Assessment of bacteriological quality and handling of bovine raw milk collected from communal areas in Mahikeng, South Africa

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DECLARATION

I, Gaboipofe Florah Mokgaotsi, hereby confirm that the work contained in this research dissertation entitled 'Assessment of bacteriological quality and handling of bovine raw milk collected from communal areas in Mahikeng.' is my own original work submitted for a Degree of Masters of Science in Animal Health, working under the supervision of Dr Lubanza Ngoma and Professor Michelo Syakalima. This dissertation has not been previously submitted to any University. Materials and evident information from any other sources have been fully acknowledged.

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ABSTRACT

According to the literature from previously done investigations, there is limited data with regard to the dangers posed by the informal milk markets, and as a result, the prevalence of zoonosis as well as chemical or drug residues in most African countries including SA is not fully understood (Karimuribo *et al*., 2005; Mosalagae *et al*., 2011). The aim of this study was to investigate the occurrence, evolutionary relationships and antibiotic resistance profiles of pathogenic bacteria isolated from raw milk obtained from communal farms in Mahikeng, North-West Province of South Africa. The study also investigated milk handling practices and antibiotic use at farm level. One hundred and sixty-eight (n=168) raw milk samples were collected from communal farms/households with lactating cows in eight villages in Mahikeng between September 2017 and January 2018. Various bacterial species including *Staphylococcus aureus, Staphylococcus equorum, Enterococcus faecium, Enterococcus faecalis, Enterococcus durans, Enterococcus casseliflavus, Enterobacter hormaechei, Enterobacter asburiae, Clostridium bifermentans, Clostridium* species, *Paraclostridium benzoelyticum, Salmonella* Typhimurium *, Escherichia coli, Shigella* species, *Bacillus* sp., *Alcaligenes faecalis, Citrobacter braakii, Proteus hauseri, Providencia* species, *Lactococcus garvieae, Lactococcus lactis* and *Weissella cibaria* were isolated and identified using morphological (Gram-staining), biochemical (catalase, oxidase, indole, coagulase and Analytical Profile Index-API) and molecular (conventional Polymerase Chain Reaction-PCR) methods. The antibiotic resistance profiles of the isolates were determined using disc-diffusion method. All raw milk samples tested (n=168) had total bacterial count (TBC) higher than the recommended level of 5.0×10^4 cfu/ml. All the isolates were subjected for API tests, whereby 56 isolates were positively identified to species level and were then subjected to PCR. Following PCR, determination of antimicrobial resistance was done for all the isolates (n=56) and it was found that only one isolate (*Weissella cibaria*) was resistant to one antibiotic while the rest were resistant to more than one antibiotic. It was further found that, most (46.4%) of the bacterial isolates were susceptible to Norfloxacin while most (53.6%) were resistant to Streptomycin. It was observed that milk handling practices at farm level were poor with lack of proper hygiene practices, example being that only 3% of the respondents indicated that they wash their hands without using soap or disinfectants before milking and the rest of the respondents reported not to wash their hands at all. It was also found that a very high number (83%) of farmers made use of antibiotics without consulting with the veterinarians, and even a higher number (93%) indicated that they did not read the instructions on pamphlets before use. Based on the results of this study which demonstrated poor quality of raw milk produced by communal dairy farmers in Mahikeng, it was therefore concluded that such milk is not safe for human consumption.

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LIST OF ABBREVIATIONS AND ACRONYMS

API	Analytic Profile Index
CFU	Colony forming units
CDC	Centers for Diseases Control and Prevention
CLIS	Clinical Laboratory Institute Standards
DAFF	Department of Agriculture, Forestry and Fisheries
DNA	Deoxyribonucleic Acid
et al.	And others
FAO	Food and Agriculture Organization
FB	Foodborne
FBD	Foodborne Diseases
I	Intermediate
PCR	Polymerase Chain Reaction
Rmp	Rounds per minutes
R	Resistant
SAgs	Staphylococcal Super antigens
S	Susceptible
sp	Species
WHO	World Health Organization

CHAPTER ONE: GENERAL INTRODUCTION

1.1 Background

Milk is an important source of nutrients for human as well as animals. It is the most important food for mammalian offspring as it is regarded to be an almost complete food (Oni and Fashogbon, 2012). It is an exceptional source of nutrients including proteins, carbohydrates, vitamins and minerals, essential for building as well as maintaining human and animal body (Kanyeka, 2014). It also contains antibodies (immunoglobulins), which provide protection to the new-born against several diseases (Pandey and Voskuil, 2011). Moreover, macro and micro nutrients of milk are essential for the improvement of bone constitution as well as reduced protein deficiency or malnutrition (Dror and Allen, 2011; Matla, 2008).

The biochemical composition of milk is complex and, its high water activity as well as nutritional value serve as perfect medium or environment for growth as well as multiplication of numerous bacteria and other microbes under a conducive environment (Parekh and Subhash, 2008). However, milk intended for consumption by human should not be contaminated by pathogens (Bertu *et al*., 2010). According to the World Health Organization (WHO) (2017), consumption of contaminated milk by human and animals may result in foodborne infections.

According to Parekh and Subhash (2008), the microbial contamination of milk can be classified as primary, secondary and tertiary microbial contamination. Primary bacterial contamination of milk mostly arises from infected or sick lactating animals while, secondary bacterial contamination takes place along the milk value-chain, which may involve factors such as contamination by milking personnel (milkers) during milking, people handling milk, unhygienic equipment as well as water used. Secondary microbial contamination also occurs during transportation and storage of milk. The tertiary microbial contamination occurs mainly as a result of milk re-contamination after it has been processed, arising from unsanitary conditions as well as inappropriate handling or storage during marketing or consumption. Therefore, milk quality is determined by its composition as well as general hygiene (Bertu *et al*., 2010).

Food Borne Diseases (FBDs), the most common health challenge nowadays with potential to significantly reduce economic productivity, have been described as either toxic or infectious in nature, arising from the consumption of contaminated food or water (WHO, 2018a; Shonhiwa

et al., 2017). According to WHO (2017), almost a third of populations residing in developed countries are affected by food borne diseases. FBDs arise from the consumption of foodstuff which has been exposed to pathogenic or biological (e.g. microorganisms), chemical (e.g. toxins) as well as physical (e.g. metal) contaminants (Schmidt *et al*., 2003). However, according to WHO (2017) a significantly higher rate of FBDs worldwide occur from microbiological origin.

According to Cantas and Suer (2014), about 61% of 1500 human pathogens are zoonotic (can be transmitted between animals and humans). Wang and Crameri (2014) further state that, 75% of all emerging diseases affecting human initially arise from domestic animals as reservoirs. Foods of animal origin including milk and milk products have been said to be responsible for a greater number of FBDs (Guerra *et al*., 2016). Therefore, the incidence of such diseases increases with increasing access to such foods, particularly with lack of proper hygiene as well as insufficient inspection for safety (Fung *et al*., 2018; Kamleh *et al*., 2012).

Pathogenic bacteria including *Salmonella*, *Brucella*, *Staphylococcus*, *Listeria*, *Proteus*, *Escherichia coli* and coliforms are spread to humans following consumption of raw milk (Vahedi *et al*., 2013). Apart from being a source of pathogenic bacteria, raw milk may also possess a critical health risk to consumers due to antibiotic resistance of these pathogenic bacteria (Jans *et al*., 2018). Antibiotics are the most regularly used antimicrobials in dairy cattle for prevention and therapy of different bacterial infections and diseases (Bengtsson and Greko, 2014; Nonga *et al*., 2010; Sharma *et al*., 2018). However, the misuse and overuse of antibiotics in smallholder farms leads to a substantial development of antibiotic resistance of pathogenic bacteria to various commonly used antibiotics. As a result, the bacterial resistance to nearly all existing antibiotics is on the increase (Chang *et al*., 2015; Zaman *et al*., 2017). As these pathogens can be transmitted from animals to human through the consumption of unpasteurized or contaminated milk or milk products, patients infected with such resistant bacteria tend to require an extended healing period and new types of antibiotics for treatment (Li and Webster, 2018; Bebell and Muiru, 2014). It is hence important to examine the prevalence and resistance profiles of pathogenic bacteria in animal husbandry as it has not been extensively studied in Mahikeng. General improvement in small-scale dairy production has the capacity to improve food safety and food security, as well as enhancing income for resource-poor farmers (Wong *et al*., 2017).

However, there is limited data on microbial pathogens and other hazards in milk produced by the communal farmers in the rural areas of Mahikeng thus, the risk to human health is mostly unknown. It is therefore imperative that, the bacteriological assessment be conducted on the milk produced in rural areas in Mahikeng. Such an assessment is necessary to establish the level of contamination in order to be able to recommend certain corrective measures with regard to ensuring the safety of milk for the society at large but mostly, for vulnerable groups in rural areas.

This particular study assessed and identified bacterial species present in raw milk from communal areas in Mahikeng. The study also investigated antibiotic resistance among milk-borne bacteria to certain commonly used antibiotics in both human and veterinary practices. The information obtained from this study is aimed at enlightening society of the dangers of consuming raw milk.

1.2 Problem Statement

According to Department of Agriculture, Forestry and Fisheries-DAFF (2017), from 2006/07 to 2015/16 there was an increase in the South African domestic dairy market in the production and consumption of milk that is not pasteurized. Moreover, milk produced and sold in the rural areas of Mahikeng is mostly from the domestic market, which is mainly informal and operated by individual smallholder producers.

The South African regulations relating to milk and dairy products (South Africa, 1997) stipulate that, raw milk meant for consumption should not contain more than 50 000 per 1ml of colony forming units (CFU). Regardless of the restrictions of this Act, a concern has been raised recently by the South African Environmental Health sector regarding an increase in the rate of informal dairy farmers who frequently do not meet the legal standards set for bacteriological quality of milk (Louw, 2013).

The informal dairy market faces various constraints which impact on the quality of milk produced, and poses a serious public health concern (Addo, *et al*., 2011). The milk produced and sold by the communal farmers is raw (unpasteurised), which does not meet the minimum statutory requirements for milk meant for consumption and in addition, the milking practices applied by the communal farmers also do not comply with best practice compliance standards (Swai and Schoonman 2011).

The yearly outbreaks of food-poisoning caused by both *Campylobacter jejuni* and *Salmonella* which can be found in raw milk, have been reported in both developed and developing countries including South Africa due to improperly processed milk (Louw, 2013).. *Staphylococcus aureus* has also been isolated from most raw milk samples and, it may as well be present in unheated or lightly heated dairy products (WHO, 2017).

According to the 2015/16 to 2019/20 DAFF (2015) strategic plan, food security for all is one of the goals to be achieved by 2020. Food safety as one of the components of food security, is explained as handling, preparing and storing food in ways that prevent foodborne illnesses (FAO, 2014). Availability of safe, nutritious and accessible food is therefore referred to as food security. The quality of food consumed by the public is therefore of importance and has to be assessed regularly, so as to achieve the goal of food safety and security for all.

Due to the misuse and overuse of antimicrobials in farm animals, there is a global ongoing concern with regard to the emergence of antibiotic resistant bacteria resulting in antibiotic resistant infections in human (Foley, 2014). Human pathogens such as *Listeria monocytogens*, which recently caused listeriosis outbreak in South Africa have been reported to be resistant to numerous antibiotics including oxacillin, cefepime, and other commonly used antibiotics (WHO, 2018b). It is therefore crucial to regularly investigate the resistance patterns of foodborne bacteria in order to contribute to the prediction of the success or failure of antibiotic therapy.

1.3 Justification

Milk is a perishable livestock product therefore, the production and handling of this product under unsanitary conditions or inadequately regulated handling practices increase the chances of spoilage (Swai and Schoonman, 2011). Munjere (2017) further states that, milk hygiene standards for unpasteurized milk and its products, is essential for the protection of human population against milk borne zoonosis. Therefore, according to Parekh and Subhash (2008), a bacteriological study of raw milk is vital in order to indicate the degree of bacterial contamination and propose relevant corrective measures. The report from this study is intended to work as basis for improving milk quality and reducing the chances of milk spoilage which pose public health risk in the rural areas around Mahikeng.

1.4 Study Objectives

1.4.1 General objective

The aim of this study was to assess bacterial quality of raw bovine milk as well as antibiotic resistance among isolates of raw bovine milk from communal farms in Mahikeng.

1.4.2 Specific Objectives

I. Assessment of milk handling practices and antibiotic use at farm level through administration of questionnaires.

II. Assessment of bacterial contamination by conventional tests including colony morphology, Gram staining and biochemical tests as well as identification of raw milk isolates using molecular (polymerase chain reaction) methods.

III. Phylogenetic analysis of isolates so as to establish phylogenetic relationships between organisms identified in this study and those in public databases.

IV. Determine antimicrobial resistance patterns of milk-borne bacteria isolated in raw bovine milk in this study.

1.5 Research questions

I. Do communal farmers in Mahikeng area practice good milk hygiene practices?

II. Is prudent antimicrobial use wide spread among communal farmers in Mahikeng?

III. What is the prevalence of pathogenic bacteria contamination of raw bovine milk from communal farms in Mahikeng?

IV. What are the antibiotic resistance profiles of pathogenic and non-pathogenic bacteria present in raw bovine milk from communal areas of Mahikeng?

V. Is raw bovine milk produced by the communal farms in Mahikeng safe for human consumption?

1.6 Chapter 1 References

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CHAPTER TWO: LITERATURE REVIEW

2.1 Composition of milk

Milk is a white-yellowish mammalian mammary gland secretion and according to Rani *et al*. (2017), 'an evolutionary successful bioactive fluid'. It is an important source of nutrients and the only food for mammalian offspring, before they are able to ingest and digest many other sorts of foods. It consists of impartial elements required for maintaining and building both the animal as well as human body (Pandey and Voskuil, 2011).

According to Guetouache *et al*. (2014), milk is composed mainly of water (87 – 88%). The remaining part is the macronutrients including carbohydrates (mainly lactose), fat, proteins as well as micronutrients including vitamins and minerals. All components are well balanced.

Milk composition is not always constant and so the average percentages of its constituents differ according to species or breeds of animals, stage of lactation, season, climate and feeds (Pandey and Voskuil, 2011). In addition, growth factors including growth regulating factors, epidermal growth factors (EGF), vascular endothelial growth factors (VEGF), neuronal growth factors, Erythropoietin (EPO) as well as immune related factors including chemokines, immune cells and cytokines exist in milk (Ballard and Morrow, 2013). Apart from these properties, there are hidden properties of milk such as the presence of miRNAs which have been recently established by researchers and are said to comprise a small non-coding RNAs group (sncRNAs) known to regulate gene expression (Zhao *et al*., 2018). This is an indication that the knowledge about the importance of milk and its properties has improved with time, more so as more research is being carried out on unknown properties of milk.

2.2 The role of milk in human diet

Bovine milk is a food product rich in essential nutrients, either consumed or used as one of the ingredients in the production of various dairy (Porcellato *et al*., 2018). Bovine milk has always been an easily accessible source of both energy and proteins for people as it is easily digestible by children as well as adults hence, its consumption is of global importance (Burger *et al*., 2007). This proves that the consumption of bovine milk complements human needs significantly well, regardless of some differences it has to human milk with regard to amounts of protein, carbohydrates and fat contents (Loss *et al*., 2015).According to Claeys *et al*. (2014), the cow's milk consumption is by far the highest globally compared to the consumption of other types of milk, thus dominating the global milk production. Gerosa and Skoet (2012)

further explain that, bovine milk production constitute 85% of global milk production while the remaining part is from other animal species including buffalo, goat, sheep as well as camel with 11%, 2.3%, 1.4% and 0.2% production respectively.

The consumption of raw bovine milk has become popular nowadays due to its known properties of lowering the risks of allergic rhinitis, pollen allergy, asthma, hay fever and atopic sensitization, associated with childhood or infanthood exposure to unpasteurized cow's milk (Godziszewska *et al*., 2018). Loss *et al*., (2011) further agree to say that, numerous epidemiological studies have previously reported the important role played by raw cow's milk consumption in lowering the risk of asthma and allergies, more especially in children. Claeys *et al*., (2013) have also mentioned that, the consumption of raw milk is gradually becoming popular as a result of the current trend known as 'consuming natural and purchasing locally', arising from the concept that heating for pasteurization reduces or destroys the health as well as nutritional benefits of milk and might also promote some dangerous effects to consumers.

Although raw milk consumption trend is developing, Verraes *et al*., (2015) argue that the consumption of raw bovine milk leads to microbial risks to consumers due to the fact that, the prevalence of food-borne pathogens including *Salmonella* species, *Escherichia coli*, *Campylobacter* and other human pathogens is constantly reported worldwide. Due to the gradual increase in foodborne diseases around the 19th century globally, health concerns regarding the consumption of raw bovine milk began at that time and it was discovered that most of these diseases were arising from bacterial contamination of such milk (Mendelson, 2011). Although these illnesses are normally limited to symptoms such as diarrhoea and nausea/vomiting, they may at times become more serious, causing chronic complications such as Irritable bowel syndrome (IBS), Post-diarrheal hemolytic uremic syndrome (D+HUS), Guillain-Barre syndrome (GBS) or even death (Food and Administration, 2011).

Due to the pathogenic risks linked to the consumption of raw bovine milk, there are no previous or current controlled intervention studies being done in children to establish the truth about the effect of such milk in lowering the risks of asthma, allergies and other health benefits correlated with the consumption of raw bovine milk (van Neerven *et al*., 2012). Thus far, every study previously done on the effect of raw bovine milk consumption was carried out using questionnaires, hence it is difficult to confirm the theory of such health benefits associated with raw milk consumption (Loss *et al*., 2011). In spite of the fact that there is a continuous public debate regarding the potential benefits of consuming raw bovine milk, public health

organizations inclusive of Centers for Disease Control and Prevention (CDC) Food and Drug Administration (FDA) have shown serious concerns about the risks linked to the consumption of raw milk possibly contaminated with pathogens (Lucey, 2015).

The market of raw milk directly to people is gradually growing globally (Bianchi, Barbaro *et al.*, 2013). Raw milk is not only sold through vending machines at certain grocery stores but also, it is a well-known and usual practice for farmers to sell the milk directly to consumers either from their respective farms or by delivering the milk to the consumer's homes (Kalmus *et al*., 2015). According to previous studies, consumers have shown a great interest in raw milk as it is said to taste better and also believed to possess high nutritional value as well as great health benefits than processed milk (Claeys *et al.*, 2013).

2.3 Smallholder dairy farmers

It is estimated that there are over 500 million small-scale farms globally, with more than 750 million people residing on dairy farms as farm workers or members of the households situated in farms (FAO, 2017). According to Lues *et al*. (2012), the creation of on-farm jobs depends on the small-scale dairy farms and it is estimated that almost 200 jobs may be created if the farm is able to produce 1 million litres of milk annually. It has also been reported that over 6 billion people throughout the world consume milk, and many of these are in developing countries (Hemme and Otte, 2010). This demonstrates the importance of dairy production and its potential to reduce poverty especially in developing countries.

Although there are factors such as level of management, cattle genotype and the holding size which set apart large and small scale farming, milk is produced by both commercial and communal dairy farmers in most countries in Africa including South Africa (Mbanjwa, 2016). Unlike in most commercial farms, dairy farming is normally merged with crop farming in smallholder farms and cattle manure is used as fertilizer for crops (Mumba, *et al*., 2011). However, the main purpose of farming for smallholder farmers is normally to provide social security for their families (Omondi, 2009).

It has been reported by the FAO (2014) that, the contribution of developing countries towards dairy production globally is gradually increasing, and is generating more profit than in developed countries. This indicates that the small scale farmers in developing countries have improved their production, which may contribute towards poverty reduction as well as creation of wealth. Sharma *et al*. (2013), are of the view that small-scale dairy farming has the potential

of enhancing economic growth as well as livelihood development in developing countries. As a results, the national governments in both Zambia and Malawi have financially stimulated small-scale dairy farming in order to reduce poverty and improve economic status of their countries (Chagunda *et al*., 2016). Such efforts improve most households in rural areas who primarily depend on farming for household income (Wouters and van der Lee, 2009).

Smallholder dairy farmers market their milk in various channels such as: marketing through the mediator, direct selling to the consumers, reverse channel and dual distribution of milk depending on the demand (Uddin *et al*., 2012). According to Ulrich *et al*. (2012), almost 90% of these farmers use more than one channel to sell their milk, with 52% of them depending on the mediator, also known as the middlemen channel. However, Dennis (2010) argues that, the most common channel used by smallholder dairy farmers is direct milk marketing to the local consumers who are mostly people residing nearby the farms. It has been reported that the informal market of milk mostly through street vending in rural areas is very high hence, most of the households in rural areas partake in dairy farming due to the high demand for fresh milk in those areas (Chagunda *et al*., 2016).

In South Africa, there are five main buyers of milk that dominate the dairy production industry with Nestle, Parmalat and Clover being the top three buyers and only two of them controlling 50% of the dairy market (Chaminuka *et al*., 2014). Moreover, the dairy industry in South Africa is dominated by commercial dairy producers and almost 89% of milk sold, is pasteurized, and is marketed through the formal channels (Banda *et al*., 2012). However, in other countries like Kenya, the dairy industry is dominated by smallholder producers, with only 30% of milk sold through the formal channels. The estimated daily production is 12.7 litres of milk per cow in South Africa compared to the average daily production of between 8 and 10 litres per cow in Kenya (Ulrich *et al*., 2012). The fact that unlike in South Africa, the milk industry is dominated by smallholder farmers in other countries reveals that the smallholder farmers in South Africa are currently facing a serious challenge in accessing the formal channels of marketing their milk.

Among the factors affecting performance in smallholder farming, market access has been identified as the leading factor (Tegegne *et al*., 2013). In order to minimize poverty and improve livelihoods of those residing in rural areas as well as small-scale farmers, market access has to be facilitated for smallholder farmers (Mbanjwa, 2016). Although this is the case, there is a concern that due to technical inability of the small scale farmers are unable to take

advantage of the availability of markets (Van Schalkwyk *et al*., 2012). Other factors that limit the small-scale farmers include low levels of education, the current use of outmoded technology and inability to adjust to new technical methods (Van den Berg, 2013). According to Baiphethi and Jacobs (2009), having low level of education with lack of technological, marketing and financial skills is a limitation for meeting the standards set by the high value markets thus, leading to low quality production.

In addition, according to Espinoza-Ortega *et al*. (2007), some of the challenges faced by the smallholder dairy producers mostly arise from policies implemented for dairy production. Some of the challenges include poor farm management especially regarding hygienic practices, improper infrastructure, poor water supply as well as poor roads leading to the farms. Inadequate availability of resources lead to production of low quantity and quality of milk in smallholder farms (Chinogaramombe *et al*., 2008). The most important challenge with the informal dairy market is public health concerns over marketing of raw milk arising from the fact that, the zoonotic controlled diseases such as tuberculosis and brucellosis are linked to consumption of such milk (Loss *et al*., 2015).

Considering that most of the smallholder farmers rely on the dairy market as their primary economic activity, it is of importance that initiatives be taken to assist smallholder dairy producers in overcoming the challenges they are faced with in the industry and one way to do so is through developing the supply chain to support the small-scale producers in order to improve their profitability (Gelan and Muriithi, 2015). According to Gebreegziabher and Tadesse (2014), the farm capital structure is determined by its financial management hence, financial management skills in small-holder market needs to be enhanced for an improved profitability.

2.4 Internal and external factors of bacterial contamination of raw milk

The existence of pathogens in raw milk may be due to a number of factors, both internal and external (Lan *et al*., 2018; Hunt *et al*., 2012). The primary milk production factors that influence the bacterial quality of raw bovine milk have been thoroughly evaluated (Msalya, 2017) and are discussed below.

2.4.1 Mastitis as an internal factor of microbial contamination of raw milk

Mastitis has been found to have a great impact not only on milk production and quality but on the health of the animal as well, leading to a significant economic impact on dairy farmers (Le Marechal *et al*. 2011). Mastitis does not only negative impact animal production, it influences the quality of milk as well as quantity and, it is among the reasons for animals to be culled at a very early age (Haftu *et al*., 2012). Mastitis is an intra-mammary infection prevalent to mammals, causing an inflammation of the mammary glands and a condition of economic importance in dairy production globally (Iraguha *et al*., 2015). The condition occurs when the pathogen has been introduced into the germ-free and most sterile part of the mammary gland (Schukken *et al*., 2011), hence considered an internal factor of milk contamination.

According to Zadoks (2014), pathogens responsible for the occurrence of mastitis are usually categorized either as contagious or environmental, depending on the pathogens causing the infection. The environmental mastitis causing pathogens include the following: coliforms including *Escherichia coli* (*Escherichia coli*), *Pseudomonas aeruginosa*, streptococci and *Klebsiella.* These are considered to be of great significance in occurrence of clinical mastitis while the pathogens responsible for sub-clinical mastitis include *Streptococcus agalactiae* bacteria and *Staphylococcus aureus* bacteria, which are able to spread among all the quarters of a cow or even to the other cows (Contreras and Rodriguez, 2011).

Contagious mastitis is caused by contagious pathogens including *Staphylococcus aureus, Streptococcus agalactiae and Mycoplasma bovis* (Taoana, 2005). These pathogens inhabit and multiply in the mammary gland of an animal and spread cow to cow mostly during milking. Mostly, infections caused by contagious pathogens are less clinical but chronic and subclinical. However, environmental mastitis is caused by pathogens from the environment invading the mammary gland (Kurjogi and Kaliwal, 2014).

Mastitis can either be clinical or subclinical whereby in clinical mastitis, the symptoms such as redness or swelling of the udder as well as abnormal milk are visible (Thompson-Crispi *et al*., 2014). With subclinical mastitis, the infection takes place without any visible signs of inflammation while causing reduction in milk production, milk quality and reproduction performance of an animal (De Vliegher *et al*., 2012).

For therapy and control of mastitis, the use of antibiotics is normally opted for. Some farms have put in place the farm-management programs whereby, antibiotics are routinely administered to the whole herd, disinfection of the teats after milking is being done, animals

showing signs of chronical mastitis are culled and other practices to maintain the mastitis free environment are being done (Zhao and Lacasse, 2008). Mastitis is both costly to the farmers and a danger to raw milk consumers as it poses a risk of consuming milk contaminated with pathogenic bacteria.

2.4.2 External factors of microbial contamination of raw milk

Generally, milk is aseptic when still in the udder of a healthy cow up to the time of milking when it gets contaminated during or after the process, mainly through poor hygiene practices such as the use of poor quality water during milking (Chege and Ndungu, 2016). Although milk contamination is mostly associated with mastitis, the bacterial contamination of raw milk primarily arise from external factors including the environment, water, human (mostly personnel involved in milking process), storage containers as well as equipment used during milking (Vacheyrou *et al*., 2011). The animal being milked may also be a source of milk contamination through its skin and soiling of the udder with faeces, which tend to lead to bacteria (pathogens and non-pathogens) diffusing in milk (Kanyeka, 2014). Mhone *et al*. (2011) further explain that, the existence of coliforms in animal products such as milk stipulates the environmental or other external sources of milk contamination as these microbes exist in large quantities in the environment.

Inappropriate hygiene practices as well as poor handling of milk are the utmost factors exposing raw milk to microbial contamination, yet a large number of smallholder farmers have little knowledge regarding the importance of observing hygiene practices during milking at farm level (Elmoslemany *et al*., 2010). Most of these farmers are not aware of that, poor hygiene practices during milking have the potential to lower the milk quality, thus posing a serious health risks to consumers (Goopy and Gakige, 2016). Proper measures therefore need to be considered when handling raw milk in order to ensure milk quality and maintain better management routines as well as an overall health of cows (Morgan, 2004).

The major point of entry for pathogens into the udder of a cow is through the teat canal, hence mastitis is mostly prevalent in cows being milked in environments where hygiene is not taken into consideration (Chege and Ndungu, 2016). According to Hovinen and Pyorala (2011), animals usually rest on the ground and that may expose the teats to all sorts of dirt including feces, urine, mud and other sorts of dirt on the ground, therefore, it is important to disinfect the udder and teats of cows before milking.

Both pathogenic and non-pathogenic bacteria may be present on the hands of persons participating in the milking process and handling the milk, therefore it is crucial that the people handling raw milk disinfect their hands before commencing with the milking process (Bereda *et al*., 2012). Moreover, proper infrastructure is of importance when milking the cows because if such a procedure is being performed in an environment where milk is exposed to insects, air, wind and extreme temperatures, quality of milk may be compromised (Olofsson, 2013).

Water is one of the most useful resources in dairy farms for purposes such as cleaning (of the equipment used or farm environment), washing (of hands, teats of the cow or personnel clothing), drinking and many other duties done on daily basis (Chege and Ndungu, 2016). For an adequate production, a large amount of water is necessary in milk producing farms as it has a great impact on both production and the farm environment (Lemma *et al*., 2018). In this case, water used at farm level is required to be clean as the level of production and animal health are determined by water quality among other aspects (Arjomandfar *et al*., 2010). Previous studies have reported the isolation of pathogenic bacteria including *Eschericia coli, Salmonella* species as well as shiga-toxin producing bacteria in water used in farms (Lambertini *et al*., 2015; Venegas-Vargas *et al*., 2016). The existence of such pathogens in water may contribute to the contamination of milk being produced, hence water quality is of importance at farm level.

2.5 Milk-borne Infections and Pathogenic Microorganisms

Most infections such as salmonellosis, *Staphylococcus* infections, *Eschericia coli* O157:H7 infections and campylobacteriosis, usually arising from the consumption of unpasteurized milk, are frequently reported worldwide with significant health effects (Moatsou and Moschopoulou, 2014). These zoonotic diseases are known to be often transmitted to humans and constitute a hazard to public health.

Most countries have developed guidelines and legislations regulating milk and milk products composition and hygienic quality, in order to protect the health of people consuming milk (Pandey and Voskuil, 2011). However, these principles are unfortunately not frequently followed especially in developing countries thus, resulting in higher public health risk due to milk-borne infections or diseases. A great example to this was made by Oliver and Murinda, (2011) who are of the view that, most (75%) of milk that is marketed in several developing countries is sold unpasteurized via informal marketing channels. Moreover, it has been

emphasized in other studies that, populations consuming raw milk are found in rural areas in abundance (Njarui *et al*., 2011; Kanyeka, 2014), consequently being exposed to the risks of being infected with zoonotic diseases.

A study done in Sari City (Iran) by Vahedi *et al.* (2013) on bacteriological quality of raw bovine milk showed that, most significant disease causing bacteria found in milk were *Brucella, Salmonella, Staphylococcus (S), Listeria (L.),* Coliforms and *Escherichia coli.* Coliforms and *Escherichia coli.* These organisms are classified as normal large intestine inhabitants and their existence in milk might indicate fecal contamination. The incidences *of Escherichia coli, Staphylococcus* species, *Salmonella* species and *Campylobacter* species as raw milk contaminants are discussed below.

2.5.1 *Escherichia coli* as milk-borne pathogens

Escherichia coli (*Escherichia coli*) is a gram-negative bacteria normally present in the gastrointestinal-tract in human as well as ruminants (Sperandio and Nguyen, 2012). Although some of the *Escherichia coli* strains are said to be non-pathogenic, some have the potential to result in gastrointestinal infections in humans, ranging from moderate to very severe symptoms that mostly advance to permanent or long-term deadly results in highly endangered patients (Ntuli, 2017). In addition, certain *Escherichia coli* strains normally acquire virulence factors making them pathogenic hence, resulting in a variety of animal as well as human infections (Kaper *et al*., 2004).

Previous studies by authors such as De Buyser *et al*. (2001) and Denny *et al*. (2008) have reported that, there are high possibilities of transmission of pathogens such as *Escherichia coli* to humans when consuming unpasteurized milk or products made from unpasteurized milk. Moreover, it has been reported that the contamination of unpasteurized milk and its products by pathogenic *Escherichia coli* occurs mostly in developing countries than in developed countries (Kotloff *et al*., 2013).

