processing units should be encouraged to cut off the informal market to ensure that all produced milk in the study area goes through cold chain to avoid milk spoilage along the market chain.
- 5) There is a need for governmental regulation and professional oversight for the prudent use of antibiotics and encouragement for regular screening of antimicrobial susceptibility.
- 6) Ceftriaxone, gentamicin and ciprofloxacin should be used in the treatment against mastitis and other staphylococcal infections in animals as well as in humans.
- 7) Further researches are needed to establish the relationship between resistant bacteria and the distribution of genes responsible for that resistance.

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APPENDICES

Appendix 1. Questionnaire for milk producers



Good morning/afternoon, I am **Dr Jean Pierre M. Mpatwenumugabo**, Master’s student from the UNIVERSITY OF NAIROBI, *College of Agriculture and Veterinary Sciences*, Faculty of Veterinary Medicine in the Department of Veterinary Pathology, Microbiology and Parasitology. We are conducting a research on “**Risk factors associated with subclinical mastitis and bacterial contamination of cow milk along the market chain and antimicrobial susceptibility patterns of isolates in Rwanda**”. The results from this study will help us to identify the negative effects of mastitis on milk production and provide the main solutions to prevent these losses. I would like to ensure you that any information provided will be used for research purpose and kept confidential.

GENERAL INFORMATION

Date of interview:.....

1. Questionnaire number:.....
2. Farm code:.....
3. Province:.....
4. District.....
5. Sector.....
6. Cell.....
7. Village.....

PART A: RESPONDENT PARTICULARS

1. Name of respondent

2. Gender: (a) Male (b) Female

3. Age (years)

- a) 15 – 20 years
- b) 21 – 30 years
- c) 31-40 years
- d) 41-50 years
- e) More than 50 years
- f) Don't know or prefer not to say

4. Which level of education have you attained?

- a) Non formal education
- b) Primary level
- c) Secondary level
- d) Tertiary level

PART B – 1: FARMING PRACTICES AND ANIMAL HEALTH MANAGEMENT

1. Type of cattle raised:

- a) Ankole
- b) Exotic (Specify)
- c) Hybrid (Specify)

2. How many cows do you keep?

3. What farming system are you practicing?

- a) Extensive (never kept indoors)
- b) Semi-intensive (kept outdoors during day and kept indoors overnight)
- c) Intensive (primarily kept indoors – zero grazing)

4. How many lactating cows do you have in this herd?

5. What is the water source for your animals?

- a) Tap water
- b) Water pans/flood water
- c) Local River/streams
- d) Local wells/boreholes

7. Do you know Mastitis?

- a) Yes
- b) No

8. At what time do you observe mastitis in your herd?

- a) The first 3 months of lactation
- b) During the whole lactating period
- c) During the dry period

9. How do you treat mastitis?

- a) Seek for veterinarians
- b) Treatment by myself
- c) If yourself, what type of drug do you use?
 - Antibiotics: Penistreptomycin Multiject Other (Specify)
 - Anti-inflammatory drugs: Phenylject
 - Antibiotics and ant-inflammatory drugs

10. How do you prevent mastitis in your herd?

- a) Use of teat dips
- b) Wash the udder and use of udder towels
- c) Early treatment of new cases
- d) Apply dry therapy

PART B – 2: MILKING PRACTICES

1. How many times do you milk per day?

- a) Once: in the morning In the evening
- b) Twice

3. Which technique do you use?

a) Stripping (Pulling the teat)

b) Squeezing Action

4. What is the milk quantity (on average) per day that you collect from your herd (in l)?

6. Where do you milk from?

a) Kraal

b) Milking shade

d) Open space

e) Other (specify)

7. Do you screen for mastitis? a) Yes

b) No

8. If yes, which method do you use?

a) Strip cup

b) California mastitis test

c) Mastitis test paper

d) Milk checks for abnormal appearance

9. If no, why.....

10. What do you do to ensure clean milk production?

a) Observe strict cleanliness

b) Strain milk

c) Milk healthy animals only

d) Use health and clean personnel for milking

11. Who primarily milks the lactating cows? (multiple choice)

a) Myself

b) Family member only

c) External employees

d) Others (specify).....