The possibility of *Escherichia coli* disseminating into raw milk meant for human consumption is normally high due to the presence of animal faeces in a milking environment, unclean dairy parlour environment or equipment as well as unhygienic farm workers (Van Kessel *et al*., 2011). According to Guh *et al*. (2010), the dissemination of faeces into milk accompanied by improper hygiene in the farm are considered the major reasons for the presence of *Escherichia coli* in such milk being produced. *Escherichia coli* found in milk is therefore regarded to as a

sign of faecal diffusion into milk and also, it indicates the high chances of other intestinal pathogens being present in such milk (De Oliveira *et al*., 2011). Harmful bacteria including *Escherichia coli* are mostly found in unpasteurized milk as well as cheese made from unpasteurized milk as the potential sources of most bacteria (Maldonado *et al*., 2014). Several researchers including Oliver *et al*. (2009), Lucey (2015) and Davis *et al*. (2014) have indicated the public health dangers and related foodborne infections outbreaks associated with unpasteurized milk and its products globally.

According to Garenaux *et al*. (2011), *Escherichia coli* responsible for infections in human are categorized into two groups (intestinal and extra-intestinal infections) depending on their definite virulence as well as the clinical signs they initiate. Kaper *et al*. (2004) reported that, diarrheagenic *Escherichia coli* also known as DEC, are the major causes of gastrointestinal infections and are further subcategorized into six groups (enteropathogenic *Escherichia coli* /EPEC, Shiga-toxin producing or enterohemorrhagic *Escherichia coli*/EHEC, enterotoxigenic *Escherichia coli* /ETEC, enteroinvasive *Escherichia coli*/EIEC, enteroaggregative *Escherichia coli*/EAEC as well as diffusely adherent *Escherichia coli*/DAEC) depending on their pathogenicity as well as the occurrence of pathotype-specific virulence genes in them. Furthermore, Garenaux *et al*. (2011) also reported that, Extra-intestinal pathogenic *Escherichia coli* (ExPEC) infections are initiated by three different strain categories (uropathogenic *Escherichia coli* /UPEC associated with urinary tract infection (UTI), meningitis-associated *Escherichia coli* /MNEC as well as necrotoxigenic *Escherichia coli*/NTEC producing cytotoxic necrotizing factors) depending on the infections they cause. However, all these *Escherichia coli* strains have been previously isolated and identified in samples collected from both ill and healthy individuals as well as animals (Belanger *et al*., 2011).

It was further reported that, Enterohemorrhagic *Escherichia coli* or EHEC strains are the major cause of both hemorrhagic colitis and haemolytic–uremic syndrome (Bugarel *et al*., 2010). Moreover, the strains identified to be responsible for such infections are distinctly virulent Shiga-toxin producing *Escherichia coli* (Beutin *et al*, 2007). However, *Escherichia coli* O157 strains are normally the most isolated and identified strains in most outbreaks, together with other *Escherichia coli* strains including *Escherichia coli* 0103, O111, O26 and 0145, which have only been linked to few incidences of hemorrhagic colitis and haemolytic–uremic syndrome (Delannoy *et al*., 2013; Fratamico and Bagi 2012). Furthermore, domestic ruminants (especially sheep) and some other farm animals have been identified as natural reservoirs of

Shiga-toxin producing *Escherichia coli* (Caprioli *et al*., 2005). The sources of human infection by EHEC are mostly unpasteurized milk and EHEC-contaminated milk products (Sargeant and Smith, 2008).

On one of the recent studies done by Ombarak *et al*. (2018), *Escherichia coli* was isolated in raw milk then tested against a number of antimicrobials, which showed resistance against the following antimicrobials: ampicillin (18.9%), sulfamethoxazole-trimethoprim (11.3%), ceftazidime (3.6%), cefotaxime (4.5%), ciprofloxacin (1.4%), kanamycin (4.1%), tetracycline (27.5%), chloramphenicol (2.3%), nalidixic acid (1.8%) and streptomycin (18.5%). Such figures show that, *Eschericia coli* is constantly acquiring virulence factors against commonly used antibiotics, which could possibly result in serious health risks for raw milk consumers.

2.5.2 *Staphylococcus* species as milk-borne pathogens

Milk is one of the most well-known nutritious food, providing a great habitat for bacterial development and growth hence, spoilage and pathogenic bacteria including *Staphylococcus* species are often present milk with *Staphylococcus aureus* (*Staphylococcus aureus*) being the most dangerous to both human and animals (Seifu *et al*., 2004). Staphylococci are gram positive bacteria appearing as clusters of cocci under the microscope (Iqbal *et al*., 2015).

Staphylococcus has been identified among the major causative agents of udder infections, resulting in inflammation of the udder which is also known as mastitis (Alian *et al*., 2012). According to Blaiotta *et al*. (2010), the genus *Staphylococcus* consists of over forty species as well as sub-species mainly categorized into two groups being coagulase-positive staphylococci known as CPS and coagulase-negative staphylococci known as CNS. However, CPS *Staphylococcus aureus* is the most pathogenic bacteria of *Staphylococcus* genus and it has been reported that, almost 4.1% to 18.0% of intra-mammary infections arise from CPS *Staphylococcus aureus* in most farms (Chambers, 2001). These microorganisms also frequently occur in milk (Cremonesi *et al*., 2007) with some having the potential to produce toxins resulting in food poisoning in human population.

Le Loir *et al*. (2003) described *Staphylococcus aureus* as one of the commonly known pathogenic aerobic organisms belonging to Gram-positive group. However, some of these pathogenic strains have the ability to produce toxins responsible for gastrointestinal infections which commonly exhibit clinical signs such as nausea or vomiting, accompanied by diarrhoea as well as abdominal cramps. Moreover, Le Loir *et al*. (2003) also reported that, food poisoning

arising from *Staphylococcus aureus* is normally caused by the ingestion of heat-stable enterotoxins, which are commonly produced by coagulase positive *Staphylococcus aureus*.

According to Irlinger (2008), *Staphylococcus aureus* are widely distributed bacterial organisms normally found on the mucous membranes or skin of human and animals as natural inhabitants but, these organisms are frequently isolated from various foodstuff (e.g. milk, cheese, fermented meat) as well as from the environmental sources (e.g. water, soil, air). *Staphylococcus aureus* bacteria are regularly found on the nasal and vaginal mucous membranes of farm animals (especially caprine), although its epidemiology has not been reported yet as it is still difficult to figure out (Spanu *et al*., 2013). Some authors including Ventola (2015) have previously reported cases of hospital acquired infections also known as nosocomial infections, arising from the CN Staphylococci infections in immunocompromised patients. It is therefore of great importance to investigate the prevalence of these organisms in food as well as analysing the safety aspects in these bacterial species so as to establish their "Qualified Presumption of Safety" as suggested by the European Food Safety Authority (Authority, 2014). However, the environment provided by milk is said to be favourable for the growth and development of *Staphylococcus aureus* since the genome sequence analysis have shown the existence of lactose phosphor-transferase systems, which allows *Staphylococcus aureus* to grow in milk (Vijayakumar, 2014).

It has been reported that, the enterotoxins produced by enterotoxin-producing *Staphylococcus aureus* have been identified as the major cause of food poisoning by *Staphylococcus aureus* (Schelin *et al*., 2011; Cretenet *et al*., 2011). For example, staphylococcal enterotoxins were found to be among the causes of the major outbreaks that occurred in the year 2000 in Japan and in 1985 in United States of America-USA, due to dairy products especially milk-chocolate consumption (Asao *et al*., 2003). However, there were six outbreaks reported in 2009 in France, arising from soft-cheese contamination with staphylococcal enterotoxins (Ostyn *et al*., 2010). According to Kadariya *et al*. (2014) as well as Fujikawa and Morozumi (2006), the enterotoxins produced by certain strains of *Staphylococcus aureus* contribute largely to the widespread foodborne intoxications as well as the toxic shock syndrome.

The staphylococcal enterotoxin H which is commonly known for causing emesis was first reported in 1995 (Su and Wong, 1995). Argudin *et al*. (2012) further state that, most of the outbreaks are normally caused by *Staphylococcus* strains conveying either one if not more of the five identified main staphylococcal Enterotoxins SEs (SEA, SEB, SEC, SED and SEE).

Staphylococcal enterotoxins SEA, SEB, SED, SEE, SEF, SEG, SEH and SEI is mostly associated with staphylococcal food poisoning (Pinchuk *et al*., 2010). Moreover, Staphylococcal enterotoxin SEC is mostly isolated from animals and SEF is associated with toxic shock. However, the newly identified SEs have also been reported in few outbreaks (Argudin *et al*., 2012). Staphylococcal enterotoxin outbreaks are often said to be the critical danger in dairy farms and, the importance of this pathogenic bacteria has been indicated by the fact that it has been frequently reported as a contaminant in raw milk products (Jamali *et al*., 2015).

Zhong *et al*. (2015) states that, *Staphylococcus aureus* as one of the *Staphylococcus* species, is the second most regular pathogenic bacterium that has for quite some time been threatening public health. It exists in many sorts of environments and spreads quickly by water, air, sustenance and biological contact, burdening individuals worldwide and mostly in developing and underdeveloped world (Raho and Abouni, 2015). However, *Staphylococcus aureus* is responsible for various types of infections including the bone infection also known as osteomyelitis, infection of the inner lining of the heart valves also known as endocarditis, blood infection also known as bacteraemia as well as abscesses in the lungs (Cross and Gould, 2011).

According to Armougom *et al*. (2009) and Jakobsen *et al*. (2011), dairy foods especially cheese made from raw milk, are the major source of staphylococcal enterotoxins and the *Staphylococcus aureus* contamination primarily comes from either the people handling the milk at farm level or the milk being obtained from lactating cows with mastitis. However, the prevalence of *Staphylococcus* species in dairy products (mostly cheese) made from pasteurized milk is less documented due to the fact that, it is rare to isolate such organisms in pasteurized milk (Landeta *et al*., 2013). According to Oliver *et al*. (2005), there are various ways in which pathogens such as *Staphylococcus aureus* can diffuse into raw milk at farm level and the two routes of contamination include the unhygienic farm environment as well as milking of infected mastitic cows. Irlinger (2008) further state that, the presence of *Staphylococcus aureus* organisms in unpasteurized milk including bulk milk, has been frequently reported by several researchers.

The *Staphylococcus aureus* prevalence in cheese produced from goat's milk in Sweden was found to be 38% (Thaml *et al*., 1990). Moreover, in a study conducted by Jorgensen *et al*., (2005) to assess the staphylococcal contamination in bulk milk in Norway, it was found that the occurrence of *Staphylococcus aureus* was 75% in bovine milk as well as 96% in goat's

milk. Cremonesi *et al*. (2007) also reported 61% of *Staphylococcus aureus* isolated from raw bovine milk and 100% prevalence of *Staphylococcus aureus* from goat's raw milk in Italy. These findings indicate the emergence of these bacterial species over the years.

However, in addition to milk producing animal, there are various factors contributing to milk contamination by *Staphylococcus aureus* including the farm environment, equipment used during milking as well as people (Ostyn *et al*., 2010). According to Scallan *et al*. (2011) and Asao *et al*. (2003), *Staphylococcus aureus* organisms are recognized to be among the major pathogens responsible for foodborne diseases globally and most of the *Staphylococcus aureus* outbreaks have been reported to be linked to various foodstuff.

Staphylococcus aureus infections have been treated with penicillin over the years in numerous countries but recently, the organism has gained resistance to penicillin (Jamali *et al*., 2015). However, the antimicrobial resistance of *Staphylococcus aureus* is gradually developing for other antibiotics including methicillin, gentamycin, tetracycline and many more (Chambers, 2001). Turkyılmaz *et al*. (2010) also mentioned that, methicillin resistant *Staphylococcus aureus* are usually isolated in high rates in hospitals. This indicates that the resistant genes of this particular organism develop gradually, which may limit the ability to treat its infection in future.

2.5.3 *Salmonella* species as milk-borne pathogens

Of all the foodborne pathogens, *Salmonella* is said to be a leading pathogen globally, with at least 2600 serotypes isolated to date (Guibourdenche *et al*., 2010). According to (Gal-Mor *et al*., 2014), *Salmonella* bacteria are facultative, anaerobic, gram-negative and rod-shaped organisms that belong to Enterobacteriaceae family. *Salmonella enterica* is the prime cause of human enteric infections and diseases, affecting animals as well and causing a number of illnesses reported globally (Kaushik *et al*., 2014). This bacteria is associated mostly with animal products including milk and meat and are said to be responsible for the transmission of the infection between humans and animals (Alvarez-Fernandez *et al*., 2012). *Salmonella enterica serovar* Typhimurium as well as *Salmonella enterica serovar* Eenteritidis are often isolated from beef, pork, chicken and their products (Vose *e*t *al*., 2011). Numerous *Salmonella* infections outbreaks linked to consumption of beef were reported by several studies (Silva *et al*., 2011).

Raw milk has been frequently identified as *Salmonella* vehicle and a source of foodborne infections arising from *Salmonella* (Robinson *et al*., 2014). Yang *et al*. (2016) further states that, farm animal produce such as milk are the main carriers of human *Salmonella.* However, *Salmonella* species have been the most reported pathogens isolated from raw milk especially bulk tank milk overtime, ranging from 11% to 66% (Oliver *et al*., 2009). A study conducted in India to assess the prevalence of *Salmonella* in raw milk reported that, the serotyping of *Salmonella* isolates showed an incidence of 2.1% of *Salmonella typhimurium* and 1.4% of *Salmonella newport in* raw milk (Kaushik *et al*., 2014). Moreover, the strains of *Salmonella* which are resistant to the most importantly used antibiotics including fluoroquinolones and cephalosporin have been isolated repeatedly (Li *et al*., 2013). According to Yang *et al.* (2016), the microbial resistance of *Salmonella* emerges with time, leading to serious public health risks.

(Kosek *et al*., 2003) reported that, *Salmonella* infections are known to cause gastroenteritis in humans especially children, which sometimes leads to death if not morbidity. Enteric fever is one of the most deadly systemic diseases mostly endemic in developing areas, caused by *Salmonella* and resulting in over 200 000 mortalities (Buckle *et al*., 2012). A study that was carried out on *Salmonella* infections in populations from four developed countries revealed that the diarrheal illnesses arising from this pathogen ranged between 0.44 and 0.99 incidents, which is estimated to reach up to 2.8 billion cases globally every year (Scallan *et al*., 2005).

2.5.4 *Campylobacter* species as milk-borne pathogens

Campylobacter are gram negative rods belonging to Campylobacteraceae family (Vandamme *et al*., 2010). According to Silva *et al*. (2011), the first reported case of campylobacteriosis was in 1988. *Campylobacter* has been one of the major causes of foodborne diseases known to cause diarrhea in human and animals globally (Vandamme *et al*., 2010). *Campylobacter* is a known to be naturally present in the intestines of human as well as animals (Nisar *et al*., 2018).

Human campylobacteriosis has been reported to spread through the consumption of contaminated meat, milk as well as direct contact with the contaminated environment (Indikova *et al*., 2015). Campylobacteriosis outbreaks are most primarily associated with the consumption of unpasteurized milk if not contaminated water (Serraino *et al*., 2013). Although some people may not show symptoms, the infection is normally denoted by abdominal pains, fever, cramps as well as diarrhea which may be bloody and accompanied by vomiting (WHO, 2018a). *Campylobacter jejuni* is the most commonly isolated *Campylobacter* species in milk (Modi *et al*., 2015). It has been reported that about 1.3 million people get infected with

Campylobacter in USA each year (CDC, 2017). Moreover, 220 000 cases were reported in 2011 in EU while the infection contributed almost 50% of the annual diseases in New Zealand in the year 2012 (Rapp *et al*., 2012).

2.6 Hygienic Practices and bacterial quality of raw milk

Parekh and Subhash (2008) described milk as an easily decomposable food-product which normally carries a smaller number of bacterial cells (500-1000 cells/ml) before getting exposed to the environmental contamination during the milking process. According to Kanyeka (2014), such high bacterial counts in milk denote poor hygienic practices or the possibility of such milk being obtained from a cow infected with mastitis (Kanyeka, 2014). However, poor hygienic practices in the farm are marked by the existence of coliforms in unpasteurized milk, specifically *Escherichia coli*, reasoning being that the presence of such intestinal bacteria indicates fecal contamination (Martin *et al*., 2016).

According to Swai and Schoonman (2011), milk however carries a temporary germicidal also known as ‘’bacteriostatic properties’’, which act as a natural defense mechanism that prevents the bacterial multiplication within the first 3hours of milking. It is therefore important to ensure that milk is cooled to at least 4ºC during the period when the temporary germicidal is active due to the fact that, there are high chances of bacterial multiplication post 3hours of milking (Siddeeg and Elamin, 2014). In order to prevent an increased bacterial multiplication, milk production has to be as hygienic as possible, cooled and heated as fast as possible (Pandey and Voskuil, 2011).

The control of bacterial contamination of unpasteurized milk can be done through eradication of microbial organisms carried by human, through improving various factors including water supplies, health education to the public, personal as well as environmental sanitation (Kanyeka, 2014). Prevention and control of bacterial infestation can also be attained through correct pasteurization or boiling of raw milk before it is presented for processing and consumption (Addo *et al*., 2011). Pathogenic bacteria arising from the milk-producing cows could be limited through developments in animal husbandry as well as maintenance of good animal management practices while, pathogens from the environments or equipment may be limited by following proper sanitary practices in the farm (Manyi-Loh *et al*., 2016).

It is of paramount to adhere to proper hygienic practices at farm level due to the fact that, the microbiological quality of raw milk is determined by farm general hygiene among other factors. Moreover, protecting the public against milk borne infections can be done by screening milk that is being sold informally.

2.7 Milk pasteurization as a microbial quality control measure

According to Guerreiro *et al*. (2005), high microbial quality and presence of pathogenic bacteria in milk is an indication of poor quality of such milk. The overall quality of milk is also influenced by many factors including improper packaging system as well as inappropriate temperature control, which tend to create suitable environments for microbial development thus altering the milk and shortening its shelf life (Ahmed and Abdellatif, 2013). A number of milk processing techniques including microfiltration, Low Temperature Long Time (LTLT) pasteurization, membrane processing, microwave treatment, sterilization, ultraviolet treatment, ultra-high temperature treatment, High Temperature Short Time (HTST) pasteurization as well as thermization have been implemented for treatment of unpasteurized milk, so as to disable microbial activities of spoilage microbes and improve the shelf-life of milk (Tremonte *et al*., 2014).

FAO/WHO (2004) explain pasteurization as "A micro-biocidal heat treatment aimed at reducing the number of any pathogenic microorganisms in milk and liquid milk products, if present, to a level at which they do not constitute a significant health hazard". According to (Sarkar, 2015), pasteurization is devised to destroy microorganisms especially *Coxiella burnetii* as well as *Mycobacterium tuberculosis*. However, Hudson *et al*. (2003) further add to say that, the pasteurization conditions were primarily implemented to deactivate *Mycobacterium tuberculosis* until it was realized that *Coxiella burnetii* was the most heat-resistant bacteria found in milk hence, pasteurization was redevised to reach 5 log decrement of *Coxiella burnetii* in milk. High Temperature Short Time pasteurization method normally destroys about 99.9% of pathogenic bacteria and is mostly effective in minimizing the level of *Mycobacterium avium* subsp. para-tuberculosis (Okura *et al*., 2012). When milk is pasteurized, its shelf-life ranges between 2-20 days depending on the quality of milk before pasteurization, hygienic conditions of milk, processing method as well as temperature maintenance throughout the whole cold chain (Rysstad and Kolstad, 2006).

The pasteurization of milk was initially initiated as a public health control measure used to eradicate human pathogenic bacteria and to destroy as well as limit the actions of spoilage microbes (Todd, 2011). However, the bacterial feasibility of milk post pasteurization process may be evaluated through three individual viability indicators being colony forming units count on plate count agar, membrane integrity based on propidium iodide exclusion and lastly by de-novo expression of a green fluorescent protein reporter gene (Li *et al*., 2015). Both phosphatase and methylene blue tests are procedures commonly utilized for the detection of bacterial existence in milk that has been pasteurized (Anderson *et al*., 2011). In order to establish the total bacterial count (total number of bacteria) in a certain volume of milk, the commonly used method used is usually the standard plate count method (Sutton, 2011). This is utilized for classifying or grading milk. For determining the total coliform count in 1 milliliter of milk, coliform plate count is used (Li *et al*., 2015).

The consumption of raw milk exposes consumers to serious health hazards as milk is a good medium for the growth and development of many microorganisms. Although maintaining lowered temperatures has an effect in lowering microbial count in raw milk, pasteurization is so far the most effective method to destroy all pathogens a well as spoilage organisms normally found in milk, while reducing even the non-pathogenic microorganisms and unwanted enzymes to improve the quality of milk (Sarkar, 2015).

2.8 The effect of milk storage as a Hazard Analysis Critical Control Point (HACCP)

The bacteriological quality of milk has direct influence on both the consumers safety and the milk or milk products shelf-life (Kalupahana and Silva-Fletcher, 2016). According to Agyei-Baffour *et al*. (2013), milk-borne zoonoses are transmitted to consumers through the consumption of milk contaminated pathogens found in milk and moreover, the presence of bacteria in abundance in milk negatively affects both the chemical as well as physical quality of such milk. However, certain countries therefore introduced safety standards to ensure minimal bacterial presence in unpasteurized milk as much as possible (Cilliers *et al*., 2014).

The ‘hazard analysis critical control point’ abbreviated as HACCP is a systematic preventative approach implemented to identify, evaluate as well as control the physical, biological and chemical hazards tampering with food production procedures (Kalupahana, 2016). It has been generally accepted that the hazard analysis critical control point has the potential of improving

the bacteriological quality of foodstuff produced including milk as well as its products (Nada *et al*., 2012). However, the development of a HACCP decision support tool also known as ‘’decision tree’’ with the proper key control as well as critical control points (CCP) should be done appropriately, with full examination or analysis as well as full local-process understanding and comprehension (Agyei-Baffour *et al*., 2013). The main hazard linked to milk or products made from milk is microbial existence hence, the dairy industry has put more effort on assuring quality as well as safety by developing and implementing proactive preventative approaches including HACCP (El-Hofi *et al*., 2010). Siddeeg and Elamin (2014) conducted a study assessing the critical control points in dairy farms and it was revealed that, majority of dairy farmers do not observe the significance of maintaining low temperatures during storage or when transporting unpasteurized milk.

A remarkable improvement of the microbial quality of milk has been reached considerably ever since the milk chilling and refrigerating procedures of bulk milk have been introduced (Xin *et al*., 2017). Nevertheless, extending the storage duration of unpasteurized bovine milk while lowering the temperature generates suitable environments for the growth and multiplication of bacterial species able to survive extremely cold temperatures (psychrotrophic or cryophilic bacteria), which therefore changes the bacterial composition of such milk (Paludetti *et al*., 2018). Generally, the presence of cryophilic bacteria in cooled raw bovine milk often account for over 90% of all the microorganisms (Samarzija *et al*., 2012). Moreover, due to the fact that the psychrotrophic bacteria are able to produce proteolytic as well as lipolytic enzymes that are resistant to heat in colder temperature or under refrigeration, these particular bacteria are therefore accountable for milk and dairy products spoilage (Xin *et al*., 2017). Although the psychrotrophic bacteria are susceptible to pasteurization, the proteolytic and lipolytic enzymes produced by these particular bacteria have the ability to survive dairy-industry pasteurization (Oliveira *et al*., 2015). The example being that of the numerous protease enzymes that the psychrotrophic bacteria produce, which usually remain stable even during UHT treatment while remaining active in milk products post UHT (Zhang *et al*., 2015).

These enzymes hydrolyze milk protein causing drastic milk quality issues like gelation of the UHT milk, the development of off-flavors (unnatural flavors) as well as reducing the ability of milk to withstand high temperatures during heat treatment, also known as ‘’heat-stability properties’’ (Caldera *et al*., 2016). The Gram negative bacterial genera that are most often represented in bacterial species isolation of raw bovine milk normally comprises of

Achromobacter, Alcaligenes, Aeromonas, Acinetobacter, Enterobacter, Flavobacterium, Pseudomonas and *Serratia,* while *Pseudomonas* bacteria is said to be the dominating genus of them all (Samarzija *et al*., 2012).

Previous studies such as the investigation done by Xin *et al*. (2017) on the occurrence of cryophilic bacteria in unpasteurized bovine milk, *Klebsiella oxytoca, Pseudomonas fluorescens, Pseudomonas fragi, Pseudomonas lundensis* and *Serratia liquefaciens* were all found to have the potential to produce proteolytic and lipolytic enzymes (Xin *et al*., 2017). However, the ability of psychrotrophic bacteria to lower the quality of milk and cause milk spoilage may also be influenced by the presence of other microorganisms (Samarzija *et al*., 2012).

2.9 The implications of the emergence of antibiotic resistance

The term ‘’Antibiotics’’ refers to a group of antimicrobials that are being utilized primarily for treating or controlling bacterial diseases in both human and animals (Odonkor and Addo, 2011). According to (Bren, 2002), these drugs work by either being bacteriostatic (stopping the bacteria from reproducing) or bactericidal (killing the bacteria). However, certain bacteria have developed the resistance against antibiotic drugs through genetic exchange with other bacteria, natural selection as well as mutation and these bacteria therefore multiply and spread gradually. The previous studies have reported that the overuse and misuse of antibiotics has enabled the antibiotic resistant bacteria to multiply and increase faster than the susceptible bacteria, consequently resulting in high chances of antibiotic-resistant bacterial infections in human (Foley, 2014; Baquero *et al*., 2008). Foley (2012) further states that, the rate at which antibiotic resistance of most bacterial species is increasing is now posing a remarkable public health hazard due to proven possibilities of ineffectiveness of antibiotics commonly used in humans and animals over time.

Numerous investigations have been previously carried out with intentions of ruling out the antibiotic resistance causes, in order to come up with strategies for the careful use of antibiotic agents in humans and animals (Landers *et al*., 2012). However, Orzech and Nichter (2008) carried out a research to examine the antibiotic resistance diversity with attention being given to three possible causes being the use of products containing antibiotics by households, the antibiotic use in animals and agriculture in broad as well as the pharmaceutical use of

antibiotics. Researchers made use of the existing literature and established a number of possible solutions including social, biological as well as evolutionary features associated with antibiotic resistance.

The identified biological solutions included the review of the definition of the word "dose" of antibiotic agents in order to refrain from the misuse of such drugs, considering the use of combined-antibiotic therapies to target the infectious pathogen as well as well as prescription of short-courses of antibiotics for treatment of bacterial infections (Orzech and Nichter, 2008). The social solutions on the other hand comprise of extension education on different approaches of lowering the bacterial load in water by keeping it clean, allowing fresh-air into the houses through proper ventilation, regular washing of hands, proper vaccination and educating patients and medical practitioners including veterinary doctors as well as farmers about the dangers of misusing antibiotics (Lee *et al*., 2013; Orzech and Nichter, 2008). With the evolutionary solutions, the environments in which the bacteria grow must be manipulated, which is known as turning the bacteria against themselves. However, research concluded that there is an existence of complex relationships among humans, animals, microorganisms as well as plants in relation with antibiotic resistance and suggested that the investigation should be carried out on these complex relationships within each group.

Salisbury e*t al*. (2002) also conducted a study seeking to address the rising concerns with regard to the possible human health risk arising from the overuse of antibiotics in animals. The study was therefore conducted using risk-development assessment model which covers three identified hazards being the antibiotic, bacteria resistant to an antibiotic together with the antibiotic resistant gene. However, in accordance with the results from the study carried out by Orzech and Nichter (2008), the researchers concluded that the antibiotic use in any situation is able to allow for the bacterial selection or amplification of the resistant bacteria and suggested that the medical practitioners, veterinarians and the society at large should be alerted on the possible risk to human (Salisbury *et al*., 2002). Moreover, the researchers suggested that the bacterial load in regard with the food chain should be minimized in order to avoid the unnecessary use of antibiotics.

Furthermore, several studies previously conducted to assess antibiotics use in livestock and food animals but still, there is limited literature on the antibiotic use on food producing animals and the attitude or beliefs of veterinarians in regard with the overuse of antibiotics in livestock (Cattaneo *et al*., 2009). According to AVMA (2012), there was an estimation of 5 thousand

horses, 8 thousand birds, 70 thousand dogs as well as 75 thousand cats owned by Americans in 2012, which were being provided with veterinary care. Therefore, this has brought attention to researchers to investigate the use of antibiotics in these species globally as well as assessing the practices, beliefs and knowledge of veterinarians treating these species in order to gather necessary information relevant to the main goal being to combat the bacterial antibiotic resistance.

According to Oliver *et al*. (2011), there is an ongoing debate with regard to antibiotic utilization in animal medicine (veterinary medicine), particularly in livestock production. However, there is a production of antibiotics worth about fifty millions of pounds every year in USA with approximately 40% percent being used in agriculture. The research results reported by Mellon *et al*. (2001) revealed that, antibiotics worth 24.6 millions of pounds of the yearly produced antibiotics in United States of America were normally used for non-therapeutic reasons, while 10.5 million pounds being used for poultry production, with 10.3 million pounds being used in swine production and 3.7 million pounds being utilized for cattle production. This proves the overuse of antibiotic drugs which needs to be addressed properly through the solutions provided.

In spite of the fact that viruses are also known to be the causative agents of food-borne diseases (FBDs), bacteria accounts for almost 75% of those illnesses in people (Mc Nulty *et al*., 2016). However, a number of bacterial species such as *Escherichia coli, Listeria, Clostridium, Campylobacter*, *Staphylococcus, Bacillus and Salmonella* are the main causative agents of FBDs and have shown an emerging resistance to most of the antibiotics (Nyenje and Ndip, 2013).

Staphylococcus species are resistant to most antibiotic drugs, except vancomycin which is considered the last hope in antimicrobial use (Doyle *et al*., 2013). Of all the diseases caused by *Staphylococcus* species, the ones caused by the methicillin resistant *Staphylococcus aureus* (MRSA) are of major concern (Chambers and DeLeo, 2009). According to Porrero *et al*. (2014), methicillin resistant *Staphylococcus aureus* are said to have been identified in environments where domesticated and food producing animals are kept as well as in different foodstuffs including milk and meat. However, MRSA is accountable for most of the hospital acquired infections (HAI) also known as a nosocomial infection globally hence, its presence in foods and animal habitats poses a serious threat to human health (Nyenje and Ndip, 2013). The

danger caused by these particular bacteria may be minimized through reduction in antibiotic use generally in order to avoid the future emergence of its resistance.

Escherichia coli (Escherichia coli) on the other hand is known for its genetic- material exchange with other bacterial species and the prevalence of beta-lactamase producing *Escherichia coli* is increasing and this bacteria has been identified in food-producing animals (Poeta *et al*., 2010). These bacterial species have been found to be resistant to cephalosporin treatment (Hawkey, 2008). However, Eagar *et al*. (2012) has reported that, the SANVAD (South African National Veterinary Surveillance and Monitoring Programme for Resistance to Antimicrobial Drugs) has identified *Escherichia coli* together with the entire *Enterococcus* species as the leading bacterial species with regard to the antibiotic resistance of various bacteria. This is a potential future human health threat as the resistance of these species is gradually increasing globally.

According to Su *et al*. (2004), the antibiotic resistance of *Salmonella* remains a leading health concern globally due to an emerging resistance of this bacteria towards commonly used antibiotics as well as its gradual resistance development towards multi-drug therapy recently. However, *Salmonella* is mostly transmitted to human through the consumption of contaminated and improperly cooked food, more especially meat (Alali *et al*., 2010).The species of importance pertaining to the antibiotic resistance within the *Clostridium* species is *Clostridium difficile* (CDC, 2013). This antibiotic resistant bacteria is responsible for a number of infections (causing symptoms ranging from diarrhoea to life threatening colon inflammation) and its rapid spread threatens human health (Spigaglia, 2016).

Although the impact of antimicrobial misuse is not fully understood due to limited literature, the main human health concern is the transmission of these antibiotic resistant bacterial species through food such as meat, milk and other foodstuff, thus resulting in foodborne infections and intestinal colonization (Landers *et al*., 2012). Most of the studies have confirmed the association of most antibiotic resistant bacteria with food and food producing animals (Marshall and Levy, 2011; Chang *et al*., 2015).

The use of antibiotics in animals is regulated by two acts being the Fertilisers, Farm Feeds, Agricultural Remedies and Stock Remedies Act (Act 36 of 1947) regulated by Department of Agriculture, Forestry and fisheries (DAFF) and the Medicines and Related Substances Control Act (Act 101 of 1965), regulated by National Department of Health (DoH) in South Africa

(Henton *et al*., 2011). Henton *et al*. (2011) further explains that, Act 36 allows for over the counter (OTC) sale of certain antibiotics with no record keeping of antibiotics sold, while Act 101 allows access to antibiotics by prescription only. However, these contradict the guidelines provided by World Health Organization (WHO) suggesting that the use of antibiotics should be granted to certified human and animal medical professionals (WHO, 2017). This shows the need for merging the Acts and guidelines regulating the antimicrobial use in South Africa so as to fulfil the main aim being, the eradication of antibiotic resistant organisms as well as prevention of their occurrence.

Listeria monocytogenes is the human pathogenic bacteria known for its potential to cause a deadly foodborne illness known as listeriosis and the bacteria has been found to be extensively distributed all over the environment (Nyenje and Ndip, 2013). WHO (2018b) recently reported the largest global listeriosis outbreak that occurred in South African in 2017, which was found to be associated with the consumption of processed foodstuff. This foodborne illness was said to have been reported from all the provinces while three provinces had many cases with Gauteng being accounting for 581 (59%), Western Cape 118 (12%), KwaZulu-Natal 70 (7%) cases and 27% of the patients died (WHO, 2018b; NICD, 2017). However, most strains of *Listeria monocytogenes* isolated from foodstuff have shown an emerging resistance against a number of antibiotics including cefepime, cefotaxime, fosfomycin, lincosamides and oxacillin (Olaimat *et al*., 2018). *Listeria monocytogenes* therefore poses a perpetual human health and food- industry threat, hence the need to minimize its antibiotic resistant has to be emphasized

2.10 Chapter 2 References

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CHAPTER THREE: STUDY METHODOLOGY

3.1 Study area description

This study was conducted in Mahikeng, North West Province of South Africa. Mahikeng is located close to South Africa's border with Botswana, with a latitude of 25.8595° and longitude of 25.6524° (SA-Venues, 2017). The area is estimated to have 3486 communal cattle. Mahikeng is dominated by unimproved grasslands and animals graze freely on natural rangeland. The vegetation type in all the areas resembles that of a savannah biome. January is the warmest month with an average temperature of 31.8°C, while July is the coldest and driest month with an average temperature of 2°C. The annual rainfall around the areas is approximately 300 - 600 mm, although most of the rain is received between November and February (SA Places, 2017).

3.2 Study design

This was a cross sectional study carried out to establish the degree of bacterial contamination of bovine milk and the resistance patterns among isolates from milk towards commonly used antibiotics. Small scale dairy farmers with lactating cows were purposefully selected to participate in the study. Each farmer was visited once for administration of questionnaires and raw milk collection.

3.3 Study populations

The animals used in the study were lactating cows from small-holder farms located throughout Mahikeng. Most calving occurs from September to December, which is the most suitable period usually with favourable conditions and abundant food and water for cows (GOmez-Brunet, Santiago-Moreno et al. 2008). As a result, sampling took place from September to December 2017.The main focus was to target farmers in rural areas who often milk their cows and consume or market the milk to the public.

3.4 Selection of Study villages and households/farms

The statistics list of all villages in Mahikeng local municipality (Census, 2011) was used to determine the number of villages used for sampling. According to Cameron (1999), in random sampling, every unit of interest in the population has the same chance of being selected at 10% representation. Therefore, from a total of 83 villages found in Mahikeng, 10% (8 villages), were randomly selected for both sociological and laboratory data collections. The selected villages for sampling are presented with their coordinates in Appendix 2. The villages were selected based on their locations on the Map and availability of small- scale dairy farmers. Households with cattle were identified with the assistance from the Mahikeng Extension-Officers and 7 farms/ households per village were selected for sampling based on the availability of lactating animals during the time of sampling as well as willingness of the farmers to participate in this study.

3.5 Sample Size Determination

A total number of lactating animals in Mahikeng during the time of sample collection was unknown and therefore, a formula by Kothari (2004) for unknown population (i.e. $n = Z^2SD^2/e^2$) was used to determine the sample size, 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),

SD (Standard deviation) = was estimated at 0.15 or 15% and

e= is the estimated error and was considered at 0.05 or 5%.

Therefore, the sample size 'n' was calculated as:

$n = \frac{(1.96)^2 \times (0.15)^2}{(0.05)^2}$= 34.6 approximately n = 35 samples per village, (35×8) = 280

A total of 280 milk samples were supposed to be collected for this study according to the formula. However, due to inadequate availability of raw cow's milk during the time of collection, 168 raw milk samples were collected from all the villages selected for testing.

3.6 Ethical consideration

Ethics clearance was approved by the ethical committee for animal and human experimentation of the North West University before the study could commence. All farmers involved in the

study voluntarily participated and a verbal consent was obtained from each of the farmers following a brief explanation of the purpose of this study as well as its importance to the society prior to interviews and collection of milk. The data obtained from this study was stored as confidential, and remains the property of the North West University. Only the authorized persons such as the principal researcher or supervisors may access the data obtained from the study.

3.7 Data collection

The study involved two types of data collection methods to address the main objectives of the study. The first data collected was sociological which addresses the first objective followed by laboratory based data collection addressing objectives 2 to 4.

3.7.1 Sociological data collection

A structured questionnaire (Appendix 1) was used to interview the selected farmers (n=100) from all eight villages mentioned. The questionnaire was pre-tested in five households before data collection in order to make corrections where needed and to improve clarity of the questions. The questionnaire consisted of 36 questions and took a maximum of twenty minutes to complete. The questionnaire was translated to Setswana, which was the language known by most of the respondents. While conducting the interview, general hygiene practices were noted through direct observation of the environment, procedures done during milking and handling of raw milk.

3.7.2. Laboratory data/samples collection and transportation

All raw milk samples were collected directly from the milk storage containers being utilized at each farm/households visited. The storage containers had to contain ready to use milk either intended to be sold to the public, stored for household consumption or both. Approximately, 50 ml of raw cow's milk were collected into sterile screw capped containers. The containers were sealed properly, coded with random numbers for identification and packed appropriately on ice for transportation to the Animal Health Microbiology laboratory at Mafikeng Campus, North West University, were analyses took place.

3.7.3 Laboratory analyses of raw milk samples

Upon arrival at the laboratory, raw milk samples were analysed for common pathogenic bacteria within 4 hours of collection. In situations where the samples were not analysed immediately, they were stored at - 80°C until analyses. Laboratory analyses was done in two steps whereby the first step involved analysis for bacterial quality which was carried out by establishing total bacterial counts (TBC) followed by isolation of possible pathogens in milk. The second step of analysis was determination of antibiotic resistance of isolated milk-borne bacteria.

3.7.4 Determination of microbial quality of raw cow's milk

3.7.4.1 Preparation and storage of media

The media used in this study were all prepared following the manufacturer's instructions and the details of the preparation are shown below.

- **Plate Count Agar**

The medium (PCA powder, Biolab, Merck-Modderfontein-1645-South Africa, HG0000C6.500, Batch 1042483) is recommended for the determination of plate counts of microorganisms in food, water, waste water and also from clinical samples. It is composed of Tryptone 5g, Yeast extract 2.5g, Dextrose (Glucose) 1g, Agar 15g and final pH (at 25°C) 7.0±0.2. The medium was prepared according to the manufacturer's instructions whereby 23.5 grams of the powder media was suspended in 1 litre of distilled water. The medium was heated to boil to dissolve completely then sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. The medium was then cooled to 45-50°C, mixed well and pour into sterile Petri plates. The prepared media plates were stored under refrigeration temperature until used.

- **Buffered Peptone Water (BPW)**

The medium (BPW powder, Biolab, Merck-Modderfontein-1645-South Africa, HG000C134.500, Batch 104667) is composed of 10 g/l Peptone, 5 g/l Sodium chloride, 3.5 g/l

Di-sodium phosphate and 1.5 g/l Potassium di-hydrogen phosphate. The medium was prepared according to manufacturer's instructions whereby 20 g of the powdered medium was dissolved in 1 liter of distilled water. The culture medium was mixed well and each 10 ml were dispensed into capped test tubes. The test tubes were then sterilized by autoclaving at 121°C for 15 minutes and cooled to 25°C before use. All the unused prepared media plates were being stored under refrigeration temperature.

- **Nutrient Agar (NA)**

The medium NA (NA powder, Biolab, Merck-Modderfontein-1645-South Africa, HG0000C1.500, Batch 1048588) is composed of 5 g/l Gelatin peptone, 3 g/l Beef extract, 15 g/l Bacteriological agar and final pH of 6.8 ± 0.2 at 25°C. The medium was prepared according to the manufacturer's instructions whereby, 23 g of the powdered medium was suspended into 1 liter of distilled water, mixed well and left on the bench to stand until the mixture was uniform. The mixed solution was then heated with gentle agitation and boiled until it was completely dissolved. The medium solution was sterilized in the autoclave at 121°C for 15 minutes then allowed to cool to 45ºC and poured onto sterile Petri dishes. The plates were left at room temperature for two hours for the media to solidify then put upside down in the incubator for 24 hours at 37°C to check for sterility and to dry the condensed vapor on the plate cover.

- **Nutrient Broth**

The medium (NB powder, Biolab, Merck-Modderfontein-1645-South Africa, HG000C24.500, Batch 1042840) is a general purpose fluid medium for the cultivation of micro-organisms not exacting in their nutritional requirements. It is composed of Lab-Lemco' powder 1g, Yeast extract 2g, Peptone 5g, Sodium chloride 5g and pH 7.4 ± 0.2 at 25°C. The medium was prepared according to the manufacturer's instructions by adding 13g to 1 litre of distilled water, mixed well and distributed into final containers. The medium was sterilized by autoclaving at 121°C for 15 minutes. The prepared media was stored below 25°C.

- **MacConkey Agar-without crystal violet.**

The medium (MA powder, Biolab, Merck-Modderfontein-1645-South Africa, HG00C141.500, Batch 1043119) is composed of 20 g/l Peptone, 10 g/l Lactose, 5 g/l Bile salts, 5 g/l Sodium chloride, 0.075 g/l Neutral red, 12 g/l Agar and final of pH 7.4 ± 0.2 at 25°C. The medium was prepared according to the manufacturer's instructions whereby 52 g of the powdered medium was suspended into 1 liter of distilled water. The medium was boiled to dissolve completely followed by sterilization through autoclaving at 121°C for 15 minutes and cooled to below 45°C, then poured onto sterile Petri dishes. The plates were left at room temperature for two hours for the media to solidify then put upside down in the incubator for 24 hours at 37°C to check for sterility and to dry the condensed vapor on the plate cover.

- **Mueller-Hinton (MH) Agar**

The medium (MHA powder, Lab M, BL9 7JJ-Lancashure- United Kingdom, Lot 147611130) is composed of 300 g/l Beef, dehydrated infusion, 17.5 g/l Casein hydrolysate, 1.5 g/l Starch, 17 g/l Agar and final pH of 7.3 ± 0.1 at 25°C. The medium was prepared according to the manufacturer's instructions whereby 38 g of the powdered medium was suspended into 1 litre of distilled water, mixed well and brought to boil to dissolve the medium completely. The medium was then sterilized by autoclaving at 121°C for 15 minutes, cooled to below 45°C and poured into sterile Petri dishes. The plates were left at room temperature for two hours for the media to solidify then put upside down in the incubator for 24 hours at 37°C to check for sterility and to dry the condensed vapor on the plate cover.

- **Mannitol Salt Agar (MSA)**

MSA is a selective medium (PCA powder, Biolab, Merck-Modderfontein-1645-South Africa, HG000C26.500, Batch 10444408) prepared for the isolation of presumptive pathogenic *staphylococci*. It is composed of `Lab-Lemco' powder 1g, Peptone 1g, Mannitol 10g, Sodium chloride 75g, Phenol red 0.025g, Agar 15g and pH 7.5 ± 0.2 at 25°C. The medium was prepared according to the manufacturer's instructions by suspending 111g in 1 litre of

distilled water, mixed well and boiled to dissolve completely. The medium was sterilized by autoclaving at 121°C for 15 minutes, cooled to below 45°C and poured into sterile petri dishes.

- **Eosin Methylene Blue (EMB) Agar**

EMB is an isolation medium (EMB powder, Biolab, Merck, KGaA-Darmstadt -1627-Germany, Lot VM693347518) for the differentiation of the *Enterobacteriaceae*. The medium was prepared according to the manufacturer's instructions by suspending 37.5g in 1 litre of distilled water and bringing it to boil to dissolve completely. It was then sterilized by autoclaving at 121°C for 15 minutes, cooled to 60°C and shaken in order to oxidise the methylene blue (i.e. restore its blue colour) and to suspend the precipitate which was an essential part of the medium.

- **Xylose lysine desoxycholate (XLD) Agar**

XLD is a selective medium (XLD powder, Biolab, Merck-Modderfontein-1645-South Africa, HG000C21.500, Batch 1041192) for the isolation of *Salmonella* and *Shigella* from clinical specimens and foods. The media is composed of Yeast extract 3g, L- Lysine HCL 5g, Xylose 3.75g, Lactose 7.5, Sucrose 7.5g Sodium desoxycholate 1g, Sodium chloride 5g, Sodium thiosulphate 6.8g, Ferric ammonium citrate 0.8g, Phenol red 0.08g, agar 12.5g and pH 7.4 ± 0.2 at 25°C. The medium was prepared according to the manufacturer's instructions whereby was suspended in 1 litre of distilled water, heated with frequent agitation until the medium boiled. The medium was transferred immediately to a water bath at 50°C, poured into sterile Petri dishes as soon as the medium cooled.

- **Normal saline solution**

The solution was prepared by dissolving 0.85 g of Sodium chloride (Sigma-Aldrich, Co., USA, Cat. S5886, Lot SLBC3215V) into 100 ml of sterile distilled water, mixed well and sterilized by autoclaving at 121°C for 15 minutes and cooled to below 45°C. The solution was then ready for use.

- **Ready to use media**

Prepared ready to use plated sheep blood agar (base) media plates provided by Merck (Pty) Ltd (Modderfontein, 1645, South Africa) were kept in the cold room prior to use.

3.7.4.2 Laboratory procedures

- **Total Bacterial Count (TBC)**

Total bacterial count (TBC) determination was done according to ISO 4833-1:2013 protocols.

- **Preparation, inoculation and incubation of samples**

Milk samples were removed from the refrigerator, thawed at room temperature then enriched in Peptone Water for 24 hours prior to serial dilution. A total of 10 small sterile test tubes were labelled 10^{-1} to 10^{-10} and dispensed with 9 ml of sterile buffered peptone water (BPW). Ten-fold serial dilution from 10^{-1} to 10^{-10} was done into sterile BPW solution using disposable sterile pipette tips whereby 1 ml of the prepared inoculum was transferred into a test tube containing 9 ml of BPW (10^{-1} dilution). From the resulting solution (10^{-1} solution), 1 ml was transferred into the second test tube and the procedure was repeated up to 10^{-10} dilution whereby 1 ml of the inoculum was discarded in the last dilution. Using a vortex, the bacterial suspensions were mixed thoroughly for 10 seconds. Prior to inoculation, the prepared plate count agar (PCA) plates were removed from the cold room and kept at room temperature then labelled. Using sterile pipettes, 2 sterile PCA plates were each inoculated with 0.1 ml of the solution from each dilution (10^{-1} to 10^{-10}) and incubated at 37ºC under aerobic condition for 24- 48 hours to allow the bacterial growth.

- **Computing and recording counts**

The plates (two plates per sample counted both to get the average) with the best countable range between 30 and 300 colony forming units per plate (cfu/plate) were selected and colonies were counted manually. Using a marker, a plate was divided into 4 quarters and the colonies were counted, recorded and their mean was calculated. The number of colony forming units

were calculated, recorded and expressed in colony forming units per ml (CFU/ml) using the following formula:

Number of bacteria = (Number of colony forming unit (CFU))/ (Volume plated (ml) × total dilution factor).

- **Detection and isolation of common bacteria in raw milk**

For each sample, the remaining initial suspension from the bacterial suspensions prepared for TBC was used to carry out bacterial detection and identification. The aim was to isolate any bacteria (pathogenic and non-pathogenic) present in milk without any limitations to certain pathogens. The detection and isolation of common bacteria in raw milk was carried out according to the protocols approved by ISO 6579:2002, which was done in the following steps:

Step 1: Enrichment in a non-selective medium

Buffered Peptone Water (BPW) liquid medium was used for pre-enrichment where by 1 ml of the initial suspension was transferred into a small bottle containing BPW and the suspensions were incubated at 37ºC for 24 hours.

Step 2: Incubation in a non-selective medium

Using a sterile pipette, 0.1 ml of the culture obtained in step 1 was inoculated onto nutrient agar using a sterile spreader and the plates were inverted then incubated 37ºC for 24 hours to allow growth.

Step 3: Plating out on several media plates

Using a sterile loop, nutrient agar cultures obtained in step 2 were inoculated on several media plates including XLD, EMB, Mannitol Salt Agar, Sheep Blood agar and MacConkey agar plates (already prepared plated media). The plates were inverted and incubated at 37ºC for 24 hours. After incubation period, colonies were purified by sub-culturing onto the new media plates and incubated again at 37ºC for 24 hours.

Each milk sample was therefore inoculated in all four different agar plates used and the procedure was done for all test samples.

3.7.4.3 Bacterial identification

The cultures obtained (Figure 9) were all subjected to Gram staining as the first technique of bacterial identification followed by various biochemical tests. Following the biochemical tests, the Analytical Profile Index tests were done for classification of bacteria and lastly, the confirmation of bacterial isolates by molecular approach.

- **Gram staining**

Gram staining was done according to the protocols approved by Cornell University whereby a pure colony from the agar plate was transferred onto a sterile slide using a sterile loop and mixed with a drop of sterile water. The slide was then heat-fixed by passing it over a flame several times using forceps. The slide was placed on a staining tray, flooded with crystal violet solution and left for 1 minute with the stain. The crystal violet stain was then rinsed off with distilled water and the slide was flooded with iodine solution and allowed to remain for 1 minute. The iodine solution was then rinsed off with distilled water. Holding the slide on a tilt, it was flooded with decolourizer (95% alcohol) for 2 seconds and rinsed off with distilled water. The slide was flooded with safranin and allowed to remain for 30 seconds then rinsed with distilled water. The slide was then dried with paper towel and placed in an upright position. The procedure was done for all the plates with pure colonies. The slide was microscopically examined under 100X objective whereby several fields on the slide where observed for bacterial organisms. Gram-positive bacteria stain deep violet to blue and gram-negative bacteria stain pink to red. The cells were observed for both colour retained and shape (cocci or rod).

- **Identification of isolates by biochemical tests**

A number of conventional biochemical tests including catalase, oxidase, indole and coagulase tests were done for bacterial identification followed by Analytical Profile Index (API) done for bacterial classification.

- **Catalase test**

Catalase test is used to differentiate bacteria that produces an enzyme catalase, such as *Staphylococcus*, from non-catalase producing bacteria such as *Streptococcus*. The slide

method was done for catalase test. Here a sterile loop was used to transfer a small amount of colony on a clean and dry glass slide. A drop of 3% hydrogen peroxide was added to the colony on the slide then observed for evolution of oxygen bubbles. Catalase positive bacteria produced bubbles and catalase negative bacteria do not produce bubbles. The procedure was done for all the plates with pure colonies.

- **Oxidase test**

Oxidase test is a biochemical test used to identify bacteria that produce cytochrome c oxidase (aerobic bacteria) which is an enzyme of the bacterial electron transport chain. A filter paper soaked with the substrate tetramethyl-p-phenylenediamine dihydrochloride was moistened with a sterile distilled water. A colony to be tested was then picked with a wooden loop and smeared in the filter paper. The inoculated area of paper was then observed for a colour change to deep blue or purple within 10-30 seconds. Oxidase positive bacteria developed dark purple colour and the negative ones had no colour change. The procedure was done throughout for all the test plates.

- **Indole test**

Indole test is used to identify the bacteria which has the ability to split amino acid tryptophan to form the compound indole, example being *Escherichia coli.* The test was done using a conventional tube method whereby sterilized test tubes containing 4 ml of tryptophan broth were inoculated with a pure colony and incubated at 37°C for 24-28 hours. Using a sterile pipette, 0.5 ml of Kovac's reagent was added to the broth culture and the reaction was observed. Positive indole bacteria formed a pink ring on top of the medium within seconds and the negative ones had no colour change.

- **Coagulase**

Coagulase test is used to differentiate *Staphylococcus aureus* (coagulase positive) from coagulase negative staphylococcus. The slide test method was used whereby a drop of

saline water was placed on each end of a slide and a portion of the isolated colony was emulsified in each drop to make two thick suspensions using a sterile wire loop. A drop of rabbit plasma was then added to one of the suspensions, well mixed and the reaction was observed within 10 seconds. Coagulase positive bacteria formed clots and coagulase negative ones had no clumping. The plasma was not added to the second suspension to be able to differentiate false positivity such as granular appearance of the organism from the true coagulase clumping.

- **Analytical Profile Index (API)**

API is a commercial miniaturized biochemical system used for the identification, characterization, and differentiation of bacterial isolates (Kipyegon, 2017). Based on the Gram-staining and biochemical tests, bacterial isolates were selected and subjected for API test. The API 20E was used for the identification of *Enterobacteriaceae* and other non-fastidious Gram-negative rods, API 20 Strep for the identification of *Streptococceae* and related organisms, API Staph for the identification of *Staphylococcus, Micrococcus* and related genera, and API 20A was used for the identification of anaerobes. The procedures were done according to the manufacturer's instructions (API, BioMerieux SA).

❖ **API 20E**

I. Preparation of the strip

An incubation box consisting of tray and lid were prepared then 5ml of distilled water was distributed into the honeycombed wells of the tray in order to create a humid atmosphere then, the strain reference was recorded on the elongated flap of the tray. The strip was removed from its packaging and placed in the incubation box.

II. Preparation of the inoculum

An ampule of API NaCl 0.85 % Medium (5 ml) was open then using a pipette, a single pure colony from an isolation plate (18-24 hours old) was picked and transferred into the suspension medium. The suspension was carefully emulsified to achieve a homogeneous bacterial suspension which was used immediately after preparation.

III. Inoculation of the strip

Using the same pipette, the tube as well as the cupule of tests citrate utilization (CIT), acetoin (VP) and gelatinase (GEL) were filled with the bacterial suspension. For the other tests, only the tubes and not the cupules were filled with the bacterial suspension. Anaerobiosis was created in the tests arginine-dihydrolase (ADH), lysine decarboxylase (LDC), ornithine decarboxylase (ODC), Hydrogen-sulfide (H_2S) and Urease (URE) by overlaying with mineral oil. The incubation box was then closed and incubated at 36°C for 18-24 hours.

IV. Reading the strip

After the incubation period, the strip was read by referring to the Reading Table provided by the manufacturer. If 3 or more tests including glucose (GLU) test were positive, all the spontaneous reactions were recorded on the result sheet and the tests which require the addition of reagents were revealed. For tryptophan deaminase (TDA) Test: 1 drop of TDA reagent was added and reddish brown colour was observed which indicated a positive reaction to be recorded on the result sheet. For indole (IND) Test: 1 drop of JAMES reagent was added and a pink colour was observed in the whole cupule which indicated a positive reaction to be recorded on the result sheet. For VP Test: 1 drop each of VP 1 and VP 2 reagents were added and left for 10 minutes to react. A pink or red colour was observed which indicated a positive reaction to be recorded on the result sheet. For those strips whereby the number of positive tests including the GLU test before adding the reagents was less than 3, the strip was re-incubated for a further 24 hours without adding any reagents then reagents were added later following reading of the strip after re-incubation.

V. Interpretation

Identification was obtained with the numerical profile. On the result sheet, the tests were separated into groups of 3 and a value 1, 2 or 4 was indicated for each. By adding the values corresponding to positive reactions within each group, a 7-digit profile number was obtained for the 20 tests of the API 20 E strip.

VI. Identification

This was performed using the database (V4.0) with the Analytical Profile Index. With the identification software, the 7-digit numerical profile was entered manually via the keyboard and the results were obtained.

❖ **API 20 Strep**

I. Preparation of the strip

An incubation box consisting of tray and lid were prepared then 5ml of distilled water was distributed into the honeycombed wells of the tray in order to create a humid atmosphere then, the strain reference was recorded on the elongated flap of the tray. The strip was removed from its packaging and placed in the incubation box.

II. Preparation of the inoculum

An ampule of API Suspension Medium (2 ml) was open then all the culture from the previously prepared subculture plate was harvested using a swab, to create a dense suspension with a turbidity greater than 4 McFarland. The suspension was therefore used immediately after preparation.

III. Inoculation of the strip

The suspension was distributed in the first half of the strip (tests VP to ADH) while avoiding the formation of bubbles by tilting the strip slightly forward then placing the tip of the pipette against the side of the cupule. For the tests VP to LAP, 100 µl of the suspension was distributed into each cupule. For the ADH test, only the tube was filled. In the second half of the strip (tests RIB to GLYG), an ampule of API GP Medium was open and the rest of the suspension (approximately 0.5 ml) was distributed into it and mixed well. The new suspension was then distributed into the tubes only and the cupule of the underlined tests (ADH to GLYG) were filled with mineral oil to form a convex meniscus. The lid was then placed on the tray which was then incubated at 36°C in aerobic conditions for 4 - 4 ½ hours to obtain a first reading and for 24 hours to obtain a second reading.

IV. Reading the strip After 4 hours of incubation:

The reagents were added for the tests requiring addition of reagents. For VP test, 1 drop of each of VP 1 and VP 2 were added and 2 drops of NIN were added to HIP test. For PYRA, αGAL, ßGUR, ßGAL, PAL and LAP tests, 1 drop of each of ZYM A and ZYM B were added. The reactions were then read after 10 minutes by referring to the Reading Table.

V. Interpretation

On the result sheet, the tests were separated into groups of 3 and a number 1, 2 or 4 was indicated for each. The values corresponding to positive reactions within each group were added to obtain a 7-digit number was obtained.

VI. Identification is obtained with the numerical profile.

This was performed using the database (V 7.0) with the apiweb identification software whereby the 7-digit numerical profile was manually entered via the keyboard.

❖ API staph

I. Preparation of the strip

An incubation box consisting of tray and lid were prepared then 5ml of distilled water was distributed into the honeycombed wells of the tray in order to create a humid atmosphere and the strain reference was recorded on the elongated flap of the tray. The strip was removed from its packaging and placed in the incubation box.

II. Preparation of the inoculum

The pure suspected cultures of Micrococcaceae family isolates (based on morphology, Gram stain, and catalase results) were subcultured onto Columbia blood agar for 18-24 hrs at 36°C. An ampule of API Staph Medium was open and a homogeneous bacterial suspension with a turbidity equivalent to 0.5 McFarland was prepared and the suspension was used immediately.

III. Inoculation of the strip

The micro-tubes (only the tube portion and not the cupules) were filled with the inoculated API Staph Medium using a pipette while avoiding the formation of bubbles by tilting the strip slightly forward and placing the tip of the pipette against the side of the cupule. The cupules were filled with mineral oil to form a convex meniscus to ensure anaerobiosis in the ADH and URE tests. The incubation box was then closed and incubated at 36°C for 18-24 hours.

IV. Reading the strip

Following the incubation period, the reactions were developed by adding reagents to those tests requiring addition of reagents and all the reactions were read by referring to the Reading Table. For VP test, 1 drop of VP 1 and VP 2 reagents were added, left to react for 10 minutes then a violet-pink colour indicating a positive reaction was observed. For NIT test, 1 drop of NIT 1 and NIT 2 reagents were added and left to react in 10 minutes before observing a red colour indicating a positive reaction. For PAL test, ZYM A and ZYM B reagents were added and left to react in 10 minutes. A violet colour indicating a positive reaction was observed.

V. Interpretation

On the result sheet, the tests were separated into groups of 3 and a number 1, 2 or 4 was indicated for each. The values corresponding to positive reactions within each group were added to obtain a 7-digit number was obtained.

VI. Identification is obtained with the numerical profile

This was performed using the database (V 4.1) with the apiweb identification software whereby the 7-digit numerical profile was manually entered via the keyboard.

❖ API 20 NE

I. Preparation of the strip

An incubation box consisting of tray and lid were prepared then 5ml of distilled water was distributed into the honeycombed wells of the tray in order to create a humid atmosphere and the strain reference was recorded on the elongated flap of the tray. The strip was removed from its packaging and placed in the incubation box.

II. Preparation of the inoculum

An ampule of API NaCl 0.85 % Medium (2 ml) was open then using a pipette, two well isolated colonies of identical morphology from an isolation plate (18-24 hours old) were picked and transferred into the suspension medium. The suspension was prepared with a turbidity equivalent to 0.5 McFarland and used immediately.

III. Inoculation of the strip

Tests NO3 to PNPG were inoculated by distributing the saline suspension into the tubes only and not the cupules using the same pipette. To avoid the formation of bubbles at the base of the tubes, the strip was slightly tilted forward and the tip of the pipette placed against the side of the cupule. An ampule of API AUX Medium was open as indicated by the manufacturer then 200 µl of the remaining saline suspension was added to the ampule, homogenised well with the pipette to avoid the formation of bubbles.

The tubes and cupules of tests GLU to PAC were all filled with the suspension. Mineral oil was added to the cupules of the 3 underlined tests (GLU, ADH and URE) until a convex meniscus was formed. The incubation box was then closed and incubated at 29°C for 24 hours.

IV. Reading the strip

After the incubation period, the strip was read by referring to the Reading Table provided. All spontaneous reactions (GLU, ADH, URE, ESC, GEL and PNPG) were recorded on the result sheet.

The assimilation tests were covered with the incubation box lid during the reading of the NO3 and TRP tests to protect them from airborne contamination.

For NO3 test: 1 drop of NIT 1 and 1 drop of NIT 2 reagents were added to the NO3 cupule then after 5 minutes, a red colour indicating a positive reaction was observed to be recorded on the result sheet.

An amount of 2-3 mg of Zn reagent was added to the NO3 cupules of negative reactions which may have been due to the production of nitrogen.

After 5 minutes, a cupule remaining colourless indicates a positive reaction to be recorded on the result sheet. If the cupule turns pink-red, the reaction is negative as nitrates were present in the tube and were reduced to nitrite by the zinc. The reaction was then read after 5 minutes and cupules remaining colourless indicated a positive reaction to be recorded on the result sheet and for those that turned pink-red, the reaction was considered negative as nitrates were present in the tube and were reduced to nitrite by the zinc.

For TRP test: 1 drop of JAMES reagent was added to TRP test. As the reaction takes place immediately, a pink colour which develops in the whole cupule indicating a positive reaction was observed to be recorded on the result sheet.

V. Interpretation

On the result sheet, the tests were separated into groups of 3 and a number 1, 2 or 4 was indicated for each. The values corresponding to positive reactions within each group were added to obtain a 7-digit number was obtained.

VI. Identification was obtained with the numerical profile.

The identification was performed using the database (V7.0) with the apiweb identification software, whereby the 7-digit numerical profile was manually entered via the keyboard.

❖ **API 20 A**

I. Preparation of the inoculum

An ampule of API 20A Medium was open then using a swab, all the growth obtained on blood agar (pure 18-24 hours old colonies) was harvested two well isolated colonies of identical morphology from an isolation plate (18-24 hours old). Holding the ampule upright, the organisms were emulsified through rubbing and rotating the swab against the side of the ampule while avoiding taking it out of the suspension medium. The final turbidity greater than 3 McFarland was maintained and the suspension was used immediately.

II. Preparation and inoculation of the strip

An incubation box consisting of tray and lid were prepared then 5ml of distilled water was distributed into the honeycombed wells of the tray in order to create a humid atmosphere and the strain references were recorded on the elongated flap of the tray. The API 20A strip was removed from its packaging and placed in the incubation box. The strip was inoculated with the suspension in the ampule, while avoiding the formation of bubbles by tilting the strip slightly forward. Both the tube and the cupule were filled with the suspension for GEL test while for IND, only the tube was filled with the suspension and the cupule was filled with mineral oil to avoid the evaporation of indole. The incubation box was then closed with the lid and incubated at 36°C for 24 hours in an anaerobic jar.