12. What do you do with milk from your animals?

- a) For family consumption
- b) Sale to milk vendors/traders
- c) Sale to milk collection centres
- d) Sale to neighbours and members of the community

13. If selling milk, for how long do you keep the milk before reaching the market?

Mention:

14. How do you store the excess of milk?

Mention:

15. What containers do you use for milking, storage and transportation of milk?

Container	Milking	Storage	Transport
1. Aluminum vessels			
2. Plastic vessels			
3. Cooking pan “ <i>isafuriya</i> ”			
4. Traditional pots			
5. Other (specify)			

16. How often do you wash your milk containers?

- a) Before milking
- b) Just after milking
- c) Both

17. What source of water do you use to clean milk handling equipment?

- a) Tapped/piped water
- b) Wells
- c) Boreholes
- d) Water stream

18. What type of water do you use to clean milk equipment?

a) Warm water

b) Cold water

c) Warm water and disinfectant (Specify the disinf.....)

d) Cold water with disinfectant (Specify the disinf)

19. When do you deliver milk to the MCC/restaurants?

1) Immediately after milking

2) One hour after milking

3) Two hours after milking

4) Three hours after milking

5) Six hours after milking

6) The following day

20. What means of transportation do you use to reach the customers, including handling facilities and storage conditions? Mention:.....

21. Did you ever encounter any rejection of your milk by customers?

Yes No

If Yes, what was the reason (s):.....

22. If you milk sick cow, what do you do with its milk?

a) Family consumption

b) Sale the milk

c) Leave for calves

d) Discard

e) Other (Specify).....

Murakoze cyane!

Appendix 2. Questionnaire survey for milk kiosks and hawkers



Good morning/afternoon, I am **Dr Jean Pierre M. Mpatswenumugabo**, Master’s student from the UNIVERSITY OF NAIROBI, *College of Agriculture and Veterinary Sciences*, Faculty of Veterinary Medicine in the Department of Veterinary Pathology, Microbiology and Parasitology. We are conducting a research on “**Risk factors associated with subclinical mastitis and bacterial contamination of cow milk along the market chain and antimicrobial susceptibility patterns of isolates in Rwanda**”. The results from this study will help us to identify the main sources of bacterial contamination of milk and identify the critical points for quality control of milk. I would like to ensure you that any information provided will be used for research purpose and kept confidential.

GENERAL INFORMATION

Date of interview:.....

1. Questionnaire number:.....
2. Sample number:.....
3. Province:.....
4. District.....
5. Sector.....
6. Cell.....
7. Village.....

PART A: RESPONDENT PARTICULARS

1. Name of respondent

2. Gender: (a) Male (b) Female

3. Age (years)

- a) 15 – 20 years
- b) 21 – 30 years
- c) 31-40 years
- d) 41-50 years
- e) More than 50 years
- f) Don't know or prefer not to say

4. Which level of education have you attained?

- a) Non formal education
- b) Primary level
- c) Secondary level
- d) Tertiary level

5. What type of business do you run?

a) Supplier b) Milk hawker c) Milk kiosk d) Restaurant

PART B: MILK HANDLING AT THE VENDOR AND RESTAURANTS/KIOSKS LEVEL

1. From where do you get your milk?

- a) Own farm
- b) Different farms
- c) MCC , specify the name of MCC:.....
- d) Market
- e) Other (specify):.....

2. Do you perform any checks for milk quality before buying?

Yes No

If yes above, what checks do you perform.....

.....

3. How do you transport the milk?

- a) Refrigerated vehicle
- b) Other (personal) vehicle
- c) Bicycle
- d) Motorcycle
- e) Public transport
- f) Other (specify):.....

4. Do you mix milk from different farms or sources?

Yes No

5. How long does it usually take to transport the milk from source to final destination?

.....