III. Reading the strip

After the incubation period, the strip was read by referring to the Reading Table provided. All spontaneous reactions (tests which do not require the additional reagents) were recorded on the result sheet. The tests requiring the additional reagents were revealed and the presence of Bromocresol purple (BCP) in the reaction medium was discoloured by reduction. A single drop of BCP reagent was added to all the micro-tubes containing carbohydrates to reveal the acidification. A positive reaction was marked by a yellow or yellow-green colour and was recorded on the results sheet. For IND test, 1 drop of XYL reagent was added into the mineral oil overlay then mixed using a wooden applicator stick and left for three minutes. A single drop of EHR reagent was then added and the results were read within five minutes. Positive reactions marked by a red colour were recorded on the results sheet. For CAT test, the determination of catalase production was done after the exposure of the strip to air for thirty minutes. Two drops

of 3% hydrogen peroxide were added to micro-tubes showing positive reactions. Positive reactions were marked with the presence of bubbles and were recorded on the results sheet.

IV. Interpretation

On the API 20A results sheet, the tests were separated into groups of 3 and a number 1, 2 or 4 was indicated for each. The values corresponding to positive reactions within each group were added to obtain an 8-digit profile number.

V. Identification was obtained with the numerical profile.

The identification was performed using the database (V4.0) with the apiweb identification software, whereby the 8-digit numerical profile was manually entered via the keyboard.

3.7.4.4 Confirmation of bacterial isolates by molecular approach

- **Extraction of genomic DNA**

A commercial DNA extraction kit was used to extract DNA for downstream identification of pathogens in raw milk samples from cow following the manufacturer's instructions.

Total genomic DNA of cultivated isolates were purified following Zymo-Research Fungal/Bacterial DNA kit instructions. Individual colonies were suspended in 1 ml sterile distilled water in Eppendorf tubes and centrifuged at 10.000 rpm for 5 min and the supernatant was discarded. Briefly, pellets were suspended in 750 µl lysis solution and vortexed at 14000 rpm for 5 min, followed by centrifugation at 10 000 rpm for 1 min. A volume of 400 µl of the upper aqueous phase was transferred into a new Eppendorf tube and centrifuged at 7000 rpm for 1 min. A volume of 1200 µl of buffer was added to the filtrate and 800 µl of the mixture was transferred to the new collection tube and centrifuged at 10.000 rpm for 1 min. The filtered DNA was pre-washed by adding 200 µl DNA pre-wash buffer and centrifuged at 10.000 rpm for 1 min. 500 µl of DNA wash buffer was added to the new collection tube and centrifuged at 10.000 rpm for 1 min. Finally, 100 µl of DNA elution buffer was added to elute the DNA in a clean 1.5 ml micro-centrifuge tube. The concentration of purified DNA was assessed using a Nano-drop instrument (Bio-Rad, South Africa).

- **Polymerase chain reaction (PCR) amplification**

Conventional PCR was used to identify pathogens in milk samples using universal primers.

The 16S rDNA nucleotide sequences was determined by PCR using Engine DYAD Peltier thermal cycler (BioRad, USA). Reaction volume of 50 µl, containing: 25 µl PCR Master Mix, 2 µl template DNA, 19 µl nuclease free water and 2 µl of each oligonucleotide primer was prepared as reported by Ntougias *et al*. (2004). The forward primer 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and the reverse 1492R (5'-TGA CTG ACT GAG GCT ACC TG-3') were commercially synthesised by Inqaba Biotechnical Industrial (Pty) Ltd, Pretoria, South Africa. Thermal cycling conditions were as follows: one cycle of initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 61°C for 30 s and extension at 72°C for 5 min, followed by final cycle of extension of 72°C for 7 min and incubated at 4°C. Amplified PCR products were resolved in 1% agarose gel stained with ethidium bromide (10 µg/ml) and visualised with Syngene Ingenius Bioimager (UK) to confirm the expected size of the product. The 16S rDNA sequences of the isolated bacteria were deposited in GenBank database.

- **Nucleotide sequence determination**

PCR products of the 16S rDNA of the strains were analysed for nucleotide sequence determination by using ABI PRISM® 3500XL DNA Sequencer (Applied Biosystems) at Inqaba Biotechnical Industrial (Pty) Ltd, Pretoria, South Africa. All confirmed sequencing results obtained in this study were cleaned using Finch-TV 1.4, analysed and edited using Bio-Edit version 7.0.4 softwares (Hall, 1999). The homology of partial sequences obtained were compared with the sequences from the DNA databases and similarity showing above 90% were retrieved by nucleotide Basic Local Alignment Search Tool (BLAST) program at the National Centre for Biotechnology Information (NCBI).

- **Phylogenetic analysis**

The 16S rDNA sequences of the strains were alligned using MEGA X software then used to search the GenBank database determine relative phylogenetic positions. To identify putative close phylogenetic relatives, multiple sequence alignments were obtained using Clustal-X version 1.83 against corresponding nucleotide sequences retrieved from GenBank. Evolutionary distance matrices were generated as described by (Jukes and Cantor, 1969). Phylogenetic analysis was performed using the program MEGA X (Tamura *et al*., 2011) and neighbour joining (Saitou and Nei, 1987), maximum-parsimony (Rzhetsky and Nei, 1992), and maximum likelihood analyses was obtained for each gene sequence (Fitch, 1986). The methods were used in order to expound on the phylogeny and for better comparison. Bootstrap analysis were performed using 1000 replications for neighbour joining, maximum-parsimony, maximum likelihood. The sequences were checked for putative chimeric artefacts using the Chimera-Buster program. No chimeric sequences were compared to the closest relatives in the NCBI GenBank database by BLAST program. Manipulation and tree editing were carried out using Tree View.

3.7.5 Determination of antimicrobial resistance of common milk-borne bacteria

For assessment of antimicrobial susceptibility tests, all the isolated milk-borne bacteria previously stored in 20% glycerol were inoculated onto Nutrient Broth, incubated for some time to activate the microorganisms then the inocula were streaked onto Nutrient agar (NA) plates. The plates were inverted and incubated at 37ºC for 24 hours. After 24 hours the microorganisms were tested for their susceptibility to a panel of eight commonly used antibiotics in veterinary and human practices. The discs used (Oxoid® Ltd., Basingstoke, Hampshire, England) were impregnated with antibiotics and their corresponding concentrations (in brackets) were as follows; Oxytetracycline (OT: 30 µg), Gentamicin (CN: 10 µg), Cephalothin (KF: 30 µg), Amoxycillin (AML: 10 µg), Sulphonamides (S3: 300 µg), Streptomycin (S: 10 µg), Erythromicin (E: 5 µg) and Norfloxacin (NOR: 5 µg). These antibiotics were selected based on availability and usage at farm level. Antibacterial susceptibility test was performed on Muller-Hinton (MH) agar by agar disc diffusion method according to Clinical and Laboratory Standards Institute (CLSI, 2009) guidelines. Well-

isolated direct colonies were suspended into bijou tubes containing 2 ml of sterile normal saline and the suspensions adjusted to a turbidity equivalent to a 0.5 McFarland standard using Vitek colorimeter (Lenexa, Kansas, USA). Sterile cotton-tipped swabs were then dipped into the suspensions to transfer the inocula onto MH agar plates and spread evenly on the entire surface to produce a confluent lawn of bacterial growth. After drying the plates with inocula for few minutes, antibiotic discs were placed over inoculated plates using disc dispenser (Oxoid® Ltd., Basingstoke, Hampshire, England) and sterile forceps. Thereafter, the plates were inverted and incubated under aerobic conditions at 37°C for 24 hours. After incubation period, the plates were examined for zones of inhibition around the discs. Diameters of inhibition zones around the discs were measured in millimeter (mm) using a Vernier metal caliper (Cat No: 121-012-14). The results were recorded and classified as resistant (1), intermediate (2) and sensitive (3) according to the general guidelines prepared by (CLSI, 2009). Standard reference strains of *Staphylococcus aureus* (ATCC® 29213) and *Escherichia coli* (ATCC® 25922) were used as quality control organisms in antimicrobial susceptibility determination (Boss *et al*., 2016).

3.8. Data analysis

3.8.1 Statistical analysis for Sociological data

The statistical analysis was performed using SPSS for Windows version 25.0. The focus of the analysis was on producing overall descriptive statistics using frequencies and percentages. Moreover, inferential statistics was performed to draw conclusions on the different views between male and female respondents towards animal health and hygiene practices through a Chi- square test.

3.8.2. Statistical analysis for laboratory data

The data collected was analysed using descriptive and inferential statistics such as means, frequency distribution and percentage using SPSS software (ver.25.0) for TBC and Antibiotic resistance data analysis. In addition, ANOVA test was used to compare the mean bacterial counts from different areas and lastly, the Post Hoc test (LSD) was used to determine where the significant differences are and their magnitudes. Furthermore, a Chi-square test was used to determine the relationship between different antibiotics and bacterial resistance and the statistical significance was confirmed using the Kruskal-Wallis test (used because the data

analysed was ordinal). Lastly, Two Stage cluster analysis was used to yield two main clusters (antibiotics).

3.9 Chapter 3 References

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CHAPTER FOUR: RESULTS

4.1 DEMOGRAPHIC CHARACTERISTICS AND PARTICULARS OF RESPONDENTS

The questionnaire (Appendix 1) was administered to 100 respondents, those who had lactating cows during sampling as well as those who normally milk when having lactating cows. The demographic characteristics and particulars of respondents are presented in Table 4.1. According to the results obtained, a large number of respondents (93%; n= 93) were found to be male. Most of the respondents were aged between 31 and 50 years and majority (70%; n= 70) had only obtained primary school education. Results reported here further show that the majority of the respondents (95%; n= 95) were not the owners of the farms, but most of them (87%, n= 87) owned cattle within the farms. In addition, 90% (n= 90) of the participants had been practicing this type of farming for more than three years and 4% (n= 4) depended on small scale dairy farming as the only source of income. Evidence from this study shows that on most farms (63%; n=63), there are more than three people working in the farm with majority (95%; n=95) being herd caretakers.

Table 4. 1: Socio-demographic characteristics of respondents

Demographic characteristics	Descriptions	Frequency	(%)
Age (years)	15-20	**0**	**0.0**
	21-30	**4**	**4.0**
	31-40	**34**	**34.0**
	41-50	**48**	**48.0**
	More than 50	**14**	**14.0**
	Don't know	**0**	**0**
Gender	Male	**93**	**93.0**
	Female	**7**	**7.0**
Education	No school education attendance	**18**	**18.0**
	Primary school level	**70**	**70.0**
	Secondary school level	**7**	**7.0**
	Tertiary education level (College, University, Technicon)	**1**	**1.0**
	Religious schooling only	**4**	**4.0**
Farm ownership	Yes	**5**	**5.0**
	No (Go to q.9)	**95**	**95.0**
Cattle ownership	Yes	**87**	**87.0**
	No	**13**	**13.0**
How long have you been farming?	Less than a year	**0**	**0.0**
	1 to 2 years	**1**	**1.0**
	2 to 3 years	**9**	**9.0**
	More than three years	**90**	**90.0**
Is farming cattle main Source of income?	Yes	**4**	**4.0**
	No	**96**	**96.0**
Number of personnel	Less than 3 people	**20**	**20.0**
	More than 3 people	**63**	**63.0**
	I work alone	**17**	**17.0**
Role in the farm	Herd care taker	**95**	**95.0**
	Manager	**5**	**5.0**

4.1.2 Animal Health and Management System

Animal health and management systems appear to be almost similar in all the farms as shown in Table 4.2. For example, on most (67%; n=67) farms, every member of the household or farm is allowed to milk. In addition, only 3% (n=3) of the participants indicated that they wash their hands before milking but none of them said that they use disinfectants or soap to wash their hands and only 24 participants wash the udder and teats of cows before milking sometimes using cold water. In almost all (99%) the farms the milking technique practiced is hand-milking through pulling of teats also known as stripping. It is also shown that majority (92%) of the respondents are able to recognise udder infections in cows mostly (70%) through the unusual warmth of the udder. The majority (84%; n=84) of respondents indicated that they only milk animals with udder infections after milking the healthy ones then discard the milk from such animals.

Table 4. 2: Mastitis related questions

Assessed Parameters	Descriptions	Frequency	(%)
Primary milker	Myself	**4**	**4.0**
	Family member only	**0**	**0.0**
	Employees	**28**	**28.0**
	All of us	**67**	**67.0**
	Others	**1**	**1.0**
Amount of milk produced	Less than 5 L	**0**	**0.0**
	Between 5-10 L	**0**	**0.0**
	Between 10-20 L	**88**	**88.0**
	More than 20 L	**12**	**12.0**
Hand washing	Yes	**3**	**3.0**
	No (go to q.14)	**68**	**68.0**
	Sometimes	**29**	**29.0**
Use of disinfectants for hand washing	Water only	**3**	**3.0**
	Water with soap	**0**	**0.0**
	Water with a disinfectant and soap	**0**	**0.0**
Washing of udder and teats	Yes	**0**	**0.0**
	No (Go to q.16)	**76**	**76.0**
	Sometimes	**24**	**24.0**
Use of disinfectants (udder and teats washing)	Warm water only	**0**	**0.0**
	Cold water	**24**	**24.0**
	Water with a disinfectant or detergent	**0**	**0.0**
Milking technique	Hand milking	**99**	**99.0**
	Machine milking (go to q.18)	**1**	**1.0**
Hand milking technique	Stripping (Pulling the teat)	**100**	**100**
	Squeezing Action	**0**	**0**
Recognition of udder infection	Yes	**92**	**92.0**
	No(go to q.23)	**8**	**8.0**
Detection of udder infection	Change of colour of the udder/teats	**5**	**5.0**
	Udder feels warm than usual	**70**	**70.0**
	Changed consistency of the udder	**9**	**9.0**
	Changed size of the udder	**0**	**0.0**
	Presence of visible lesion on the udder	**0**	**0.0**
	Udder veins are engrossed	**0**	**0.0**
	Changed milk consistency and colour	**8**	**0.8**
	Others	**0**	**0.0**
Milking of animals with udder infections	Yes	**84**	**84.0**
	No (go to q.23)	**8**	**0.8**
	Sometimes	**0**	**0.0**
Time of milking cows with udder infection	Before the healthy animals	**0**	**0.0**
	After the healthy animals	**84**	**84.0**
Purpose of milk from an infected udder	Discard	**84**	**84.0**
	Consume in your household	**0**	**0.0**
	Sale to the market	**0**	**0.0**

	Feed to calves	**0**	**0.0**
	Others	**0**	**0.0**

4.1.3 Milk production and practices that could expose one to hazards

Most respondents (86%; n=86) indicated that raw milk was produced for household consumption, followed by 14% (n=14) who indicated that they sell milk to the neighbors. In most (89%; n=89) farms, plastic containers are used for milk storage and majority (91%; n=91) of the respondents do not keep their milk under the shade during milking process. Furthermore, milk is stored for less than 2 hours at room temperature by most respondents (81%; n=81) after milking. Majority (95%; n=95) of the households indicated that they consume raw milk. A large number (80%; n=80) of households consume milk from sick animals, and over half (69%; n=69) of them sell milk from recently treated animals to the public. It is evident that very few (11%; n=11) respondents follow the withdrawal periods of drugs administered to animals. The results are presented in table 4.3 below.

Table 4. 3: The purpose of production of raw-milk production and practices that could result in exposure to hazards like zoonotic diseases and drugs

Assessed Parameters	Descriptions	Frequency	(%)
Purpose of milk production	Consume the milk as a household	**86**	**86.0**
	Sell to milk vendors	**0**	**0.0**
	Sell to local businesses	**0**	**0.0**
	Sell to milk processing company	**0**	**0.0**
	Sell to neighbours and members of the community	**14**	**14.0**
	Other	**0**	**0.0**
Raw milk consumption in the family	Always (regarded as yes)	**95**	**95.0**
	Sometimes (regarded as yes)	**5**	**5.0**
	No	**0**	**0.0**
Boiling of milk	Yes	**29**	**29.0**
	No	**71**	**71.0**
Containers used to store milk	Calabash (Wooden)	**0**	**0.0**
	Plastic container	**89**	**89.0**
	Glass bottle	**0**	**0.0**
	Metal can	**11**	**11.0**
	Don't know	**0**	**0.0**
Storage under shade during milking	Yes	**9**	**9.0**
	No	**91**	**91.0**
Milk storage duration under room temperature	Less than 2 hrs	**81**	**81.0**
	Between 2 – 6 hrs	**16**	**16.0**
	Between 6 – 12 hrs	**3**	**3.0**
	More than 12 hrs	**0**	**0.0**
Milk from sick animals	Don't milk the animal	**16**	**16.0**
	Sell the milk	**4**	**4.0**
	Consume it in the family	**80**	**80.0**
	Use it for the calves	**0**	**0.0**
	Other (specify)	**0**	**0.0**
Milk obtained from recently treated animals	Don't milk the animal	**13**	**13.0**
	Sell the milk	**69**	**69.0**
	Milk the animal and discard the milk	**18**	**18.0**
	Consume it in the family	**0**	**0.0**
	Use it for the calves	**0**	**0.0**
	Other	**0**	**0.0**
Withdrawal periods	I follow the withdrawal period of the medication	**11**	**11.0**
	For as long as I want to	**70**	**70.0**
	Never wait at all	**19**	**19.0**

4.1.4 Veterinary antibiotic use

The results in Table 4.4 indicate that a relatively large proportion (87%; n=87) of respondents were aware without being informed on what antibiotics are, while the rest (13%; n=13) could only express awareness after they had been educated. However, all the participants confirmed to have administered antibiotics to their animals and majority (83%) administer antibiotics when the animals are showing signs of sickness while only few (17%) make use of antibiotics when instructed by the veterinarian. Moreover, only few (2%) always read instructions before antibiotic administration.

Table 4. 4**:** Veterinary antibiotic use

Assessed Parameters	**Descriptions**	**Frequency**	**(%)**
Antibiotic awareness	Yes	**87**	**87.0**
	No (question elaborated)	**13**	**13.0**
Administration of antibiotics	Yes	**100**	**100.0**
	No	**0**	**0.0**
Instances of administration	Only when the animal is sick or showing signs of bacterial infection	**83**	**83.0**
	Only when instructed by a Veterinarian	**17**	**17.0**
	There is a period set for administration of antibiotics	**0**	**0.0**
	Anytime I feel like administering antibiotics	**0**	**0.0**
Reading of instructions	Always	**2**	**2.0**
	Never	**5**	**5.0**
	Sometimes	**93**	**93.0**

4.1.5 Inferential statistics

From the results above, it is evident that most (68%) of the respondents do not wash their hands and most (92%) are able to recognise udder infections. However, the demographic characteristics of the farmers may be influencing the attitude of respondents towards the two variables. Inferential statistics was therefore performed to determine the possible relationship between the variables mentioned and some of the demographic characteristics of the farmers.

For inferential analysis, a Chi- square test was run at p-value >0.05 to test if washing of hands and recognition of udder infections depend on gender and level of education of farmers. However, there is no significant relationship between the tested variables as the p-value is greater than 0.5 throughout. Therefore, both washing of hands and recognition of udder infections do not depend on gender and level of education of farmers.

The Chi- square results are as follows (Table 4.5 and Table 4.6):

Table 4. 5**:** Cross tabulation of washing of hands before milking vs Gender and Education

Association between washing of hands and:	**Chi-Square Tests**	**Value**	**df**	**Asymp.Sig/P-value (2-sided)**
Gender	Likelihood Ratio	1.441	2	0.487
Education	Likelihood Ratio	13.095	8	0.109

Table 4. 6**:** Cross tabulation of recognition of udder infections vs Gender and Education

Association between recognition of udder infections and:	**Chi-Square Tests**	**Value**	**df**	**Asymp.Sig (2-sided)**
Gender	Likelihood Ratio	2.884	1	0.089
Education	Likelihood Ratio	7.125	4	0.129

4.2 Bacteriological Quality of Raw Cow Milk

4.2.1 Total Bacterial Counts (TBC)

A total of 168 milk samples from the selected villages were cultured for total bacterial count (TBC). Following 10^{-10} dilutions, it was found that 100% (n=168) of the samples had bacterial growth while petri-dishes from 10^{-5} dilutions were the most suitable for a count between 30 and 300 colonies throughout, which were then selected as representatives for TBC of each sample. The total bacterial count Table (Appendix 2) shows the results of all samples tested for TBC. The results showed that the overall TBC ranged from 1.94 x 10^7 to 4.36 x 10^7 cfu/ml. Mean TBC for all the samples tested (Table 4.7) was 3.1 x 10^7cfu/ml which is higher than the maximum recommended level of 5.0 x 10^4 cfu/ml according to the Foodstuffs, Cosmetics and Disinfectants Act, No. 54 of 1972: Regulations relating to milk and dairy products, No. R.1555. As stipulated by the results, all (100%) milk samples tested had higher TBC than the maximum acceptable level of 5.0x10^4cfu/ml. This is an indication that the quality of raw cow's milk from all the selected areas of Mafikeng was poor.

Table 4. 7**:** Statistical data for Total Bacterial Count of all test samples (n= 168)

Variable	**Figure (×10^7)**
Mean	**3.0893**
Std. Deviation	**0.52875**
Minimum	**1.94**
Maximum	**4.36**

4.2.2 Statistical Analysis

The Statistical Package for the Social Sciences (SPSS) version 25.0 was used to summarize the data distribution of TBC results for each village (8 villages) as shown in figures 1 to 8. In addition, the ANOVA and Post Hoc (LSD) tests were used for further analysis. Lastly, the two stage cluster analysis of antibiotics was done (Table 4.8 and Table 4.9) as shown below.

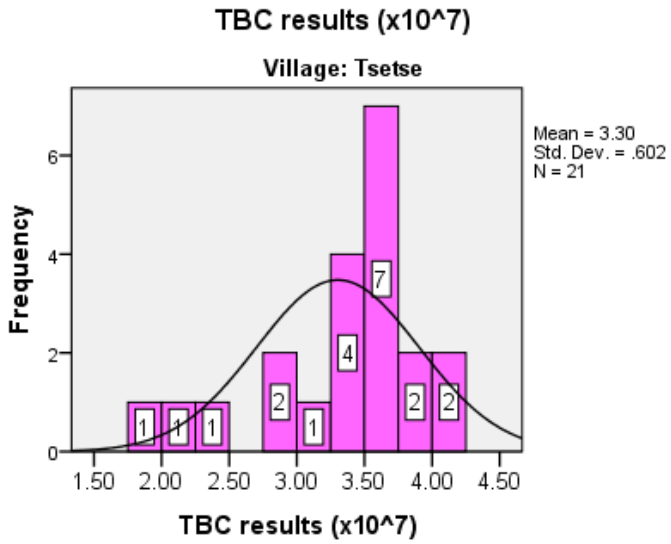

Figure 1 shows the statistical analysis of TBC in Tsetse village

Figure 1 shows that the mean TBC in Tsetse village was $3.3*10^7$ with N=21 and the distribution of the bacteria count is slightly skewed to the left, indicating that there are more upper counts of bacteria than lower counts in the area. The modal class of bacteria count is $3.5*10^7$ to $<3.75*10^7$.

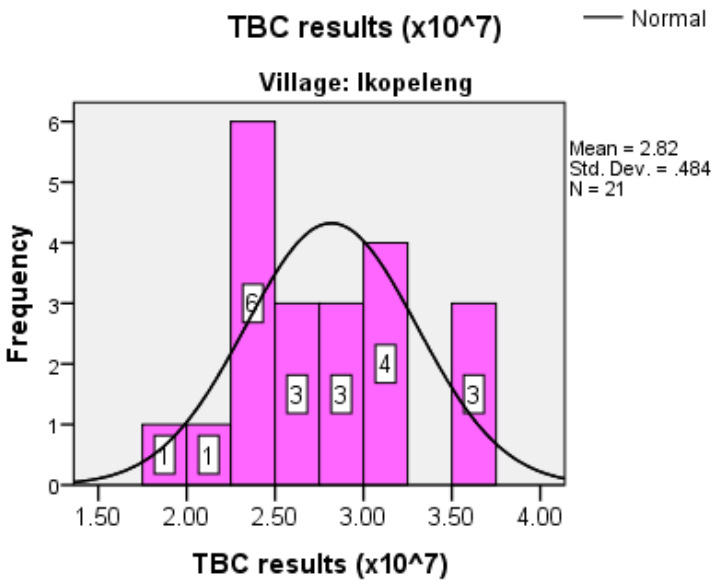

Figure 2 shows the statistical analysis of TBC in Ikopeleng village

Figure 2 shows that the mean TBC in Ikopeleng village was $2.82*10^7$ with N=21 and the distribution of the bacteria count is almost normally distributed indicating that there are more counts of bacteria distributed in between the lowest and highest counts in the area. The modal class of bacteria count is $2*10^7$ to $<2.25*10^7$.

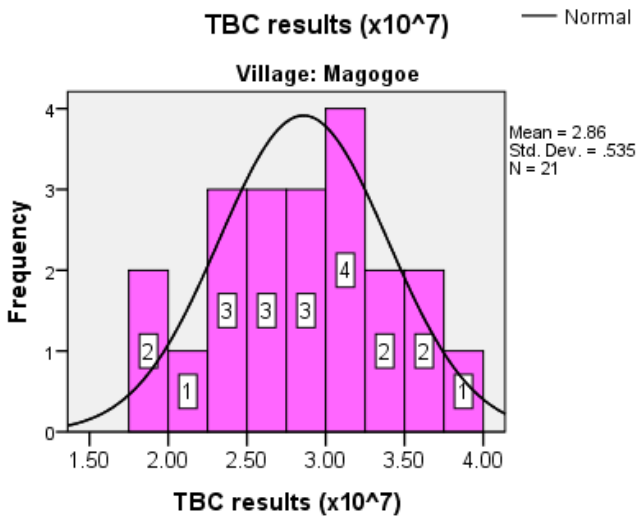

Figure 3 shows the statistical analysis of TBC in Magogoe village

Figure 3 shows that the mean TBC in Magogoe village was $2.86*10^7$ with N=21 and the distribution of the bacteria count is almost normally distributed indicating that there are more counts of bacteria distributed in between the lowest and highest counts in the area. The modal class of bacteria count is $3*10^7$ to $<3.25*10^7$.

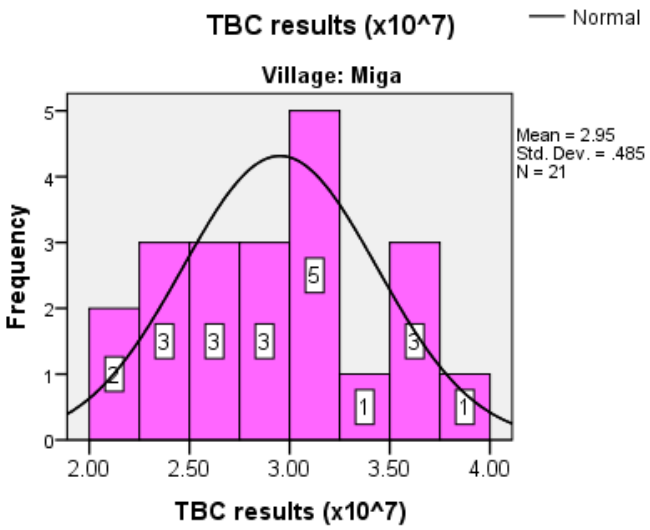

Figure 4 shows the statistical analysis of TBC in Miga village

Figure 4 shows that the mean TBC in Miga village was $2.95*10^7$ with N=21 and the distribution of the bacteria count is very close to a normal distribution indicating that there are more counts of bacteria distributed in between the lowest and highest counts in the area. The modal class of bacteria count is $3*10^7$ to $<3.25*10^7$.

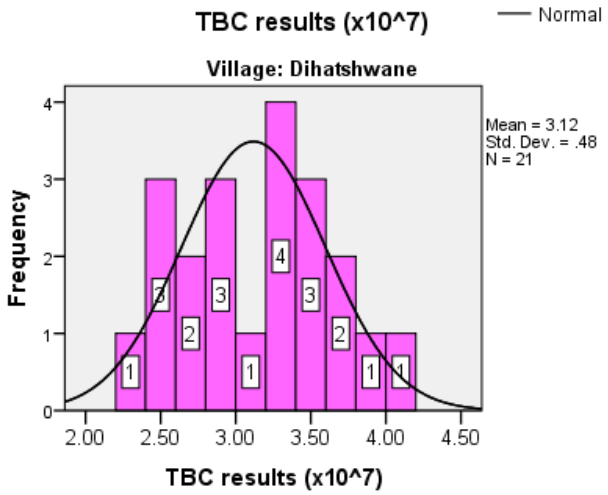

Figure 5 shows the statistical analysis of TBC in Dihatshwane village

Figure 5 shows that the mean TBC in Dihatshwane village was $3.12*10^7$ with N=21 and the distribution of the bacteria count is almost normally distributed indicating that there are more counts of bacteria distributed in between the lowest and highest counts in the area. The modal class of bacteria count is $3.25*10^7$ to $<3.5*10^7$.

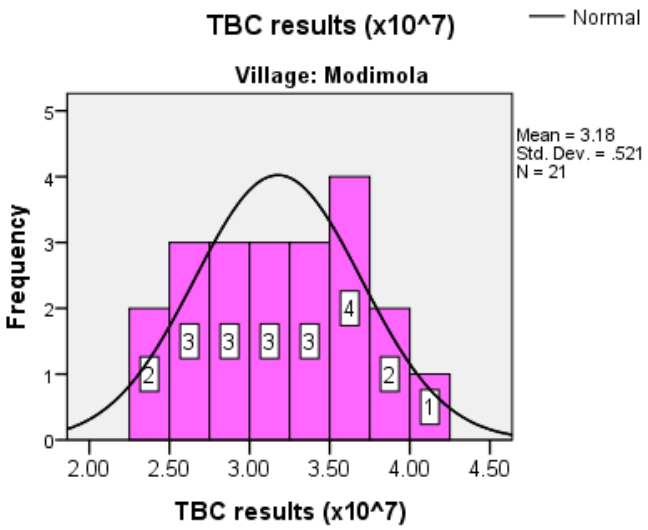

Figure 6 shows the statistical analysis of TBC in Modimola village

Figure 6 shows that the mean TBC in Modimola village was $3.18*10^7$ with N=21 and the distribution of the bacteria count is almost normally distributed indicating that there are more counts of bacteria distributed in between the lowest and highest counts in the area. The modal class of bacteria count is $3.5*10^7$ to $<3.75*10^7$.

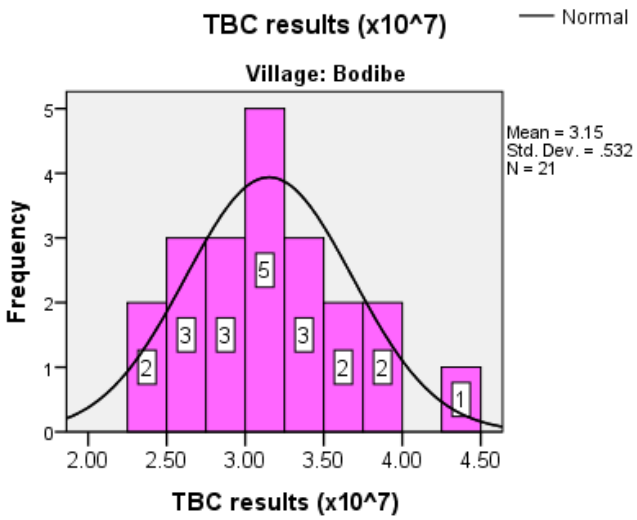

Figure 7 shows the statistical analysis of TBC in Bodibe village

Figure 7 shows that the mean TBC in Bodibe village was $3.15*10^7$ with N=21 and the distribution of the bacteria count is almost normally distributed indicating that there are more counts of bacteria distributed in between the lowest and highest counts in the area. The modal class of bacteria count is $3*10^7$ to $<3.25*10^7$.

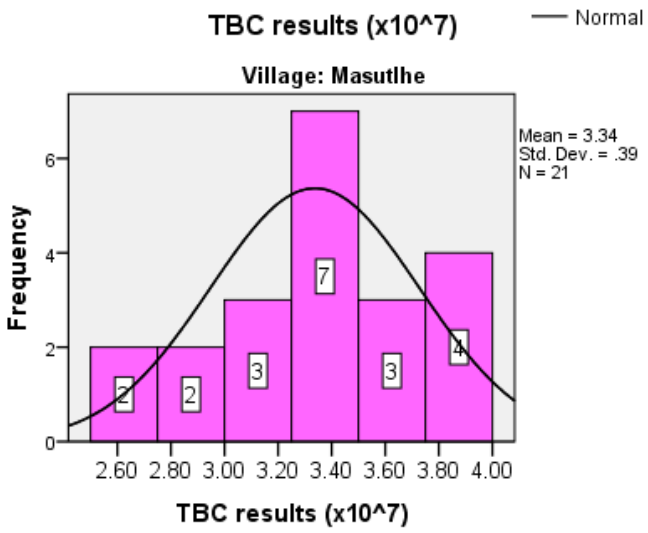

Figure 8 shows the statistical analysis of TBC in Masutlhe village

Figure 8 shows that the mean TBC in Masutlhe village was $3.34*10^7$ with N=21 and the distribution of the bacteria count is very close to a normal distribution indicating that there are more counts of bacteria distributed in between the lowest and highest counts in the area. The modal class of bacteria count is $3.25*10^7$ to $<3.5*10^7$.

In summary, by visual inspection of the histograms in Figures 1-8, the means and modal classes of the count of bacteria differ from one area to the other, , but an ANOVA test of difference in means is used next to confirm the statistical significance of these visual differences.

Table 4. 8**:** ANOVA test results for TBC

ANOVA					
TBC results (x10^7)					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5.575	7	.796	3.099**[1]	.004
Within Groups	41.114	160	.257		
Total	46.689	167			

The p-value corresponding to the ANOVA test is less than 0.05, therefore, the mean bacteria count differ significantly across the areas. The following table of Post Hoc test (LSD) determines where these significant differences are and their magnitude.

[1] **significant at 1%

Table 4.9: Post Hoc Test (LSD) results for TBC

Some areas differ significantly (p-value < 0.05) in terms of the mean bacteria count (Table 4.9). For example, the mean bacteria count from Tsetse is significantly higher than the ones from Ikopeleng, Magogoe, Miga (Table 4.9) respectively. Also, the mean bacteria count from Ikopeleng village is significantly lower than that of Modimola, Bodibe and Masutlhe (Table 4.9). The results also show that the mean bacteria count from Magogoe is significantly less than that of Modimola by $0.479*10^7$, and the mean count from Miga village is less than that from Masutlhe by $0.386*10^7$. The other pairs do not show any statistical significance (p-values >0.05).

Table 4. 9**:** Post Hoc Test (LSD) results for TBC

(I) Village	**(J) Village**	Mean Difference (I-J)	**Std. Error**	**Sig.**
Tsetse	Ikopeleng	.48476*[2]	0.15644	0.002
	Magogoe	.44476*	0.15644	0.005
	Miga	.35143*	0.15644	0.026
	Dihatshwane	0.185	0.15644	0.239
	Modimola	0.126	0.15644	0.423
	Bodibe	0.151	0.15644	0.335
	Masutlhe	-0.034	0.15644	0.827
Ikopeleng	Magogoe	-0.040	0.15644	0.799
	Miga	-0.133	0.15644	0.395
	Dihatshwane	-0.300	0.15644	0.057
	Modimola	-.35905*	0.15644	0.023
	Bodibe	-.33333*	0.15644	0.035
	Masutlhe	-.51905*	0.15644	0.001
Magogoe	Miga	-0.093	0.15644	0.552
	Dihatshwane	-0.260	0.15644	0.098
	Modimola	-.31905*	0.15644	0.043
	Bodibe	-0.293	0.15644	0.063
	Masutlhe	-.47905*	0.15644	0.003
Miga	Dihatshwane	-0.167	0.15644	0.288
	Modimola	-0.226	0.15644	0.151
	Bodibe	-0.200	0.15644	0.203
	Masutlhe	-.38571*	0.15644	0.015
Dihatshwane	Modimola	-0.059	0.15644	0.706
	Bodibe	-0.033	0.15644	0.832
	Masutlhe	-0.219	0.15644	0.163
Modimola	Bodibe	0.026	0.15644	0.87
	Masutlhe	-0.160	0.15644	0.308
Bodibe	Masutlhe	-0.186	0.15644	0.237

[2] * significant at 5%

4.2.2 Biochemical tests and Molecular approach

The results of the Gram stain, catalase, oxidase, indole and coagulase for the 168 samples in the biochemical tests are in Appendix 3, followed by Analytical Profile Index (API) for identification of bacteria. It was found that 56 out of 168 isolates were responsive to the API tests. Following the API tests, fifty-six (56) bacterial isolates identified by API tests were subjected for molecular approach by PCR. The results revealed the presence of the PCR product bands of the isolates as presented in Figures 10 and 11. The desired 1kb base pairs fragments were obtained after running the gel on electrophoresis for 65 minutes. Table 4.10 shows the API and Molecular approach results. Phylogenetic analysis was then done to assess the relationships of 56 16S rDNA nucleotide sequences isolated from raw milk in this study, with 21 most similar sequences obtained from NCBI GenBank as shown on the phylogenetic tree (Figure 12).

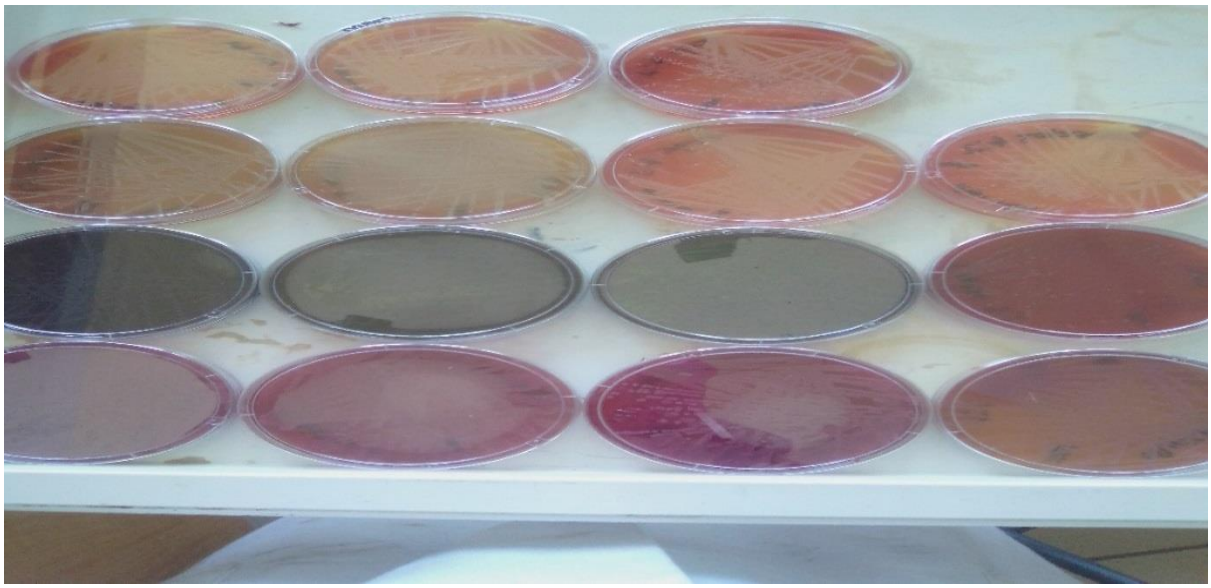

Figure 9 shows the bacterial cultures in different media plates (XLD, EMB, Mannitol Salt Agar, Sheep Blood agar and MacConkey agar plates).

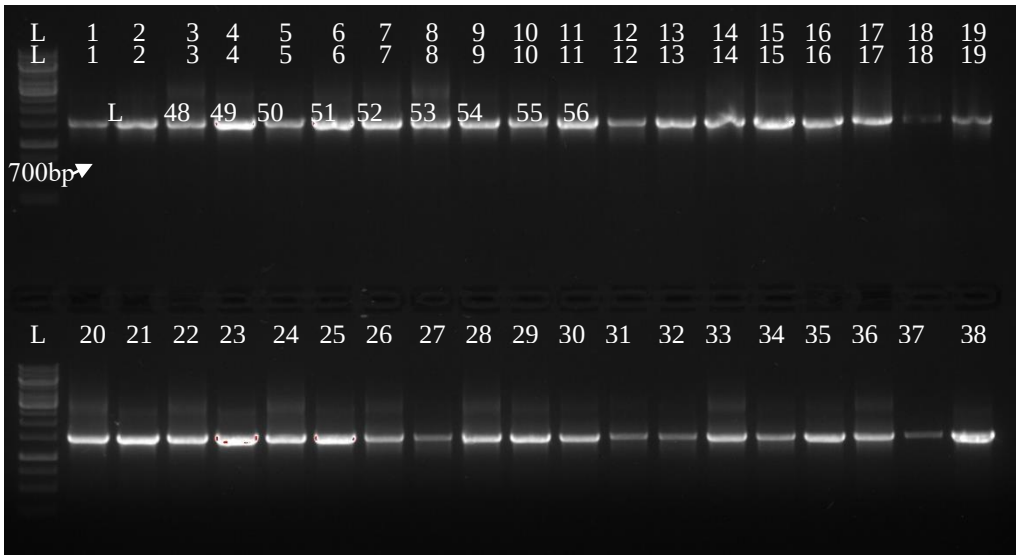

Figure 10 shows agarose Gel electrophoresis of PCR products using 16S rDNAprimers of presumed bacterial isolates. L is the 1.5kb ladder whereas numbers 1-38 represents different isolates.

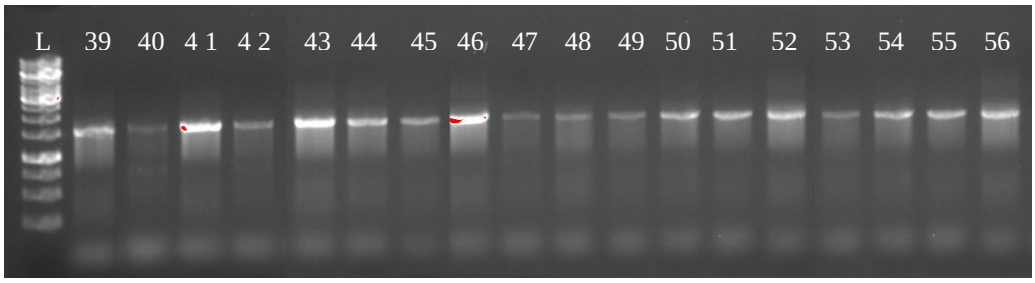

Figure 11 shows agarose Gel electrophoresis of PCR products using 16S rDNA primers of presumed bacterial isolates. L is the 1.5kb ladder whereas numbers 39-56 represents different isolates

BLAST results for the 16S region showed that the isolates had 95-100% similarity to the following species: *Staphylococcus aureus (n=9), Staphylococcus equorum (1), Enterococcus faecium (6), Enterococcus faecalis (n=9), Enterococcus durans (n=1), Enterococcus casseliflavus (n=1), Enterococcus sp (n=1), Enterobacter hormaechei (2), Enterobacter asburiae (1), Enterobacter sp. (2), Clostridium bifermentas (5), Clostridium sp. (4), Paraclostridium benzoelyticum (1), Salmonella* Typhimurium *(1), Escherichia coli (2),Shigella sp. (2), Bacillus sp. (1), Alcaligenes faecalis (1), Citrobacter braakii (1),Proteus hauseri (1), Providencia sp. (1), Lactococcus garvieae (1), Lactococcus lactis (1) and Weissella cibaria (1).*

Table 4. 10**:** API and Molecular approach results of 56 isolates

Sample ID	API	16sDNA	Gene Bank Accession no.	Homology	Obtained Accession no.
Ma 1	*Staphylococcus aureus*	*Staphylococcus aureus*	KR085871.1	99	MH346228
Ma 2	*Enterococcus faecium*	*Enterococcus faecium*	KJ571212.1	97	MH346229
Ts 1	*Proteus* sp.	*Proteus hauseri*	KY072916.1	99	MH346230
Mi 1	*Enterococcus faeium*	*Enterococcus* sp.	KJ210577.1	98	MH346231
Ts 2	*Enterococcus* sp.	*Enterococcus faecalis*	KY697082.1	98	MH346232
Ik 1	*Enterobacter hormaechei*	*Enterobacter hormaechei*	KF015738.1	99	MH346233
Ik 2	*Citrobacter* sp.	*Citrobacter braakii*	HQ288930.1	99	MH346234
Mi 2	*Shigella dysenteriae*	*Shigella* sp.	DQ337523.1	99	MH346235
Mi 3	*Staphylococcus aureus*	*Staphylococcus aureus*	MG818959.1	99	MH346236
Ts 3	*Enterococcus durans*	*Enterococcus faecalis*	AY692453.1	95	MH346237
Ik 3	*Staphylococcus aureus*	*Staphylococcus aureus*	MF144454.1	99	MH346238
Di 1	*Enterococcus faecalis*	*Enterococcus faecalis*	AB669429.1	99	MH346239
Ik 4	*Enterococcus faecalis*	*Enterococcus faecalis*	KT260655.1	97	MH346240
Di 2	*Enterococcus casseliflavus*	*Enterococcus casseliflavus*	KM096606.1	99	MH346241
Mi 4	*Enterococcus faecalis*	*Enterococcus faecalis*	KX018438.1	99	MH346242
Mi 5	*Staphylococcus aureus*	*Staphylococcus aureus*	CP019590.1	98	MH346243
Ts 4	*Enterobacter hormaechei*	*Enterobacter hormaechei*	KP119816.1	99	MH346244
Ik 5	*Enterococcus faecalis*	*Enterococcus faecalis*	KY697083.1	98	MH346245
Di 3	*Enterobacter asburiae*	*Enterobacter* sp.	JQ726698.1	99	MH346246
Ma 3	*Enterobacter asburiae*	*Enterobacter asburiae*	KF360066.1	99	MH346247
Di 4	*Clostridium biffermentans*	*Clostridium* sp.	LC194786.1	99	MH346248
Di 5	*Clostridium bifermentas*	*Clostridium bifermentans*	KT633856.1	97	MH346249
Ma 4	*Micrococcus* sp.	*Bacillus* sp.	KF870418.1	95	MH346250
Ik 6	*Staphylococcus aureus*	*Staphylococcus aureus*	JN092619.1	99	MH346251
Ik 7	*Enterococcus faecium*	*Enterococcus faecium*	JN792505.1	98	MH346252

Ts 5	*Clostridium* sp.	*Clostridium bifermentas*	DQ978211.1	99	MH346253
Ts 6	*Clostridium* sp.	*Clostridium bifermentans*	NR_113323.1	99	MH346254
Di 6	*Staphylococcus aureus*	*Staphylococcus aureus*	CP020467.1	99	MH346255
Di 7	*Enterococcus* sp.	*Enterococcus durans*	KX129817.1	95	MH346256
Di 8	*Enterococcus faecium*	*Enterococcus faecium*	JN792502.1	99	MH346257
Mi 6	*Enterococcus* sp.	*Enterococcus faecium*	GU727821.1	97	MH346258
Mi 7	*Weissella* sp.	*Weissella cibaria*	KU302758.1	99	MH346259
Mi 8	*Enterococcus faecium*	*Enterococcus faecium*	KJ571216.1	99	MH346260
Ik 8	*Escherichia coli*	*Escherichia coli*	MH196345.1	98	MH346261
Mi 9	*Salmonella* Typhimurium	*Salmonella* Typhimurium	MH196335.1	98	MH346262
Ik 9	*Alcaligenes* sp.	*Alcaligenes faecalis*	JF682513.2	99	MH346263
Ik 10	*Staphylococcus xylosus*	*Staphylococcus equorum*	KJ920933.1	99	MH346264
Mi 10	*Clostridium bifermentans*	*Clostridium* sp.	MG592375.1	98	MH346265
Ma 5	*Enterococcus faecium*	*Enterococcus faecium*	KM095647.1	99	MH346266
Di 9	*Enterococcus faecalis*	*Enterococcus faecalis*	KY697084.1	99	MH346267
Ik 11	*Clostridium* sp.	*Paraclostridium benzoelyticum*	NR_148815.1	99	MH346268
Mi 11	*Staphylococcus aureus*	*Staphylococcus aureus*	CP021105.1	99	MH346269
Ts 7	*Escherichia coli*	*Escherichia coli*	AB604196.1	99	MH346270
Ma 6	*Providencia stuartii*	*Providencia* sp.	KR232641.1	99	MH346271
Ik 12	*Clostridium* sp.	*Clostridium* sp.	EF052864.1	99	MH346272
Ma 7	*Staphylococcus aureus*	*Staphylococcus aureus*	CP020467.1	99	MH346273
Di 10	*Staphylococcus aureus*	*Staphylococcus aureus*	MG971398.1	99	MH346274
Ts 8	*Shigella dysenteriae*	*Shigella* sp.	GU968176.1	99	MH346275
Ma 8	*Enterococcus faecalis*	*Enterococcus faecalis*	KX261530.1	99	MH346276
Ts 9	*Enterobacter asburiae*	*Enterobacter* sp.	KJ499995.1	99	MH346277
Ma 9	*Clostridium* sp.	*Clostridium* sp.	LN846800.2	99	MH346278
Ts 10	*Lactococcus lactis*	*Lactococcus lactis*	KU291415.1	99	MH346279
Di 11	*Enterococcus faecalis*	*Enterococcus faecalis*	MF582338.1	99	MH346280

Di 12	*Clostridium* sp.	*Clostridium bifermentans*	AF320283.1	99	MH346281
Ts 11	*Lactococcus garvieae*	*Lactococcus garvieae*	LK985397.1	99	MH346282
Ma 10	*Clostridium* sp.	*Clostridium bifermentans*	AB538434.1	99	MH346283

Phylogenetic Analysis of isolates from raw milk

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 3.97447402 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site. This analysis involved 77 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1650 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018).

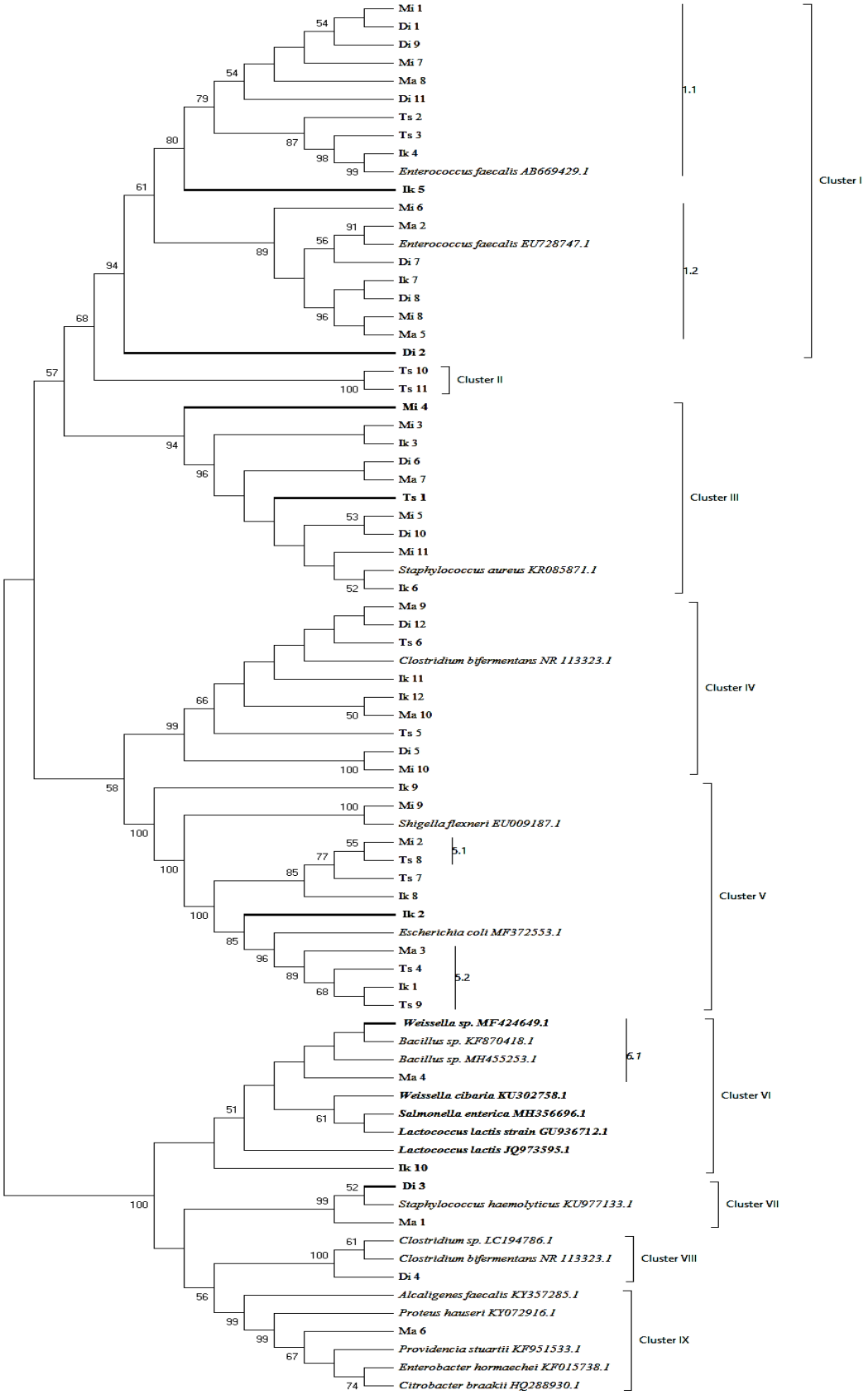

Figure 12 Phylogenetic tree showing the relationships among 16S rDNA gene sequences from raw milk with the most similar sequences obtained from the GenBank.

4.2.3 Antibiotic resistance of isolates

Table 4.11 shows that most (46.4%) of the isolates were susceptible to Norfloxacin while a large proportion (53.6%) was resistant to Streptomycin. Table 4.12 shows resistance profiles of isolates from milk that were multidrug resistant (MDR). *Staphylococcus aureus* isolates had the highest (0.75) margin while *Weissella cibaria* had the lowest (0.13) margin of antibiotic resistance.

The association between antibiotics and bacterial resistance was determined using Chi-square test (Table 4.13) and the association was shown on a clustered bar-chart (Figure 15). The resistance was further analyzed for each antibiotic against all the isolates and the results are presented in Figures 16 to 23. Table 4.14 shows the Kruskal-Wallis test confirming that the distribution of resistance across categories of antibiotics presented in Figures 16 to 23 are significantly different (p-value < 0.05). As presented in Figure 24, the Post-Hoc test confirms that there is a significantly higher number of bacterial isolates that are resistant to S10 than they are to S3-300, KF30 and AML10. The Post-Hoc test further confirms that there is a significantly higher number of bacterial isolates that are susceptible to NOR 5 than they are to OT30, S3-300, CN10, KF30 and E5. Lastly, a two stage cluster analysis (Figures 25 to 28) shows that, the highest bacterial antibiotic resistance was to S10 (53%) and the highest bacterial antibiotic susceptibility was to NOR (46.4%).

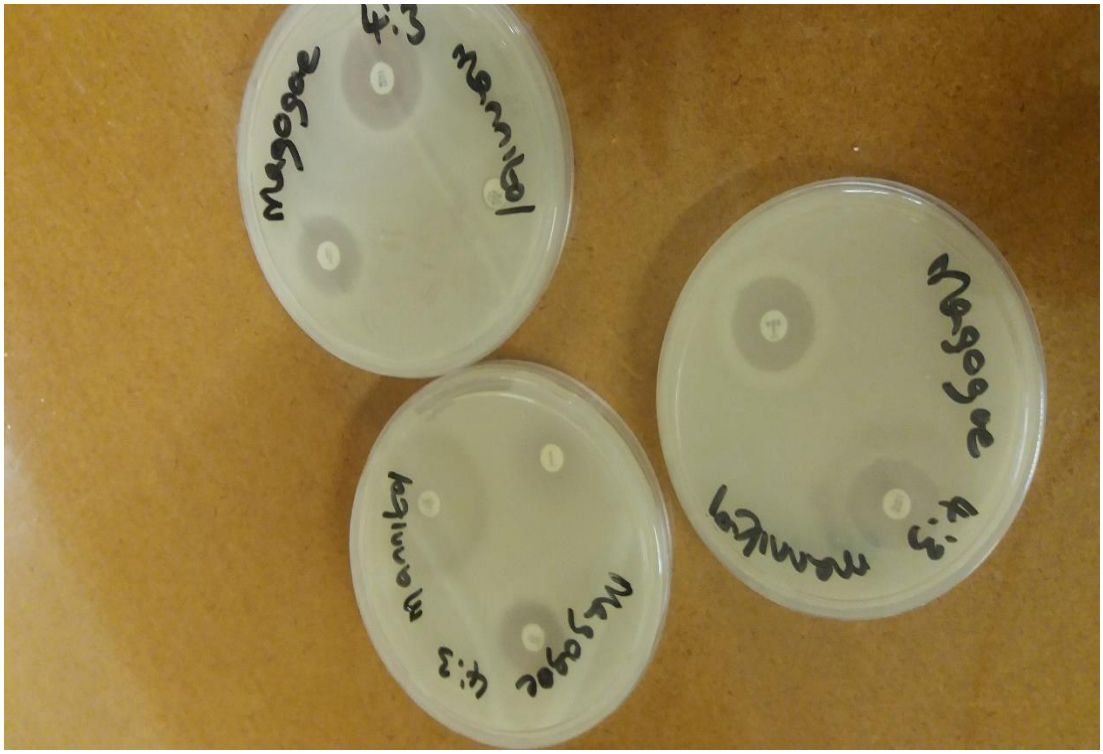

Figure 13 Disc diffusion method for antibiotic resistance test of isolates against Oxytetracycline (OT: 30 µg), Gentamicin (CN: 10 µg), Cephalothin (KF: 30 µg), Amoxycillin (AML: 10 µg), Sulphonamides (S3: 300 µg), Streptomycin (S: 10 µg), Erythromicin (E: 5 µg) and Norfloxacin (NOR: 5 µg).

Table 4. 11**:** Showing the resistance patterns of isolates to different antibiotics

Isolates	**Status**	**KF 30**	**S3 300**	**AML 10**	**S10**	**CN 10**	**OT 30**	**E5**	**NOR 5**
		N(%)	**N(%)**	**N(%)**	**N(%)**	**N(%)**	**N(%)**	**N(%)**	**N(%)**
***Staphylococcus aureus* (9)**	**Resistant**		5 (55.5)	9 (100)	9 (100)	6 (66.7)	9 (100)	9 (100)	
	Intermediate		2 (22.2)			3 (33.3)			
	Susceptible	9 (100)	2 (22.2)						9 (100)
***Staphylococcus equorum* (1)**	**Resistant**		1 (100)	1 (100)	1 (100)	1 (100)	1 (100)		
	Intermediate							1 (100)	
	Susceptible	1 (100)							1 (100)
***Enterococcus faecium* (6)**	**Resistant**	6 (100)			6 (100)	6 (100)	5 (83.3)		
	Intermediate		3 (50)	2 (33.3)			1 (16.7)	2 (33.3)	
	Susceptible		3 (50)	4 (67.7)				4 (67.7)	6 (100)
***Enterococcus faecalis* (9)**	**Resistant**	9 (100)			9 (100)	9 (100)	9 (100)	4 (44.45)	
	Intermediate		1 (11.1)					1 (11.1)	
	Susceptible		8 (88.9)	9 (100)				4 (44.45)	9 (100)
***Enterococcus durans* (1)**	**Resistant**	1 (100)			1 (100)	1 (100)		1 (100)	
	Intermediate						1 (100)		
	Susceptible		1 (100)	1 (100)					1 (100)
***Enterococcus casseliflavus* (1)**	**Resistant**	1 (100)			1 (100)	1 (100)		1 (100)	
	Intermediate		1 (100)				1 (100)		
	Susceptible			1 (100)					1 (100)

***Enterococcus* sp. (1)**	**Resistant**	1 (100)			1 (100)		1 (100)		
	Intermediate		1 (100)	1 (100)		1 (100)			
	Susceptible							1 (100)	1 (100)
***Enterobacter hormaechei* (2)**	**Resistant**	2 (100)		2 (100)	2 (100)		2 (100)	2 (100)	
	Intermediate								
	Susceptible		2 (100)			2 (100)			2 (100)
***Enterobacter asburiae* (1)**	**Resistant**	1 (100)		1 (100)	1 (100)		1 (100)	1 (100)	
	Intermediate								
	Susceptible		1 (100)			1 (100)			1 (100)
***Enterobacter* sp. (2)**	**Resistant**	2 (100)		2 (100)	2 (100)		2 (100)	2 (100)	
	Intermediate								
	Susceptible		2 (100)			2 (100)			2 (100)
***Clostridium bifermentas* (5)**	**Resistant**		5 (100)		5 (100)		1 (20)		1 (20)
	Intermediate								
	Susceptible	5 (100)		5 (100)		5 (100)	4 (80)	5 (100)	4 (80)
***Clostridium* sp. (4)**	**Resistant**		4 (100)		3 (75)		1 (25)		
	Intermediate								
	Susceptible	4 (100)		4 (100)	1 (25)	4 (100)	3 (75)	4 (100)	4 (100)
***Paraclostridium benzoelyticum* (1)**	**Resistant**		1 (100)		1 (100)				1 (100)
	Intermediate								
	Susceptible	1 (100)		1 (100)		1 (100)	1 (100)	1 (100)	
***Salmonella typhimurium* (1)**	**Resistant**		1 (100)		1 (100)		1 (100)		
	Intermediate								

	Susceptible	1 (100)		1 (100)		1 (100)		1 (100)	1 (100)
***Escherichia coli* (2)**	**Resistant**		2 (100)		2 (100)		1 (50)		
	Intermediate								
	Susceptible	2 (100)		2 (100)		2 (100)	1 (50)	2 (100)	2 (100)
***Shigella* sp. (2)**	**Resistant**				2 (100)		2 (100)	1 (50)	
	Intermediate								
	Susceptible	2 (100)	2 (100)	2 (100)		2 (100)		1 (50)	2 (100)
***Bacillus* sp. (1)**	**Resistant**	1 (100)	1 (100)	1 (100)	1 (100)				
	Intermediate								
	Susceptible					1 (100)	1 (100)	1 (100)	1 (100)
***Alcaligenes faecalis* (1)**	**Resistant**				1 (100)		1 (100)		
	Intermediate								
	Susceptible	1 (100)	1 (100)	1 (100)		1 (100)		1 (100)	1 (100)
***Citrobacter braakii* (1)**	**Resistant**		1 (100)		1 (100)		1 (100)		
	Intermediate								
	Susceptibe	1 (100)		1 (100)		1 (100)		1 (100)	1 (100)
***Proteus hauseri* (1)**	**Resistant**		1 (100)		1 (100)		1 (100)		
	Intermediate								
	Susceptibe	1 (100)		1 (100)		1 (100)		1 (100)	1 (100)
***Providencia* sp. (1)**	**Resistant**						1 (100)		1 (100)
	Intermediate								
	Susceptibe	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)		1 (100)	
***Lactococcus garvieae* (1)**	**Resistant**		1 (100)	1 (100)		1 (100)	1 (100)		

	Intermediate							
	Susceptibe	1 (100)		1 (100)			1 (100)	1 (100)
***Lactococcus lactis* (1)**	**Resistant**		1 (100)	1 (100)		1 (100)		
	Intermediate							
	Susceptibe	1 (100)	1 (100)		1 (100)		1 (100)	1 (100)
***Weissella cibaria* (1)**	**Resistant**		1 (100)					
	Intermediate			1 (100)				
	Susceptibe	1 (100)	1 (100)		1 (100)	1 (100)	1 (100)	1 (100)

Table 4. 12**:** Resistance profile of Multi-drug resistant isolates

Resistance Profile		**Antibiotic**	
Isolates	**Number of isolates**	**Resistance Patterns**	**Margin**
Staphylococcus aureus (9)	5	S3-AML-S-CN-OT-E	0.75
	1	AML-S-CN-OT-E	0.63
	3	AML-S-OT-E	0.5
Staphylococcus equorum (1)	1	S3-AML-S-CN-OT	0.5
Enterococcus faecium (6)	5	KF-S-CN-OT	0.5
	1	KF-S-CN	0.38
Enterococcus faecalis (9)	4	KF-S-CN-OT-E	0.63
	5	KF-S-CN-OT	0.5
Enterococcus durans (1)	1	KF-S-CN-E	0.5
Enterococcus casseliflavus (1)	1	KF-S-CN-E	0.5
Enterococcus sp. (1)	1	KF-S-CN-OT	0.5
Enterobacter hormaechei (2)	2	KF-AML-S-OT-E	0.63
Enterobacter asburiae (1)	1	KF-AML-S-OT-E	0.63
Enterobacter sp. (2)	2	KF-AML-S-OT-E	0.63
Clostridium bifermentans (5)	3	S3-S	0.25
	1	S3-S-OT	0.38
	1	S3-S-NOR	0.38
Clostridium sp. (4)	3	S3-S	0.25
	1	S3-OT	0.25
Paraclostridium benzoelyticum (1)	1	S3-S-NOR	0.38
Salmonella Typhimurium (1)	1	S3-S-OT	0.38
Escherichia coli (2)	1	S3-S-OT	0.38
	1	S3-S	0.25
Shigella sp. (2)	1	S-OT-E	0.38
	1	S-OT	0.25
Bacillus sp. (1)	1	KF-S3-AML-S	0.5
Alcaligenes faecalis (1)	1	S-OT	0.25
Citrobacter braakii (1)	1	S3-S-OT	0.38
Proteus hauseri (1)	1	S3-S-OT	0.38
Providencia sp. (1)	1	OT-NOR	0.25
Lactococcus garvieae (1)	1	S3-AML-CN-OT	0.5
Lactococcus lactis (1)	1	S3-S-OT	0.38
Weissella cibaria (1)	1	S3	0.13

Table 4. 13**:** Association between Antibiotics and Bacterial Resistance

Chi-Square Tests			
	Value	**df**	**Asymp. Sig. (2-sided)**
Likelihood Ratio	**157.133****	**14**	**.000**

The chi-square test of association (Table 4.13) shows that a significant association (p-value < 0.01) exists between the antibiotic and resistance. The following clustered bar chart shows this association.