6. How long do you keep the milk from transport until sale?.....

7. Have you got any kind of formal training on milk handling and marketing?

Yes No

8. Which equipment do you use to store the milk?

- a) Aluminum vessels
- b) Plastic vessels
- c) Cooking pan "*isafuriya*"
- d) Traditional pots
- e) Other (specify)

9. How do you keep the milk until sale?

- a) At room temperature
- b) Refrigerator
- c) Other (Specify).....

10. What type of milk do you sell ?

- 1) Raw milk

- 2) Boiled milk
- 3) Fermented milk
- 4) African tea

11. How frequently do you clean the milk containers?

- a) Sometimes
- b) Monthly
- c) Weekly
- d) Daily
- e) Other (specify):.....

12. What type of water do you use to clean milk containers?

- a) Cold water only
- b) Hot water only
- c) Hot water with detergent/soap
- d) Hot water with detergent/soap

13. What is your source of water for cleaning?

- a) Tap water
- b) Water tank
- c) Local River/streams
- d) Local wells/bore holes
- e) Other (specify):.....

14. What do you use for washing your hands?

- a) Cold water only
- b) Warm water only
- c) Cold water and soap
- d) Warm water and soap
- e) Other (specify):.....

15. If you don't sell all the milk in 24 hours, what do you do with the remaining milk?

- a) Sell the following day
- b) Discard
- c) Home consumption
- d) Other (specify):.....

Direct observation

16. Cleanliness of the vendor/server

Well-clean Dirty

17. Storage equipment status

Clean Dirty

19. Is the storage equipment covered?

Yes No

18. Type of container used to fetch milk from the large container

- a) A cup with handle
- b) A cup without handle
- c) Other (specify):.....

19. How is the milk served?

- a) From a large container/thermal flask and pour into a cup
- b) By immersing a cup in the large container/cooking pan (Scooping)
- c) Cold from the fridge
- d) Other(s) specify.....

20. Type of the floor in the milk kiosks:

- a) Tiles
- b) Cement only
- c) Soil

Murakoze cyane!

Appendix 3. Susceptibility patterns of all isolates

Classification of isolates: No. of isolates (%)									
Antibiotics	CNS (n=45)			<i>E. coli</i> (n=19)			<i>Staph. aureus</i> (n=19)		
	Susceptible	Intermediate	Resistant	Susceptible	Intermediate	Resistant	Susceptible	Intermediate	Resistant
Nalidixic acid	0.0%	0.0%	100.0% ^a	78.9%	10.5%	10.5%	0.0%	0	100.0% ^a
Gentamicin	100.0%	0.0%	0.0%	100.0%	0.0%	0.0%	100.0%	0	0.0%
Amoxicillin/clavulanate	93.3%	0.0%	6.7%	68.4%	0.0%	31.6%	100.0%	0	0.0%
Penicillin	42.2%	0.0%	57.8%		<i>b</i>		26.3%	0	73.7%
Erythromycin	88.9%	0.0%	11.1%		<i>b</i>		94.7%	0	5.3%
Ceftriaxone	100.0%	0.0%	0.0%	100.0%	0.0%	0.0%	94.7%	0	5.3%
Doxacillin	93.3%	2.2%	4.4%	94.7%	5.3%	0.0%	94.7%	0	5.3%
Tetracycline	60.0%	0.0%	40.0%	78.9%	10.5%	10.5%	78.9%	0	21.1%
Ampicillin	51.1%	2.2%	46.7%	26.3%	15.8%	57.9%	26.3%	0	73.7%
Ciprofloxacin	97.8%	2.2%	0.0%	94.7%	5.3%	0.0%	100.0%	0	0.0%

a: All Staphylococci have an intrinsic resistance towards Nalidixic acid (CLSI, 2014)

b: Breakpoints are not provided in the CLSI guidelines (2014).

CNS: Coagulase Negative Staphylococci

E.: *Escherichia*

Staph. -*Staphylococcus*