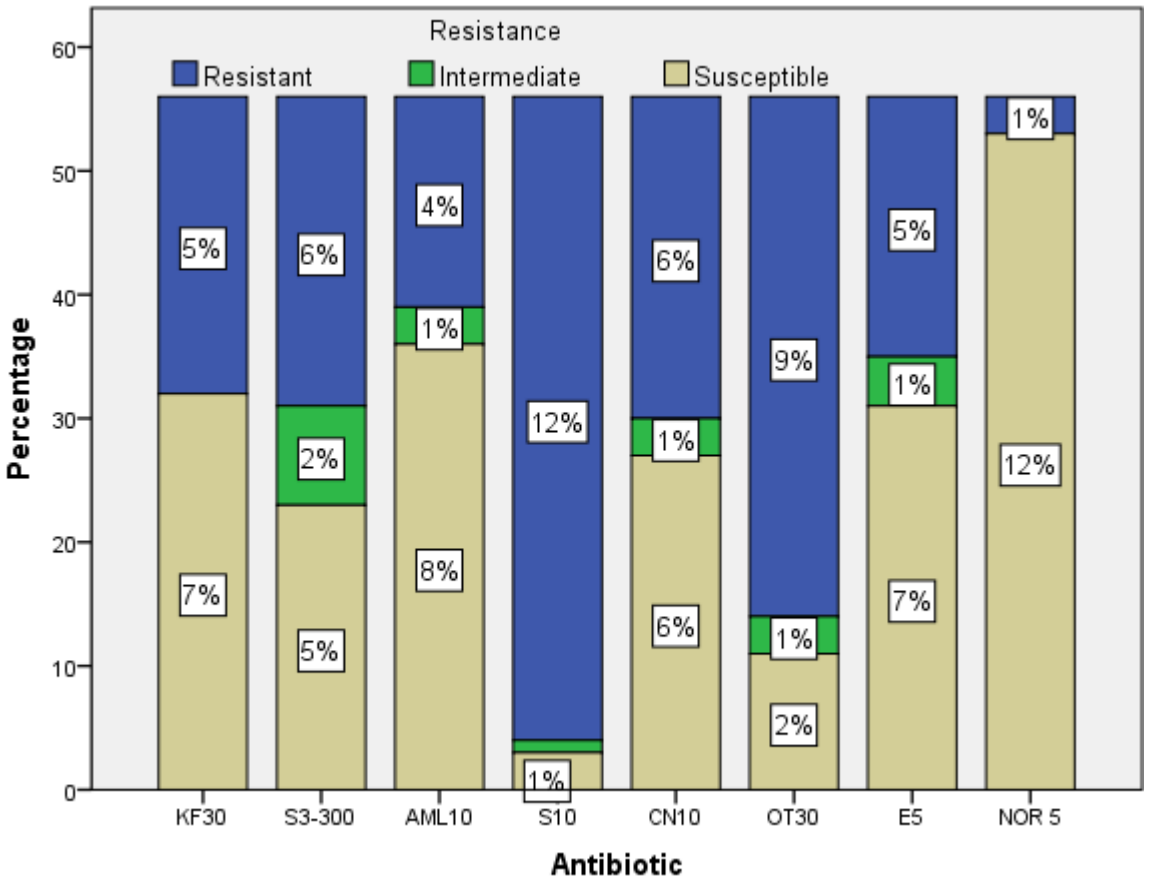

Figure 14 shows a significant association between the antibiotics and bacterial resistance

Figure 14 shows that most bacterial strains were resistant to S10, and most bacteria were susceptible to NOR5. A very small percentage (0% - 2%) were intermediate to each of the antibiotics.

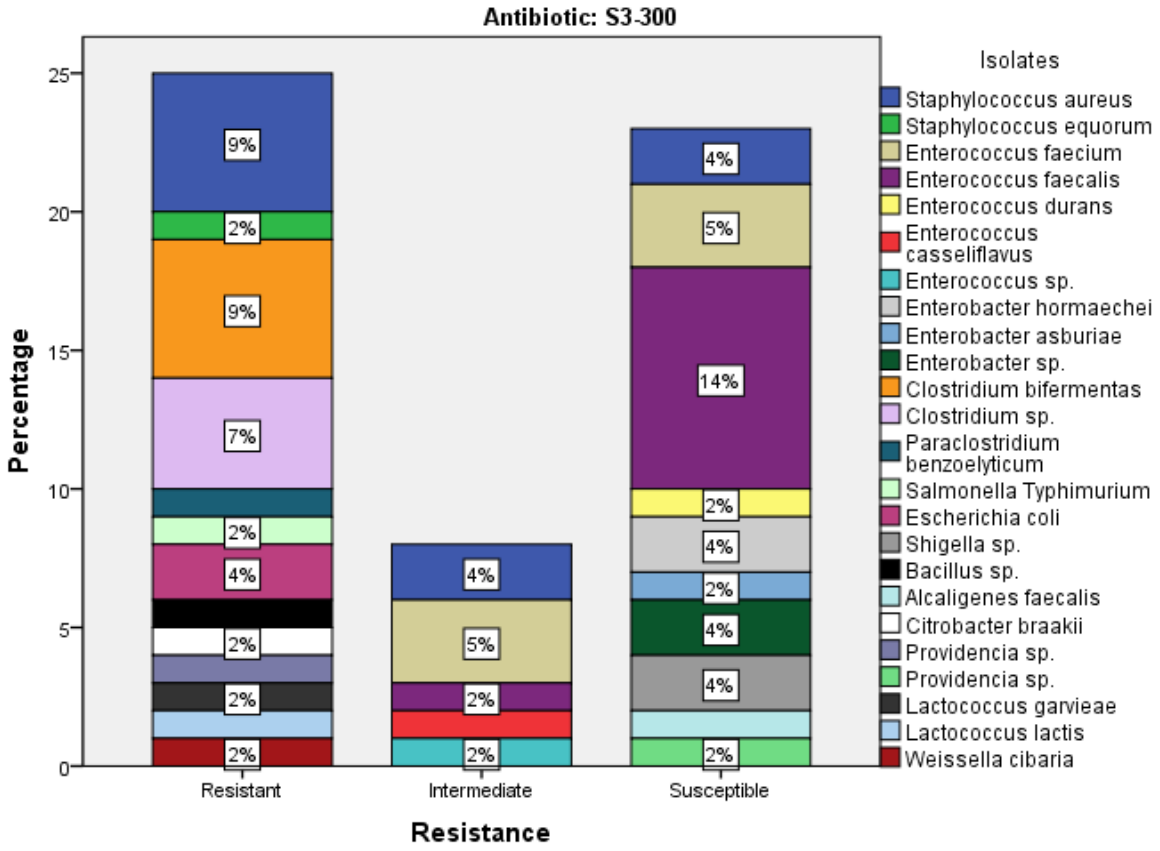

Figure 15 shows the bacterial resistance to S3-300

Figure 15 shows that most of the strains were resistant to S3-300, the second highest number of strains were susceptible to this antibiotic whereas a relatively very few number of strains were intermediate to S3-300.

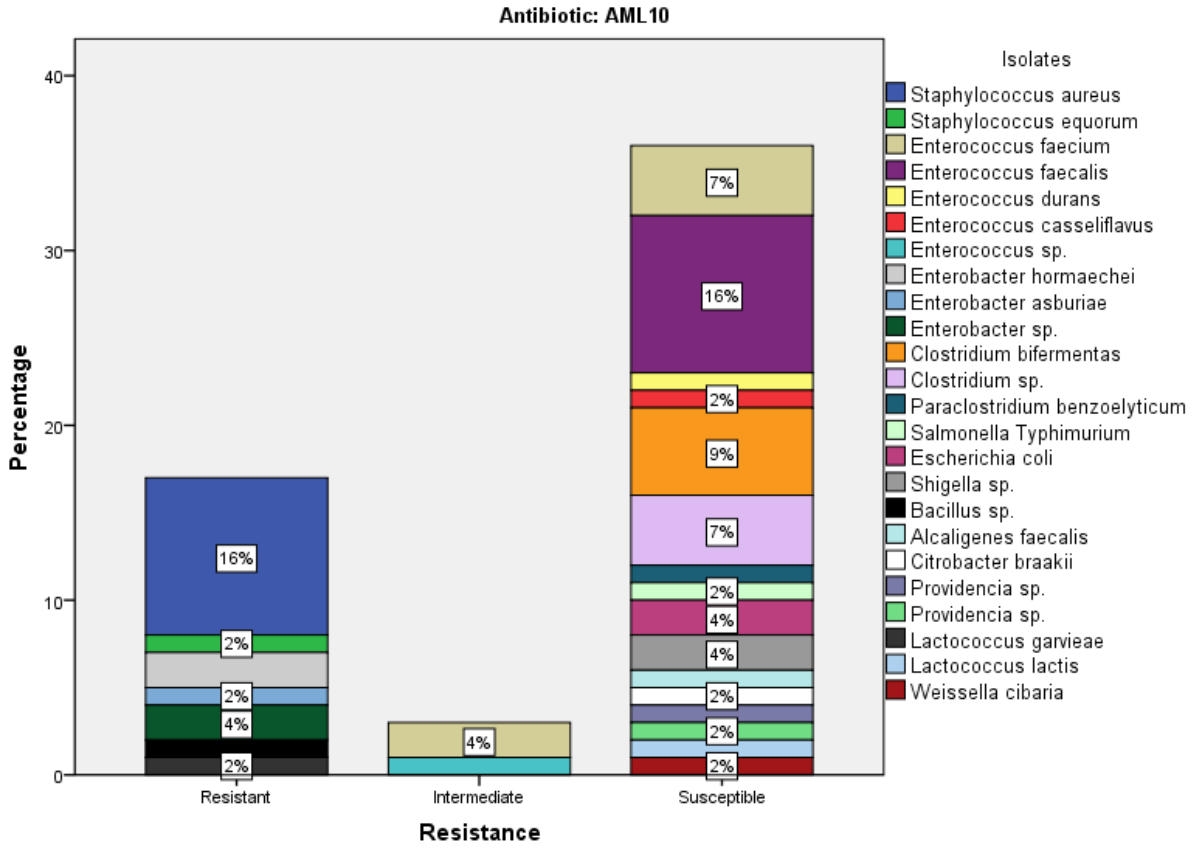

Figure 16 shows the bacterial resistance to AML

Figure 16 shows that most of the strains were susceptible to AML 10, the second highest number of strains were resistant to this antibiotic whereas a relatively very few number of strains were intermediate to AML 10.

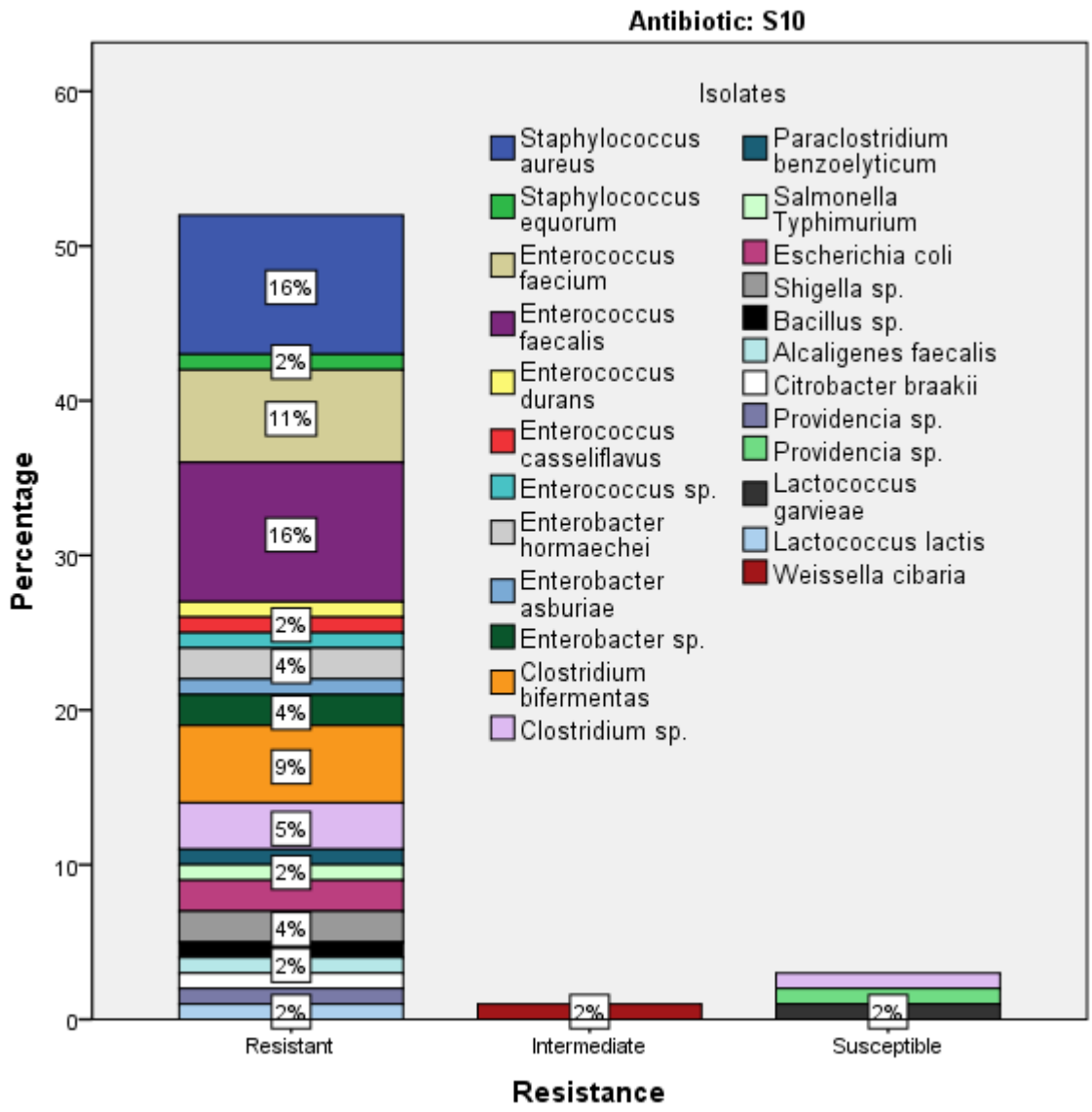

Figure 17 shows the bacterial resistance to S10

Figure 17 shows that almost all the strains were resistant to S10, the second highest number of strains were susceptible to this antibiotic whereas only 1 strain was intermediate to S10.

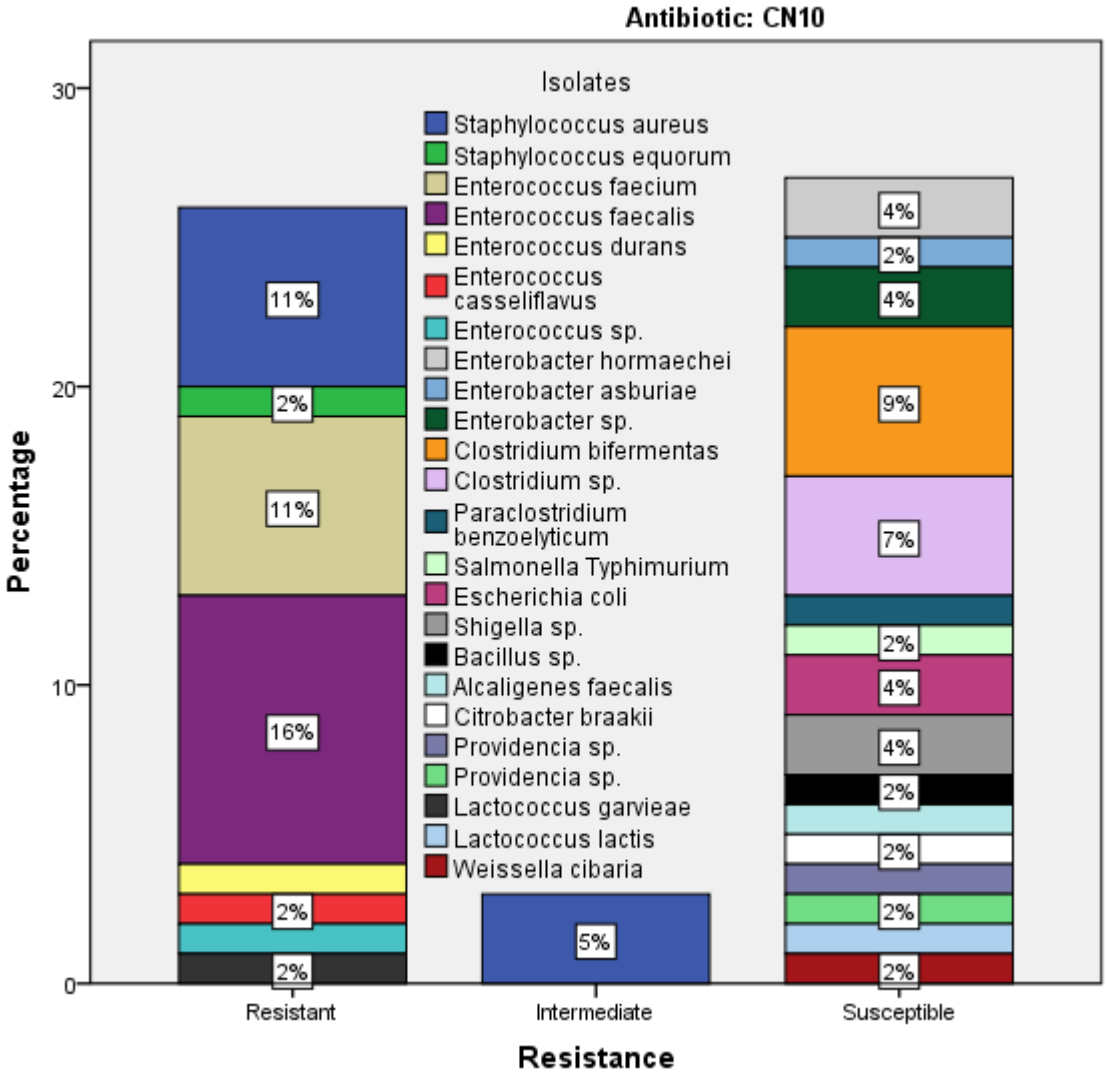

Figure 18 shows the bacterial resistance to CN10

Figure 18 shows that most of the strains were susceptible to CN10, the second highest number of strains were resistant to this antibiotic whereas only one of these strains was intermediate to the antibiotic.

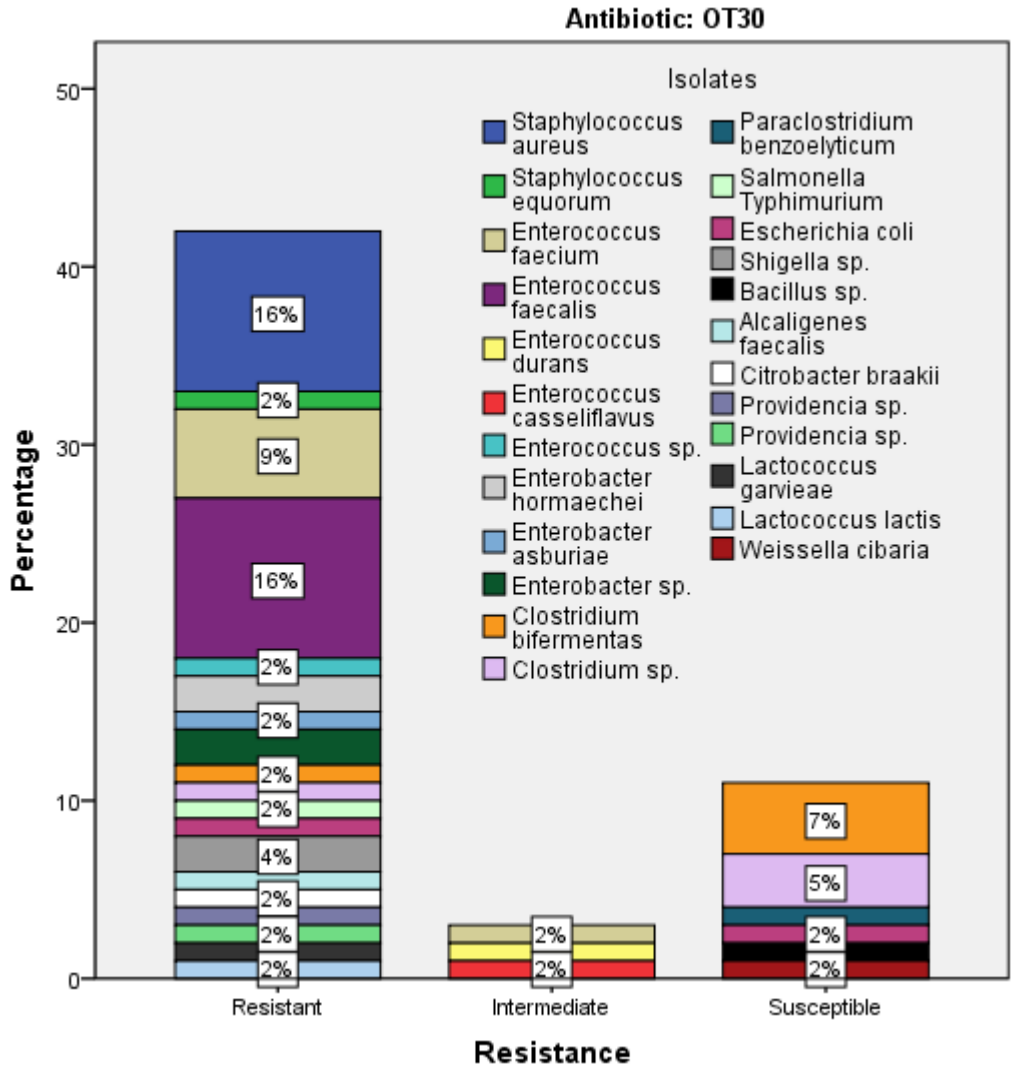

Figure 19 shows the bacterial resistance to OT30

Figure 19 shows that a lot of the strains were resistant to OT30, the second highest number of strains were susceptible to this antibiotic whereas a relatively very few number of strains were intermediate to this antibiotic.

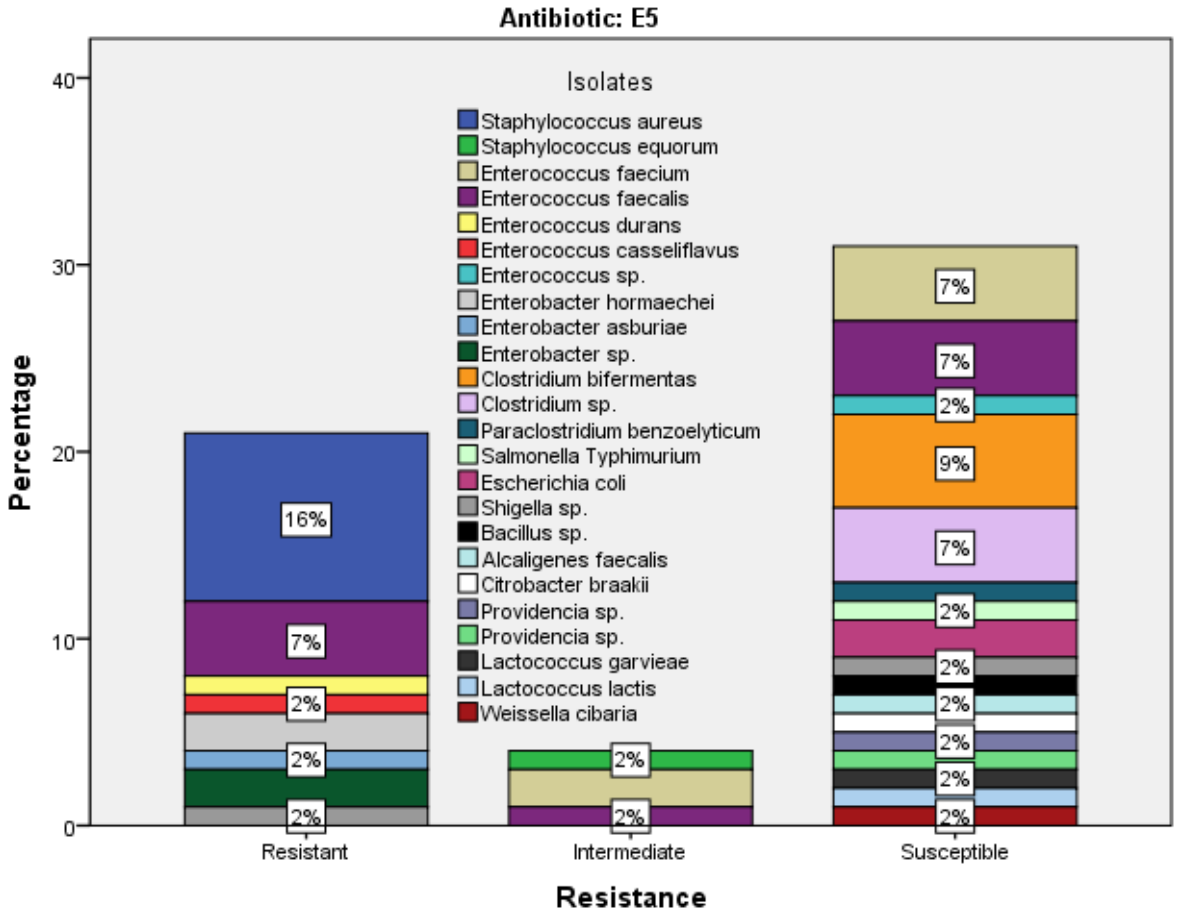

Figure 20 shows the bacterial resistance to E5

Figure 20 shows that most of the strains were susceptible to E5, the second highest number of strains were resistant to this antibiotic whereas a relatively very few number of strains were intermediate to E5.

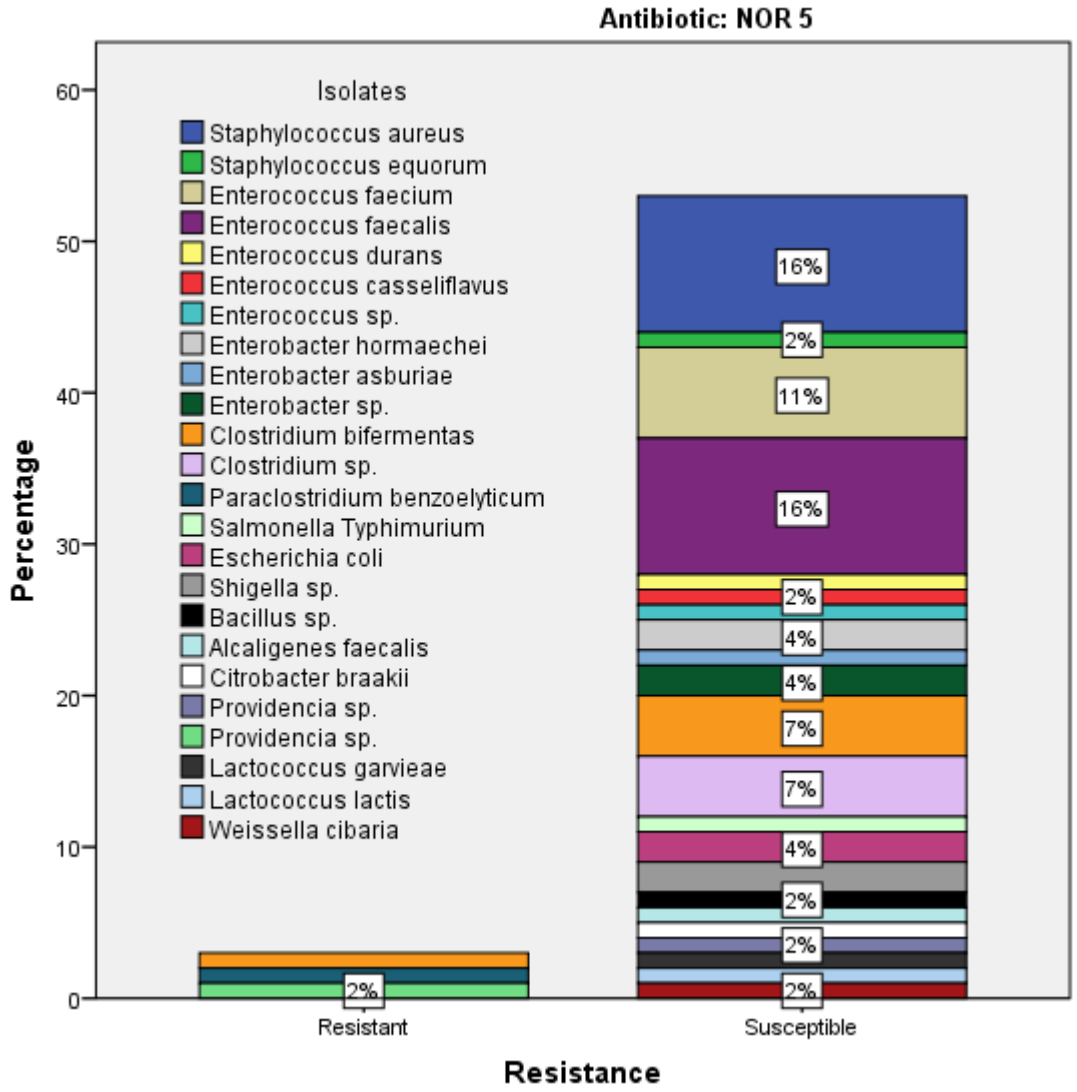

Figure 21 shows the bacterial resistance to NOR 5

Figure 21 shows that almost all the strains were susceptible to NOR 5 whereas only 3 of these strains were resistant to this antibiotic.

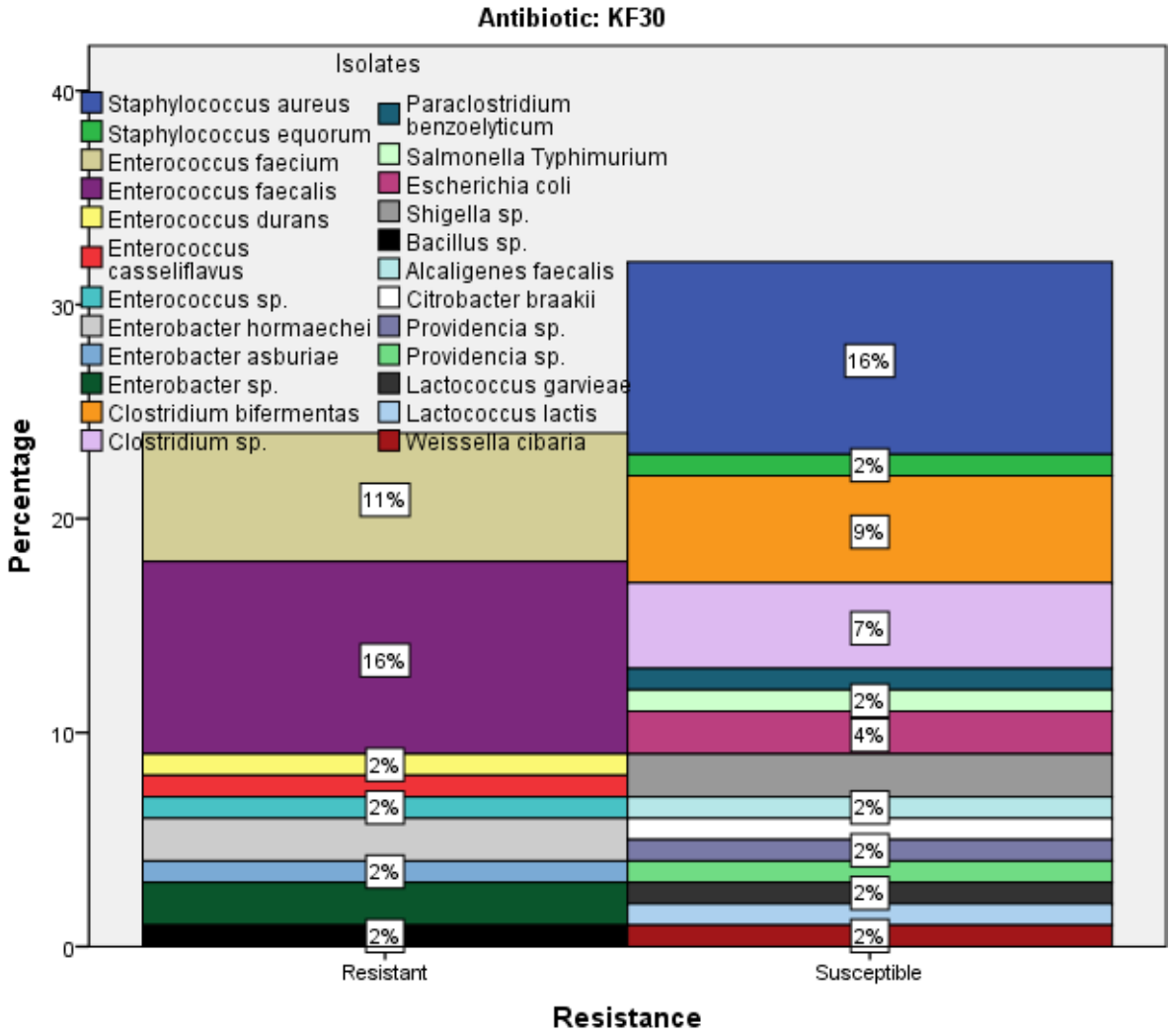

Figure 22 shows the bacterial resistance to KF 30

Figure 22 shows that most of the strains were susceptible to KF 30 whereas a little less than that were resistant to this antibiotic.

In summary, the number of strains which are either resistant, intermediate or susceptible is different across the antibiotics as shown in Figures 16 to 23. To confirm the statistical significance of these differences, the Kruskal-Wallis test was used and the results presented next:

Table 4. 14**:** Kruskal-Wallis test to confirm the statistical significance of antibiotic resistance/susceptibility differences per isolate to antibiotics tested

Hypothesis Test Summary				
	Null Hypothesis	**Test**	**Sig.**	**Decision**
1	**The distribution of Resistance is the same across categories of Antibiotic.**	**Independent-Samples Kruskal-Wallis Test**	**.000**	**Reject the null hypothesis.**

The p-value for the Kruskal-Wallis test is less than 0.05, therefore the resistance depicted in Figures 16 to 23 are significantly different across the antibiotics. The following post-hoc test confirms where these differences are present as well as the direction of such.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
S10-S3-300	98.429	21.676	4.541	.000	.000
S10-CN10	-104.857	21.676	-4.838	.000	.000
S10-KF30	119.625	21.676	5.519	.000	.000
S10-E5	-123.714	21.676	-5.707	.000	.000
S10-AML10	142.625	21.676	6.580	.000	.000
S10-NOR 5	-207.750	21.676	-9.584	.000	.000
OT30-KF30	81.911	21.676	3.779	.000	.004
OT30-E5	-86.000	21.676	-3.968	.000	.002
OT30-AML10	104.911	21.676	4.840	.000	.000
OT30-NOR 5	-170.036	21.676	-7.845	.000	.000
S3-300-NOR 5	-109.321	21.676	-5.043	.000	.000
CN10-NOR 5	-102.893	21.676	-4.747	.000	.000
KF30-NOR 5	-88.125	21.676	-4.066	.000	.001
E5-NOR 5	-84.036	21.676	-3.877	.000	.003

Figure 23 shows the post-hoc test that confirms where the significant differences in Figures 15 to 22 are present as and the direction of such differences

Figure 23 shows the exact pairs of antibiotics where the resistance is significantly different across the antibiotics (p-values <0.05). A positive “Std. test Statistic” indicates that “Sample 1” has a significantly higher number of strains which are resistant to it compared to “Sample 2”. For example, there are more strains that are resistant to S10 than there are for S3-300 (See Figure 15 and Figure 17). A negative “Std. test Statistic” indicates that “Sample 2” has a significantly higher number of strains which are susceptible to it compared to “Sample 1”. For

example, the number of strains which are susceptible to NOR 5 is significantly higher than that of the strains which are susceptible to OT30.

Two Stage cluster analysis of antibiotic resistance

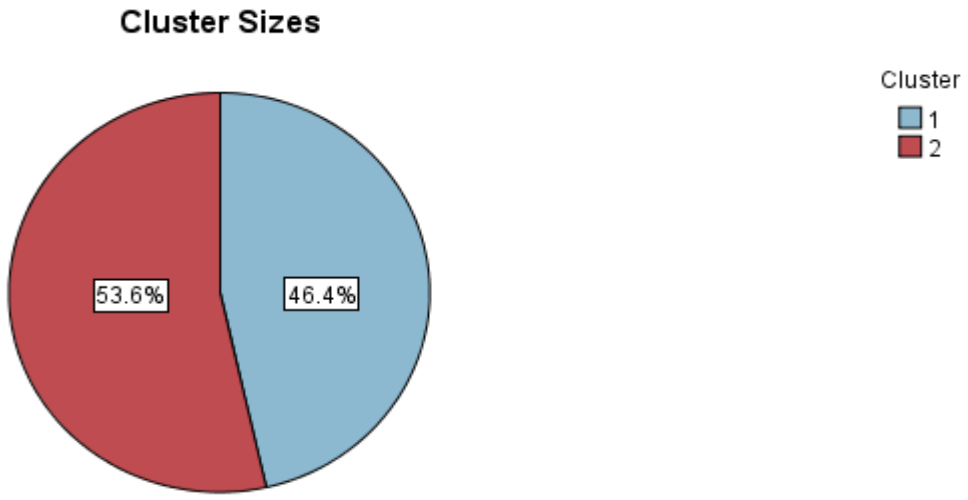

Figure 24 shows the Two-stage cluster analysis of antibiotic resistance

Figure 24 shows that the Two-stage cluster analysis yielded two main clusters with slightly more than half of the strains (53.6%) belonging to cluster 2 and the rest to cluster 1 (46.4%). These clusters are described below:

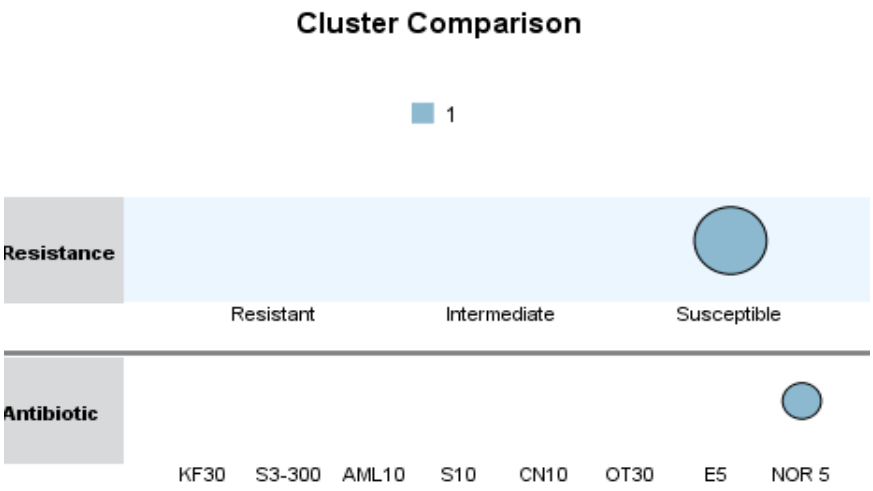

Figure 25 shows that cluster 1 comprise strains which are susceptible to NOR 5.

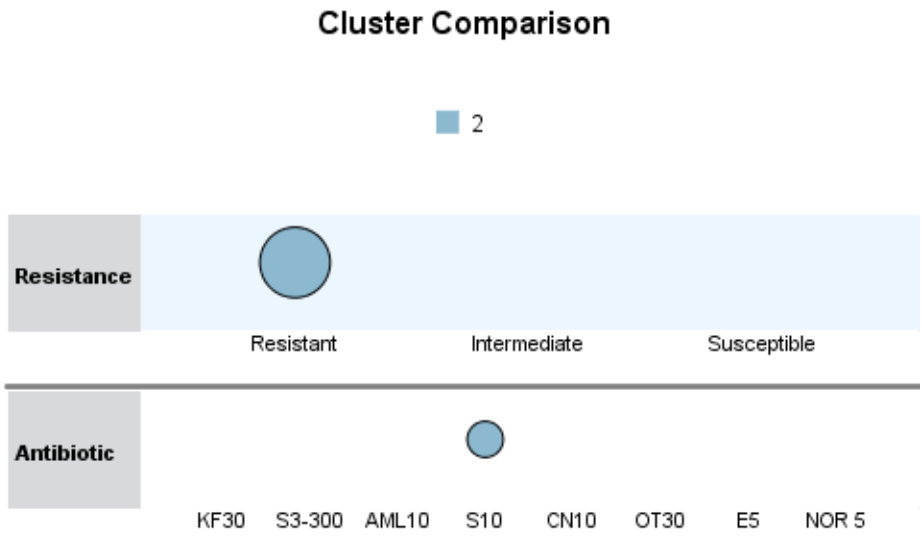

Figure 26 shows that the second cluster comprise strains which are resistant to S10.

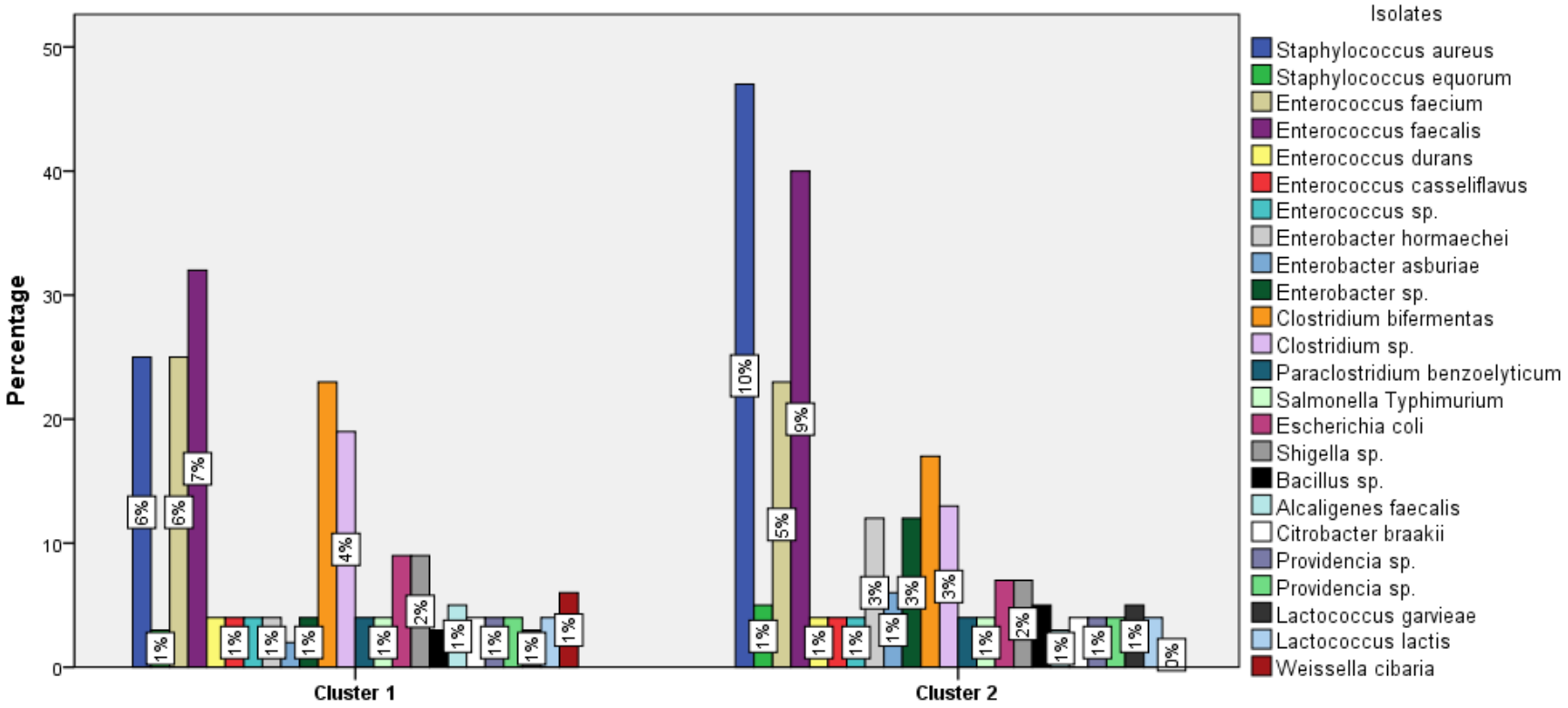

Figure 27 shows the distribution of bacterial isolates between two clusters

Figure 27 demonstrates the distribution of isolated bacteria between the two main clusters. For example, of the 16% of all the bacterial isolates which comprise *Staphylococcus aureus*, 10% is susceptible to NOR 5 (i.e belong to cluster 1) whereas 6% is resistant to S10 (i.e. belong to cluster 2).

Chapter 5: DISCUSSION

Normally, raw milk produced from healthy animals is free of bacteria, but could get contaminated from various factors including the farm environment, animal skin and external teat surface, the hands of milkers, and poor hygiene practices generally on the farm (Muhammad *et al*., 2009). This study assessed the possible factors leading to raw milk contamination, evaluate the bacterial quality of raw bovine milk as well as determining the antibiotic resistance of milk-borne bacteria isolated from communal dairy farms in Mahikeng. According to the findings of this study, general observation of hygiene practices on smallholder farms during milking revealed that contamination of raw milk may arise at step of milk production process. This could explain number of pathogens isolated in the study. Moreover, the isolates from raw milk were resistant to most of the antibiotics, with most of these bacteria showing great margins of resistance patterns against various antibiotics tested.

5.1 Sociological Study (Questionnaire)

This study established that most of the farmers had primary school level of education or no education at all while only few had obtained secondary school, tertiary and religious education. Low level of education is identified by Khapayi and Celliers (2016) as one of the factors limiting the performance in small holder farm. As a result, farmers with low formal education lack proper management and marketing skills. Farm management solely depends on level of knowledge and skills obtained through various levels of education (Kahan, 2013). This has been exemplified by a large number of farmers in this study who reported that everyone in the farm is allowed to milk animals and only few have primary milkers in their respective farms. Such poor management practices make it difficult to trace the major sources of contamination in cases where there is a disease outbreak arising from milk-borne pathogens from such farms as everyone is allowed to perform any role in the farm. Ferreira (2018) also support by stating that, education has an impact in helping farmers improve their decision making in farms as well as improving production. Moreover, a study carried out to evaluate factors preventing the progression of emerging farmers to commercial farming in Eastern Cape (South Africa) also identified the main challenges to be low levels of education, lack of farming skills and poor management (Khapayi *et al*., 2016).

According to the literature, udder infections and failure to wash hands before milking are both said to be the important factors influencing the microbial quality of raw milk (Le Marechal *et al*., 2011; Bereda *et al*., 2012). It was observed in this study that the majority of smallholder farmers do not wash their hands prior to milking, and the few that did wash their hands only use water with no soap or disinfectant. In addition, it was also found that most of the farmers practice hand-milking technique with unwashed hands. This is in contrary to the findings by Sabapara *et al*. (2016) who reported that, most farmers wash their hands before milking. Among the rules set for clean hand-milking, DAFF (2008) emphasizes that washing of hands prior to milking and ensuring cleanliness of the persons milking the cows is essential in order to obtain the healthiest and clean milk. This is due to the fact that bacteria such as *Corynebacterium, Staphylococcus, Micrococcus* and *Propionibacterium* have been isolated on human skin, with *Staphylococcus aureus (Staphylococcus aureus)* being reported as the main skin commensals in humans (Christensen and Bruggemann 2013). However, a number of *Staphylococcus aureus* bacteria have been isolated in this study, which points out dirty milkers' hands as the possible source of *Staphylococcus aureus* contamination.

In addition to the factors most likely to be responsible for bacteriological contamination of milk, it was further found that none of the farmers washed or disinfected the animal udder and teats before and after milking. This is in agreement with the report by Kanyeka (2014) who observed that majority of the smallholder farmers do not practice washing of udder and teats of cows prior to milking. On the other hand, the results contradict those reported by Sheikh (2016), who observed that majority of the farmers wash the udder and teats prior to and post milking. It is essential to ensure that the udder and teats are properly washed and disinfected before milking due to the fact that, the mastitis causing agents including *Staphylococcus aureus* and *Streptococcus agalactiae* are frequently isolated from the udder quarters of cows (Carrillo-Casas and Miranda-Morales, 2012).

However, the findings in this study revealed that majority of the farmers are able to recognize as well as detect udder infections and the animals showing udder infections are milked after the healthy ones with such milk being discarded. This is in agreement with the findings reported by Taoana (2005) that most of the farmers have knowledge on mastitis and are able to recognize udder infections. It is of great importance for smallholder farmers to be able to recognise udder infections as the first step of mastitis control in the farm (Abrahmsen *et al*., 2014). Milk from cows with udder infections is not healthy for both human and animal consumption thus, it has to be discarded (Abb-Schwedler *et al*., 2014).

The farmers in this study further reported that the main purpose of milk from their farms is consumption in the household and majority consume milk from sick animals while milk from recently treated animals is sold to the public. Moreover, it was observed that the farmers do not comply with the withdrawal periods of drugs used to treat their animals. Similar observations have been reported by Kanyeka (2014) that most of the farmers use milk from sick and recently treated animals for consumption, and the withdrawal periods are not adhered to.

Milk production meant for consumption requires proper hygienic practices including adequate cleaning of storage containers used, in order to minimize the microbial build-up consequently contaminating the milk (Getachew, 2003). In the current study, it was also observed that plastic containers were used by majority of farmers for storage during milking, which were not properly cleaned before use. Similar observations were reported by Zewdu (2015) that majority of the smallholder farmers use plastic containers for storage and transportation of milk and cleaning of the storage containers was not done adequately. Majority of the farmers in this study do not store their milk under the shade during the milking process. This is in contrary with the findings reported by Kanyeka (2014) that most of the small-scale farmers use calabash containers for milk storage. It is essential to store milk under the shade during milking in order to maintain cool temperatures due to the fact that fresh milk normally spoil within 3 to 4 hours of milking if not kept in cool environments (FAO, 2018).

The current study further found that most of the smallholder farmers were aware of antibiotics and all of them administer antibiotics to their animals with most of the farmers administering antibiotics when the animals show signs of sickness or bacterial infections while the others only administer antibiotics when instructed by the veterinarians. Moreover, majority of the farmers in this study have reported not to always read the instructions. This poses a serious health risk to consumers as they may end up consuming antibiotic residues as well as antibiotic resistant bacteria due to failure to adhere to instructions guiding individuals on proper routes, dosage and withdrawal periods of such antibiotics used (Russ, 2011). Consequently, most of the isolated organisms in this study showed resistance against a number of commonly used antibiotics tested. This indicates that milk-borne pathogens found in Mahikeng are gradually gaining resistance against most antibiotics. However, Kumar and Gupta (2018) also reported that most of the smallholder farmers use antibiotics for curative purposes. Moreover, Redding *et al.* (2014) also reported that the farmers use antibiotics over 83% of the time for treatment of diseases.

5.2 Bacteriological Quality of Raw Milk

5.2.1 Analysis of Total Bacterial Count (TBC)

Total Bacterial Count described by NICD (2018) as a primary indicator of hygiene management used to assess the number of microorganisms existing in samples, was performed in this study as an important indicator of bacteriological quality of raw milk. As shown in the findings of this research, it was established that all (100%) milk-samples tested had TBC that was more than the approved amount of 5.0 x 10^4 cfu/ml in South Africa as stipulated by Foodstuffs, Cosmetics and Disinfectants Act, No. 54 of 1972. As shown in Figure 1 to Figure 8, TBC was higher than the recommended level in all the villages. This suggests that the milk is not suitable for human consumption mainly for those who have indicated that they consume such milk without boiling it to destroy hazardous microorganisms. These results indicate that the microbial quality of raw bovine milk from the communal areas in Mahikeng is poor. These high bacterial loads in raw milk may be resulting from contamination possibly from poor general farm hygiene, milk storage containers, milking equipment, unclean udder and teats, unclean hands of persons handling, the milk and the lactating cows (Bukuku 2013). Based on the practices observed from milking to storage at each farm, high bacterial counts were expected due to the fact that the general level of hygiene was poor among all the farms studied. Findings from this study are in agreement with those reported by Rwehumbiza *et al.* (2013), Kanyeka (2014), Addo *et al.* (2011) and Schoder *et al.* (2013) in which the majority of raw milk samples which were tested were reported to have TBC higher than the recommended levels.

5.2.2 Analysis of isolated bacteria in raw milk samples

Milk safety due to microbial hazards, is of great concern globally (Camino Feltes *et al*., 2017). The current study evaluated the bacteriological quality of unpasteurized milk produced by communal farms in the study area for human consumption. Results show that the milk contains a diversity of pathogenic bacteria such as: *Staphylococcus aureus, Salmonella Typhimurium, Escherichia coli, Shigella* sp., *Citrobacter braakii, Providencia* sp., *Enterobacter hormaechei, Enterobacter asburiae, Enterococcus faecium, Enterococcus faecalis, Enterococcus durans, Enterococcus casseliflavus* and *Alcaligenes faecalis*. Previous studies conducted in Tanzania

(Kanyeka, 2014) and in China (Lan *et al*., 2017) to evaluate the microbial quality of raw milk have also reported similar organisms isolated from their samples

The presence of *Staphylococcus aureus* in raw milk indicates the possibility of clinical mastitis in lactating cows (Titouche *et al*., 2016). It was found that, *Staphylococcus aureus* accounts for 16.1% of contamination in this study. This is lower than the percentage of 52% contamination of *Staphylococcus aureus* in raw milk from Malayer city of Iran reported by Pourhassan and Najafabadi (2011). The possibility of interchanging *Staphylococcus aureus* strains may occur as a result of close association between the cows and handlers during milking together with poor hygienic practices (Adjlane-Kaouche., 2014). Moreover, nasal carriage of *Staphylococcus aureus* in both pets and human is considered the primary reservoir of this bacteria, which may contribute to the contamination of milk (Rahimi *et al*., 2015). A study done by Kamal *et al*. (2013) revealed that, 80% of hand swabs used by dairy workers had high frequency of coagulase-positive *Staphylococcus aureus* (CPS), which may also contribute to the contamination of raw milk with CPS present on their skin. The use of hand swabs instead of proper disinfection, absence of refrigeration and lack of pasteurization of raw milk may also constitute to contamination by *Staphylococcus aureus.* According to Louw (2013), certain mastitis *Staphylococcus aureus* produce enterotoxins, which may pose a health risk to consumers of raw milk contaminated with mastitis *Staphylococcus aureus.*

The prevalence of *Escherichia coli* and other Enterobacteriaceae in raw milk suggests the environmental as well as fecal contamination. Moreover, these organisms have been associated with food safety and spoilage (Mhone *et al*., 2011; Baylis *et al*., 2011). This study found that, from a total of 56 bacterial isolates identified, *Escherichia coli* contamination in raw milk account for 3.6%, *Shigella* account for 3.6%, *Salmonella* Typhimurium account for 1.8%, *Providencia sp*. accounts for 1.8%, *Citrobacter braakii* accounts for 1.8% and *Enterobacter* species account for 8.9%. The prevalence of *Escherichia coli* and *Salmonella* contamination in raw milk in this study is closer to the reported prevalence of *Escherichia coli* (4.1%) and *Salmonella* (2%) in raw bovine milk from Kilosa and Mvomero Districts in Tanzania (Kanyeka, 2014). *Providencia sp.* in this study was found to be responsible for 1.8% of milk contamination, while Saidi *et al*. (2013) has reported 4% prevalence of *Providencia* sp. in raw milk from Algeria. Moreover, *Citrobacter braakii* and *Enterobacter* species have been reported to be accountable for a total of 3.3% and 21.4% of raw milk contamination (Ntuli *et al*., 2016). It was further found that, the prevalence of *Shigella* (3.6%) in raw milk in this study was less than the prevalence of *Shigella* in raw milk obtained from Jigjiga City in Eastern Ethiopia

reported to be 17.5% (Reta *et al*., 2016). Although the prevalence of pathogenic *Enterobacteriaceae* in this study as well as the reference studies may seem to be lower, the presence of these pathogens in milk remains a threat to the consumers (FSANZ, 2009).

Enterococci bacteria are normally found in the gastrointestinal tract as well as the mammalian female genital tract causing no harm, and they have been mostly isolated from fermented food, food of animal origin (beef, swine and poultry carcass), soil and water (Nilsson, 2012; Economou and Gousia, 2015). These bacteria have been considered probiotics and appreciated for fermentation and preservation of food over the previous years but have recently been recognized as emerging human pathogens (Ike, 2017). Enterococci is responsible for a variety of infections including urinary tract, pelvic, intra-abdominal and soft tissue infections (Higuita and Huycke, 2014). The prevalence of different *Enterococcus* species including *Enterococcus faecium* (10.7%), *Enterococcus faecalis* (16.1%), *Enterococcus durans* (1.8%), *Enterococcus casseliflavus* (1.8%) and *Enterococcus sp.* (1.8%). *Enterococcus faecalis* and *Enterococcus faecium* isolated in this study have shown the highest prevalence compared to other bacterial species isolated. However, *Enterococcus faecalis* and *Enterococcus faecium* have been considered the opportunistic pathogens in human, capable of causing infections in immune-deficient patients (Jha *et al*., 2005; Hammerum, 2012). Mrkonjic-Fuka *et al* (2017) have reported the prevalence of *Enterococcus* isolates from raw milk cheese as follows; *Enterococcus faecium* (53.8%), *Enterococcus faecalis* (42.4%), *Enterococcus durans* (2.84%) and *Enterococcus casseliflavus* (0.95%). However, *Enterococcus durans* and *Enterococcus casseliflavus* from their study and the ones from this study have lower prevalence compared to *Enterococcus faecalis* and *Enterococcus faecium* prevalence from both studies. The presence of this bacteria in raw milk may therefore arise from soil contamination or the use of contaminated water in the farm.

This study further established that the prevalence of *Alcaligens faecalis* in raw milk was 1.8%. *Alaligens faecalis* is the human intestinal flora most commonly found in soil and water (Tena *et al*., 2014). *A.faecalis* are commonly isolated in hospital environments and the infections caused by this bacteria are said to be nosocomial, often associated with hospital equipment contamination (Al Laham *et al*., 2017). Moreover, this organism has been reported to be responsible for sporadic cases of chronic otitis, peritonitis, endocarditis, endophthalmitis, abscess and pyelonephritis (Tena *et al*., 2014). However, *Alcaligens faecalis* bacteria are very rare hence, there is limited literature available about this bacteria (Tena *et al*., 2014). Zhang

(2015) has also isolated *Alcaligens faecalis* in raw bovine milk with no data reported on the prevalence of this bacteria.

Moreover, non-pathogenic bacteria including *Staphylococcus equorum, Clostridium bifermentans, Paraclostridium benzoelyticum, Bacillus* sp, *Proteus hauseri, Lactococcus garvieae, Lactococcus lactis* and *Weissella cibaria* were also isolated from raw milk. *Staphylococcus equorum* is a coagulase negative (CNS) bacteria, normally present in fermented food and known to be benign bacteria with no reported evidence of pathogenicity (Jeong *et al*., 2016; Calcutt *et al*., 2013). These bacteria are known for being an inhabitant of healthy horse skin (Novakova *et al*., 2006). *Clostridium bifermentans* and *Paraclostridium benzoelyticum* on the other hand are mostly isolated from marine sediments and have been reported to be non-pathogenic (Jyothsna *et al*., 2016). *Proteus* species are known to cause urinary infection in human but so far, *Proteus hauseri* has not been classified among the pathogenic *Proteus* species (Armbruster *et al*., 2018; Armbruster *et al*., 2017). Moreover, *Bacillus* species most commonly isolated from raw milk are Bacillus cereus, which have been reported to have the ability to survive pasteurization but so far, they have not been reported to be pathogenic (Gopal, 2015). *Lactococcus garvieae* and *Lactococcus lactis* are lactic acid bacteria normally found in milk and used in the manufacturing of dairy products (Florez and Mayo, 2015; Fallico *et al*., 2011; Mermod *et al*., 2010). *Weissella* species mostly *Weissella cibaria,* play an essential role in most fermentation processes including silage and food production (Abriouel *et al*., 2015).

5.2.3 Phylogenetic analysis

Phylogenetic analysis is the means of investigating the evolutionary relationships of species (Kalaniemi, 2013). A phylogenetic tree was constructed to assess the relationships of nucleotide sequences isolated from raw milk in this current study with the most similar sequences obtained from NCBI GenBank as presented in Figure 12. Most of the organisms showed great percentages of identity with each other ranging from 52% to 100%. The phylogenetic tree shows nine (9) main clusters, with the first cluster (cluster I) having 2 sub-clusters (1.1 and 1.2), the fifth cluster (cluster V) having two sub-clusters (5.1 and 5.2), the sixth cluster (cluster VI) having one sub-cluster (6.1) and the rest of the clusters (II, III, IV, VII, VIII and IX) showing no sub-clusters. Cluster I shows a close relationship among *Enterococcus* species isolated from this study with the *Enterococcus* species isolated

elsewhere. Sub-cluster 1.1 shows that isolates Mi 1 to IK 4 (*Enterococcus* species) from this study show a close relationship with *Enterococcus faecalis* (AB669429.1) isolated from pickled peaches in Taiwan, also showing 79% identity to their common ancestor. Sub-cluster 1.2 shows a close relationship among isolates Mi 6 to Ma5 (*Enterococcus* species) with *Enterococcus faecalis* (EU728747.1) isolated from Porcine, with 89% identity to their common ancestor. Although isolate IK 5 (*Enterococcus faecalis*) is an outlier of sub-cluster 1.1, it shows 80% identity to the organisms in sub-cluster 1.1. However, isolate Di 2 (*Enterococcus casseliflavus*) shows to be an outlier to the rest of the isolates in Cluster I but 94% identical to these organisms. The closest relationship demonstrated in Cluster I was between isolate Ik 4 (*Enterococcus faecalis*) from this study with *Enterococcus faecalis* (AB669429.1) showing 99% similarity. A study done by Alnakip *et al*. (2016) also presented a phylogenetic tree demonstrating a close relationship between *Enterococcus* species identified in their study and the related *Enterococcus* species in GenBank.

It was further found that the second cluster (Cluster II) consisted of two *Lactococcus* species which were isolated from unpasteurized milk in this study. The two isolates being Ts 10 (*L.lactis*) and Ts 11 (*L. garvieae*) did not cluster with any of the *Lactococcus* species obtained from the GenBank, and a similarity of 100% was shown for the two isolates. This high similarity might be due to the fact that the two isolates were from milk samples obtained from the same geographical location (Tsetse village). González-Rocha et al (2017) also reported two isolates yielding 100% identity match in their phylogenetic analysis of the bacterial species isolated from the Fildes Peninsula.

The third cluster (Cluster III) was made up of mostly *Staphylococcus* species. The organisms isolated in this study have clustered with *Staphylococcus aureus* (KR085871.1) isolated from Chandra Tal Lake (Indian Trans-Himalayas), showing lower similarities among each other but a high similarity (94%) with their common ancestor. This is in conflict with the phylogenetic analysis results from the study done by Aplin (2014), which demonstrated high percentage similarities between Staphylococcus species isolated from dairy products in their study and the related GenBank obtained sequences. However, isolates Mi 4 (*Enterococcus faecalis*) and Ts 1 (*Proteus hauseri*) did not cluster with other organisms in the study, hence they are both considered to be the outliers. This might be due to the fact that, both the organisms are non-*Staphylococcus* but may be sharing some genetic similarities with the other organisms within the cluster, hence they are part of Cluster III. Generally, the similarity percentages among the

organisms within Cluster III were lower with the highest being between isolates Mi 5 and Di 10 at 53%.

The phylogenetic tree further established the fourth cluster (Cluster IV) consisting of *Clostridium* species only. The isolates Ma 9 to Mi 10 within Cluster IV have shown genetic similarities among each other and have clustered with *Clostridium bifermentans* (NR 113323.1). Although most of the *Clostridium* species within this cluster have shown lower percentages of similarities, isolates Di 5 and Mi 10 have clustered together at 100% similarity. Both isolates Di 5 and Mi5 were isolated from milk samples isolated from different locations. Drouin and Lafreniere (2012) have identified soil as the major reservoir of *Clostridium* in most farms, and further emphasized animal feeds as well as raw milk contamination by *Clostridium* species in farms. The similarity of 100% between isolates Di 5 and Mi 5 might therefore arise from the possibility of organisms originating from the same location, then one being moved with the soil by wind to the other location. However, a high similarity of 99% was shown between organisms in Cluster IV and their common ancestor.

Interest, the diversity of organisms belonging to the *Enterobacteriaceae* family have clustered together forming the fifth cluster (Cluster V). In this cluster, most of the *Enterobacteriaceae* organisms isolated from raw milk in this study including isolates Ik 9, Mi 9, Mi 2, Ts 8, Ts 7, Ik 8, Ik 2, Ma 3, Ts 4, Ik 1 and Ts 9 have clustered with *Shigella flexneri* (EU009187.1) and *Escherichia coli* (MF372553.1), also showing a great degree of similarity among each other. Isolate Mi 9 from raw milk in this study has shown the highest similarity of 100% with *Shigella flexneri* (EU009187.1). The main cluster further formed two sub-clusters (5.1 and 5.2). Sub-cluster 5.1 shows a close relationship between Mi 2 and Ts 8 which are both *Shigella* species while, sub-cluster 5.2 was made demonstrates a close relationship among isolates Ma 3, Ts 4, Ik 1 and Ts 9 which are all *Enterobacter* species. Moreover, isolate Ik 2 has shown to be an outlier of both sub-clusters within Cluster V, located in between the two sub- clusters. All the organisms within Cluster V show 100% similarity to their common ancestor. In contrary to the results from this study, phylogenetic analysis of bacteria isolated from cows with mastitis in a study done by Mustopa *et al.* (2018), demonstrated lower similarity percentage of most isolated Enterobacteriacea species.

Of great interest, the 6th cluster (Cluster VI) have shown relationships among various organisms belonging to different families. The cluster consists of two organisms (Ma 4 and Ik 10) isolated from this study and eight organisms from the GenBank. Moreover, Cluster VI is made up of

sub-cluster 6.1 organisms and a diversity of other organisms. Sub-cluster 6.1 shows a close relationship among isolate Ma 4 (*Bacillus sp.*) and *Weissella sp.* (MF424649.1), *Bacillus* sp. (KF870418.1) and *Bacillus* sp. (MH455253.1). Coorevits (2011) also demonstrated close relationships among various *Bacillus* species in a study done to assess the spoilage potentials of bacilli and *Pseudomonas*. However, *Weissella sp.* (MF424649.1) is an outlier of sub-cluster 6.1 as the sub-cluster consists of *Bacillus* species only and it is the only different organism within the sub-cluster.

The phylogenetic tree has further demonstrated a high degree of 99% similarity of organisms forming Cluster VII to their common ancestor. However, isolate Di 3 (*Enterobacter* sp.) clustered with *Staphylococcus haemolyticus* KU977133.1, showing a moderate similarity of 52%. Moreover, *Clostrium* sp. (Di 4) from this study has clustered with *Clostridium* sp. (LC194786.1) and *Clostridium bifermentans* (NR 113323.1), showing the highest degree of similarity (100%) to their common ancestor. The 9th cluster (Cluster IX) consists of a diversity of Gram- negative *Enterobacteriaceae* organisms. Within this cluster, isolate Ma 6 from has clustered with *Alcaligenes faecalis* KY357285.1, *Proteus hauseri* KY072916.1, *Providencia stuartii* KF951533.1, *Enterobacter hormaechei* KF015738.1, *Citrobacter braakii* HQ288930.1.

5.3 Antibiotic resistance profiles of bacteria isolated from raw milk

The emerging rate of bacterial resistance to numerous antibiotics has become a global threat (Ventola, 2015). In this study, it was found that majority of the isolated bacterial species showed resistance to Streptomycin and, majority showed susceptibility to Norfloxacin. Our findings have shown that of all the isolated strains from raw milk, 5% showed resistance to cephalothin, 6% were resistant to sulphonamides, 4% were resistant to amoxicillin, 12% showed resistance to streptomycin, 6% showed resistance to gentamicin, 9% showed resistance to oxytetracycline, 5% showed resistance to erythromycin and only 1% showed resistance to norfloxacin. Tetracycline and streptomycin have been said to be the most commonly used antibiotics in both communal and commercial farms around Mahikeng due to the fact that they are freely available over the counter (OTC) and they have both been detected in meat from farm animals (Moneoang and Bezuidenhout, 2009; Ateba, *et al*., 2010). This explains the leading proportions of both tetracycline and streptomycin resistance.

The results have also shown that 46.4% of the isolates where susceptible to norfloxacin while a large proportion of 53.6% of the isolates were resistant to streptomycin. However, other microbiological studies of raw milk done elsewhere found that both norfloxacin and streptomycin were effective against the isolated organisms (Islam *et al*., 2016; Tiruchengode, 2016), while the findings from other studies revealed that the two antibiotics where not effective against the islotated organisms (Disassa *et al.,* 2017; Aliyu *et al*., 2018). This shows that the antibiotic resistance patterns of different bacteria may vary among locations depending on factors such as low levels of education, improper prescription practices, lack of diagnostic facilities, non-accredited sale of antibiotics and easy accessibility of antibiotics (Ayukekbong *et al.,* 2017).

In this study, it was further found that *Staphylococcus aureus* isolates from raw milk ranged between 50% and 75% margins of multidrug resistance. From nine isolates of *Staphylococcus aureus* isolated in this study, five were resistant to sulfonamides, amoxicillin, streptomycin, gentamicin, oxytetracycline and erythromycin, three were resistant to amoxicillin, streptomycin, oxytetracycline and erythromycin, and one was resistant to amoxicillin, streptomycin, gentamicin, oxytetracycline and erythromycin. A study done by Zdolec *et al.* (2016) assessing the antimicrobial susceptibility of milk bacteria has revealed that *Staphylococcus aureus* was resistant to a number of antibiotics tested. Moreover, Ayele *et al*. (2017) also reported a multidrug resistance of *Staphylococcus aureus* that was isolated in raw milk in Ethiopia. These indicate that treatment of *Staphylococcus aureus* is gradually becoming difficult due to its ability to rapidly develop multidrug resistance (Makgotlho, 2009). However, *Staphylococcus aureus* in this study has been found to be susceptible to both cephalothin and norfloxacin. The susceptibility of *Staphylococcus aureus* to these two antibiotics may arise from the fact that they are rarely used compared to other antibiotics in communal farms in Mahikeng and therefore, the overuse of cephalothin and norfloxacin should be avoided as *Staphylococcus aureus* is already resistant to most of the commonly used antibiotics.

Although *Staphylococcus equorum* was resistant to sulfonamides, amoxicillin, streptomycin, gentamicin and oxytetracycline, it is considered a non-pathogen as there is no reported evidence of its pathogenicity (Jeong *et al*., 2017).

Enterococcus species have also been said to be harmless commensal bacteria but recently, they have been associated with intra-peritoneal and urinary tract infections (Kajihara *et al*., 2015). *Enterococcus* species isolated including *Enterococcus faecalis, Enterococcus faecium, E*

durans, Enterococcus casseliflavus and another *Enterococcus* sp. have shown common resistance against cephalothin, streptomycin and gentamicin, with five isolates of *Enterococcus faecium*, nine isolates of *Enterococcus faecalis* and one *Enterococcus* sp. resistant to oxytetracycline; four isolates of *Enterococcus faecalis*, one isolate of *E durans* and one isolate of *Enterococcus casseliflavus* resistant to erythromicin. *Enterococcus* species isolates in this study have shown a multidrug resistance profile range of 38% to 63%. Previous studies have also reported that *Enterococcus* species to be intrinsically resistant to most of the antibiotics including sulfonamides, cephalosporins, ß-lactams, erythromycin, tetracycline, fluoroquinolones, glycopeptides and clindamycin (Fair and Tor, 2014; Trivedi *et al*., 2011; Gimenez-Pereira, 2005). Although *Enterococcus* species have been reported to be resistant to most of the commonly used antibiotics, most of the strains of this bacterium are susceptible to vancomycin, ampicillin and penicillin (Kristich *et al*., 2014). The emergence of *Enterococcus* species resistance to most commonly used antibiotics poses a health risk to human consuming raw milk and other foodstuff contaminated with such bacteria as its infections might be among the most difficult to treat infections in future.

Enterobacteriaceae bacteria have been associated with food spoilage problems and most of the members from this family have also been reported to have an emerging pathogenicity due to their potential to acquire virulence and antibiotic resistant factors carried on their genetic elements (Baylis *et al*., 2011). The members of Enterobacteriaceae family including *Enterobacter* species, *Salmonella* Typhimurium, *Escherichia coli* (*Escherichia coli*), *Shigella* species, *Alcaligenes faecalis, Citrobacter braakii, Proteus hauseri and Providencia species* isolated in this study have shown resistance to various antibiotics tested. *Enterobacter* species have all shown resistance to cephalothin, amoxicillin, streptomycin, oxytetracycline and erythromycin, with a multidrug resistance margin of 63%. However, a study previously done by Villegas and Quinn (2002) reported that *Enterobacter* species have been gradually showing resistance to cephalosporins and aminoglycosides which were considered the drugs of choice against *Enterobacter* infections. Although the *Enterobacter* species in this study were susceptible to gentamycin (aminoglycosides), the resistance of all the isolated *Enterobacter* species to cephalothin (cephalosporins) indicates the rapid resistance development of this bacteria to cephalosporins. Findings from the study done by Yagoub *et al.* (2005) to assess raw milk pathogens has also indicated that, *Enterobacter* species are resistant to erythromycin and amoxicillin.

Moreover, *Salmonella* Typhimurium, *Escherichia coli* (*Escherichia coli*) and *Shigella* species have also been isolated in this study. CDC (2010) has reported that *Salmonella, Escherichia coli* and *Shigella* species are among the most important foodborne pathogens causing illnesses in humans globally hence, it is of paramount to assess their response to commonly used antibiotics. According to the findings of this study, *Salmonella* Typhimurium and one of the two isolated *Escherichia coli* bacteria showed resistance to sulphonamides, streptomycin and oxytetracycline. The other *Escherichia coli* isolate showed resistance to sulphonamides and streptomycin only. This is in agreement with the findings reported by other researchers that, *Salmonella* and *Escherichia coli* have revealed multidrug resistance to a number of antibiotics including streptomycin, tetracycline, sulphonamides, chloramphenicol, kanamycin as well as certain β-lactam antibiotics (Economou and Gousia, 2015; Frank, 2017). One of the two *Shigella* species isolated in this study was resistant to streptomycin, oxytetracycline and erythromycin while the other isolate was resistant to streptomycin and oxytetracycline only. These results are in-line with the results obtained by Onuoha *et al*, (2016) who reported *Shigella* species to be resistant to various antibiotics including streptomycin, oxytetracycline and erythromycin. Multiple Antibiotics Resistance (MAR) in major foodborne pathogens such as *Salmonella, Escherichia coli* and *Shigella* bacteria has been said to be the emerging threat of importance to both human and animal health globally (Dobiasova *et al.* 2013).

It was further found that *Proteus hauseri* and *Citrobacter braakii* isolated from raw milk in this study were resistant to sulphonamide, streptomycin and oxytetracycline. Several studies have reported that *Proteus* is an environmental bacteria associated with pneumonia in children as well as human urinary tract infections(UTIs) but, *Proteus hauseri* has not been isolated as one of the *Proteus* pathogens responsible for such diseases (Drzewiecka, 2016; Whary *et al*., 2015; Crawford and Daum, 2008; Ala'Aldeen and Wooldridge, 2012). Its pathogenicity is unknown hence, there is no literature about its antibiotic resistance. On the other hand, *Citrobacter braakii* has been identified as one of the pathogens causing nosocomial infections and UTIs in humans and has been isolated in raw milk in South Africa (Ntuli *et al*., 2016; Liu *et al*., 2017). A study conducted by Straley *et al.* (2006) meant to assess the antimicrobial resistance of gram-negative bacteria in raw bulk tank milk has shown that *Citrobacter* species isolated were resistant to tetracycline, gentamicin, cephalothin and other antimicrobials used. The variation between the results from this study and the results from the study conducted by Straley *et al.* (2006) might be due to difference in locations, time of sample collection or variation in resistant genes of bacteria.

Moreover, it was found that *Providencia* species isolated in this study was resistant to oxytetracycline and norfloxacin. Oliveira *et al*. (2017) have also mentioned levofloxacin as one of the antibiotics that *Providencia* species has shown resistance towards. Both levofloxacin and norfloxacin are fluoroquinolones. This is an indication that, *Providencia* species' resistance to fluoroquinolones is emerging. Moreover, *Providencia* species have been declared naturally resistant to tetracycline (Feyzioglu, *et al.*, 2013). However, Adeleke and Omafuvbe (2011) found some of the *Providencia* species isolated in their study to be susceptible to tetracycline while some were resistant to tetracycline. This might be due to presence of acquired resistant genes which were absent in other bacteria of the same genus. *Providencia* species isolated in their study therefore might have also acquired resistant genes over time through over-use of tetracycline and streptomycin as the most frequently used antibiotics in communal farms around Mahikeng.

Alcaligenes faecalis has shown resistance to streptomycin and oxytetracycline in this study. Although there is limited data available with regard to the existence and activities of *A.faecalis* due to its scarcity, Tena *et al*. (2014) have reported its susceptibility to amoxicillin and gentamicin. However, the results from this current study have also shown the susceptibility of this pathogen to amoxicillin and gentamicin. This shows that, the two antibiotics are capable of preventing the emergence of *A.faecalis*.

Moreover, this organism has been reported to be responsible for sporadic cases of chronic otitis, peritonitis, endocarditis, endophthalmitis, abscess and pyelonephritis (Tena *et al*., 2014). However, *Alcaligens faecalis* bacteria are very rare hence, there is limited literature available about this bacteria (Tena *et al*., 2014). Zhang (2015) has also isolated *Alcaligens faecalis* in raw bovine milk with no data reported on the prevalence of this bacteria.

It was further found that all ten *Clostridium* species isolated in this study were resistant to sulphonamide. Moreover, all *Clostridium bifermentans* were resistant to streptomycin, one being resistant to oxytetracycline and the other was resistant to norfloxacin. Out of four *Clostridium sp.* isolated, three were resistant to streptomycin while one was resistant to oxytetracycline. *Paraclostridium benzoelyticum* was further resistant to streptomycin and norfloxacin. A study done by Fourie (2017) in South Africa has also reported that *Clostridium* species have shown resistance to tetracycline among other antibiotics tested. In addition, Pirs *et al*. (2013) also reported that, *Clostridium* species tested for antimicrobial resistance in their study revealed resistance to a number of antibiotics including tetracycline and streptomycin.

However, there is limited literature with regard to the antibiotic resistance/susceptibility of *Clostridium* species isolated in this study.

Although the antibiotic resistance patterns of *Bacillus, Lactococcus* and *Weisella* species have been presented in the findings, these organisms are non-pathogenic and therefore, their antibiotic resistance is not of paramount (Tuazon, 2010; Fessard and Remize, 2017). The findings of this study have shown that most of the bacterial isolates were resistant to majority of the antibiotics tested, which indicates a serious threat to both human and animal health. This high bacterial-resistance prevalence could be due to the unrestricted availability of over the counter (OTC) antibiotics, as well as the misuse or over-use of antibiotics as a result of lack of knowledge with regard to antimicrobial use and the possibilities of bacterial antibiotic-resistance development (Ayukekbong *et al*., 2017; Asante *et al*., 2017). According to the findings, most of the farmers administer antibiotics without the veterinarian's instructions and rarely read or follow the instructions. This problem is therefore exacerbated by the fact that, the commonly used antibiotics are uncontrollably available at the market.

5.4 Chapter 5 References

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CHAPTER 6: CONCLUSION, RECOMMENDATIONS AND LIMITATIONS OF THE STUDY

6.1 Conclusion

The primary objectives of this current study was to assess the bacteriological quality of raw bovine milk produced, consumed and sold by the communal dairy farmers in Mahikeng as well as to evaluate the antibiotic resistance profiles of foodborne pathogenic bacteria isolated from such milk. As demonstrated by the results from this study, raw bovine milk produced by communal dairy farmers in Mahikeng is of poor quality, which makes it unsafe for human consumptiondue to the presence of milk-borne pathogens including *Staphylococcus aureus*, *Enterococcus* species, *Escherichia coli* and other *Enterobacteriaceae* species isolated in such milk. Poor hygienic practices, inappropriate handling as well as poor storage during and post milking at the farm have a substantial impact on the bacterial contamination of unpasteurized milk.

As demonstrated in the results of phylogenetic analysis, organisms isolated in this study showed great relationships among themselves and with organisms isolated elsewhere. In addition, high similarity between organisms and their common ancestors was also being demonstrated by the phylogenetic tree. This means that the bacterial organisms evolve with time and they share similar traits, which therefore makes it difficult to control or treat certain bacterial infections as such pathogens possess foreign traits. It is therefore imperative to improve hygienic practices in farms because, bacterial contamination in food is primarily associated with poor hygiene.

Moreover, the observed high resistance rates of isolated pathogens to streptomycin as well as the multi-drug resistance of isolated pathogens to regularly used antibiotics indicate the inappropriate or overuse of antibiotics in communal farms, which poses a health risk to the consumers of such milk and may result in most if not all commonly used antibiotics being ineffective in the future if the matter is not taken into consideration.

6.2 Recommendations

There is a need for routine assessment of raw or unpasteurized milk normally produced by the small-scale farmers for consumption. Such information is of public importance because, milk-borne pathogens may lead to severe illnesses or even death if not adequately controlled.

Educating smallholder farmers and dairy farm workers on the importance of proper hygienic practices when handling raw milk in the farm may assist in enhancing the quality of raw milk. Moreover, the importance of pasteurization of milk should be emphasized to the smallholder dairy farmers and the society at large, through education on procedures of pasteurizing milk at home. Although there are individual who believe that raw milk has many health benefits compared to pasteurized milk, the consumption of unpasteurized milk should be discouraged by alerting the society about the potential health dangers of consuming such milk. Extension officers, Veterinarians as well as the Para-veterinarians should improve their surveillance visits to the communal farms to educate, advice and train the farmers on how to maintain good farm management and animal health.

With regard to the use of antibiotics in smallholder farms, the habit of farmers administering antibiotics without consulting with the veterinarians should be discouraged. The government should review the laws allowing the availability of antibiotics without prescription in order to minimize the use of antibiotics in smallholder farms. Moreover, the government could establish food-safety programs as part of the national strategies on antimicrobial resistance, in order to promote the careful use of antimicrobials.

6.3 Limitations of the study

During the period of collecting milk samples, there were only few households/farms with lactating cows. The number of samples collected therefore was lower than expected. The isolation of specific bacteria could not be done due to limited availability of specific media to isolate such bacteria hence, the isolation of bacteria was carried out using general methodology.

This being an observational study, it is susceptible to short comings associated with observational studies. In addition, the findings of this study cannot extrapolated to other areas or the whole of South Africa

Appendices

Appendix 1: Questionnaire

Assessment of bacteriological quality and handling of raw bovine milk collected from communal areas in Mahikeng, South Africa.

Hi. My name is Gaboipofe Florah Mokgaotsi, an MSc (Animal Health) student from North West University-Mafikeng Campus. I am doing a survey regarding the possible risk factors of bacteria as well as antibiotic resistance in milk at farm level.
This questionnaire aims at finding people's knowledge and awareness of risk factors that could lead to bacterial contaminations and antibiotic resistance of such bacteria in milk at farm level. It will take less than fifteen minutes to complete this questionnaire. Please note that answering the questionnaire is voluntarily. Your answers will be kept completely confidential and your name will not be included in any reports of these results. Your answers to the following questions will not be shared with anyone.

SECTION A: RESPONDENT PARTICULARS

1. How old are you?

a) 15 – 20 yrs

b) 21 – 30 yrs

c) 31-40 yrs

d) 41-50 yrs

e) More than 50 yrs

f) Don't know or prefer not to say

2. Respondent's gender:

a) Male

b) Female

3. Respondent’s level of education:

a) No school education attendance

b) Primary school level

c) Secondary school level

d) Tertiary education level (College, University, Technicon.)

e) Religious schooling only

4. Are you the owner of this herd?

a) Yes

b) No (Go to question 9)

5. Do you own all the cattle in this herd?

a) Yes

b) No

6. For how long have you been keeping cattle?

a) Less than a year

b) 1 to 2 years

c) 2 to 3 years

d) More than three years

7. Is this herd the only source of income for your family?

a) Yes

b) No

8. How many people work here?

a) Less than 3 people

b) More than 3 people

c) I work alone

9. What is your role in the farm?

a) Herd care taker

b) Manager

c) General worker

d) Other (specify)……

SECTION B: MASTITIS RELATED QUESTIONS

10. Who primarily milks the lactating cows?

a) Myself

b) Family member only

c) External employees

d) All of us

e) Others (specify)……

11. How much milk on average do you collect from this herd per day in litres?

a) Less than 5 L

b) Between 5-10 L

c) Between 10-20 L

d) More than 20 L

12. Do you wash your hands before milking?

a) Yes

b) No (go to question 14)

c) Sometimes

13. What do you use to wash your hands?

a) Water only

b) Water with soap

c) Water with a disinfectant and soap

14. Do you wash udder and teats of the animal before and after milking?

a) Yes

b) No (Go to question 16)

c) Sometimes

15. What do you use to wash udder and teats?

a) Warm water only

b) Cold water

c) Water with a disinfectant or detergent

16. Which milking technique do you use?

a) Hand milking

b) Machine milking (move to question 18)

17. If hand milking, which technique do you use?

a) Stripping (Pulling the teat)

b) Squeezing Action

18. Can you recognize if your cow has an infection/ a problem in the udder?

a) Yes

b) No (go to question 23)

19. How do you detect?

a) Change of colour of the udder/teats

b) Udder feels warm than usual

c) Changed consistency of the udder

d) Changed size of the udder

e) Presence of visible lesion on the udder

f) Udder veins are engrossed

g) Changed milk consistency and colour

h) Others (specify)……

20. Do you milk animals with udder problem?

a) Yes

b) No (go to question 23)

c) Sometimes

21. When do you milk animals with udder infection?

a) Before the healthy animals

b) After the healthy animals

c) Others (specify)……..

22. What do you do with the milk obtained from cows with udder infection?

a) Discard

b) Consume in your household

c) Sale to the market

d) Feed to calves

e) Others (specify)……

SECTION C: GENERAL ZOONOSES EXPOSURE PRACTICES

23. What do you do with milk from your cattle herd?

a) Consume the milk as a household

b) Sell to milk vendors

c) Sell to local businesses (restaurants, hotels schools, etc)

d) Sell to milk processing company

e) Sell to neighbours and members of the community

f) Other (specify) ……

24. Do you cover the milk at household?

a) Yes

b) No

c) Sometimes

25. Is the milk sample boiled?

a) Yes

b) No

26. What is the type of milk storage container used?

a) Calabash (Wooden)

b) Plastic container

c) Glass bottle

d) Metal can

e) Don’t know

27. Is the milk stored under shade?

a) Yes

b) No

28. At what time did you milk? (Milk storage duration)

a) Less than 2 hrs

b) Between 2 – 6 hrs

c) Between 6 – 12 hrs

d) More than 12 hrs

29. When the animal is sick, what do you do with the milk?

a) Don’t milk the animal

b) Sell the milk

c) Consume it in the family

d) Use it for the calves

e) Other (specify)……

30. If the sick animal is treated with a medicine, what do you do with the milk?

a) Don't milk the animal

b) Sell the milk

c) Milk the animal and discard the milk

d) Consume it in the family

e) Use it for the calves

f) Other (specify)……

31. For how long do you wait after treatment to milk again (withdrawal periods)

a) I follow the withdrawal period of the medication

b) For as long as I want to

c) Never wait at all

32. Do you and your family consume raw milk?

(Note: Raw being unprocessed milk, NOT BOILED, not pasteurized or homogenized)

a) Always (regarded as yes)

b) Sometimes (regarded as yes)

c) No

SECTION D: VETERINARY ANTIBIOTIC USE

33. Do you know of veterinary antibiotics and their function?

a) Yes

b) No (The question to be elaborated in an explanatory manner of what antibiotics are and if the respondent has no idea of what antibiotics are and has never encountered their use in the farm then they do not have to answer the following questions)

34. Do you administer antibiotics for treatment of animals in the farm?

a) Yes

b) No

35. How often do you administer antibiotics?

a) Only when the animal is sick or showing signs of antibiotic infection

b) Only when instructed by a Veterinarian

c) There is a period set for administration of antibiotics

d) Anytime I feel like administering antibiotics

36. Do you read the instructions of the antibiotic before administering it?

a) Always

b) Never

c) Sometimes

Thank you* very *much for devoting your time to participate in this study.

Appendix 2: Selected villages for sampling

Villages	**Coordinates**
1. Tsetse	25.7314° S, 25.6702° E
2. Ikopeleng	25.6836° S, 25.6022° E
3. Magogoe	25.8950° S, 25.5977° E
4. Miga	25.6495° S, 25.5711° E
5. Dihatshwane	25.9085° S, 25.7548° E
6. Modimola	25.8578° S, 25.4141° E
7. Bodibe	26.0579° S, 25.8348° E
8. Masutlhe	25.8049° S, 25.4171° E

Appendix 3: Total Bacterial count (TBC) results table

Sample number	**Dilutions selected**	**Number of colonies observer**	**TBC results ($n\times10^7$)**
1.	10^{-5}	180	3.6
2.	10^{-5}	192	3.84
3.	10^{-5}	115	2.3
4.	10^{-5}	150	3
5.	10^{-5}	175	3.5
6.	10^{-5}	163	3.26
7.	10^{-5}	166	3.32
8.	10^{-5}	168	3.36
9.	10^{-5}	210	4.2
10.	10^{-5}	140	2.8
11.	10^{-5}	175	3.5
12.	10^{-5}	140	2.8
13.	10^{-5}	110	2.2
14.	10^{-5}	98	1.96
15.	10^{-5}	167	3.34
16.	10^{-5}	175	3.5
17.	10^{-5}	208	4.16
18.	10^{-5}	190	3.8
19.	10^{-5}	182	3.64
20.	10^{-5}	184	3.68
21.	10^{-5}	180	3.6
22.	10^{-5}	154	3.08
23.	10^{-5}	181	3.62
24.	10^{-5}	112	2.24
25.	10^{-5}	120	2.4
26.	10^{-5}	162	3.24
27.	10^{-5}	118	2.36
28.	10^{-5}	134	2.68

29.	10^{-5}	162	3.24
30.	10^{-5}	124	2.48
31.	10^{-5}	122	2.44
32.	10^{-5}	99	1.98
33.	10^{-5}	180	3.6
34.	10^{-5}	162	3.24
35.	10^{-5}	130	2.6
36.	10^{-5}	148	2.96
37.	10^{-5}	184	3.68
38.	10^{-5}	144	2.88
39.	10^{-5}	120	2.4
40.	10^{-5}	146	2.92
41.	10^{-5}	133	2.66
42.	10^{-5}	124	2.48
43.	10^{-5}	162	3.24
44.	10^{-5}	115	2.3
45.	10^{-5}	180	3.6
46.	10^{-5}	142	2.84
47.	10^{-5}	97	1.94
48.	10^{-5}	121	2.42
49.	10^{-5}	176	3.52
50.	10^{-5}	128	2.56
51.	10^{-5}	160	3.2
52.	10^{-5}	130	2.6
53.	10^{-5}	99	1.98
54.	10^{-5}	166	3.32
55.	10^{-5}	184	3.76
56.	10^{-5}	142	2.84
57.	10^{-5}	123	2.46
58.	10^{-5}	130	2.6
59.	10^{-5}	168	3.36
60.	10^{-5}	110	2.2
61.	10^{-5}	160	3.2
62.	10^{-5}	162	3.24
63.	10^{-5}	142	2.84
64.	10^{-5}	126	2.52
65.	10^{-5}	154	3.08
66.	10^{-5}	162	3.24
67.	10^{-5}	142	2.84
68.	10^{-5}	154	3.08
69.	10^{-5}	110	2.2
70.	10^{-5}	180	3.6
71.	10^{-5}	124	2.48
72.	10^{-5}	162	3.24
73.	10^{-5}	120	2.4
74.	10^{-5}	180	3.6
75.	10^{-5}	162	3.24
76.	10^{-5}	110	2.2

77.	10^{-5}	124	2.48
78.	10^{-5}	130	2.6
79.	10^{-5}	164	3.28
80.	10^{-5}	136	2.72
81.	10^{-5}	142	2.84
82.	10^{-5}	180	3.6
83.	10^{-5}	192	3.84
84.	10^{-5}	145	2.9
85.	10^{-5}	144	2.88
86.	10^{-5}	128	2.56
87.	10^{-5}	124	2.48
88.	10^{-5}	172	3.44
89.	10^{-5}	168	3.36
90.	10^{-5}	190	3.8
91.	10^{-5}	172	3.44
92.	10^{-5}	166	3.32
93.	10^{-5}	180	3.6
94.	10^{-5}	160	3.2
95.	10^{-5}	128	2.56
96.	10^{-5}	200	4
97.	10^{-5}	188	3.76
98.	10^{-5}	176	3.52
99.	10^{-5}	120	2.4
100.	10^{-5}	150	3
101.	10^{-5}	161	3.22
102.	10^{-5}	130	2.6
103.	10^{-5}	142	2.84
104.	10^{-5}	140	2.8
105.	10^{-5}	135	2.7
106.	10^{-5}	115	2.3
107.	10^{-5}	132	2.64
108.	10^{-5}	160	3.2
109.	10^{-5}	120	2.4
110.	10^{-5}	140	2.8
111.	10^{-5}	126	2.52
112.	10^{-5}	142	2.84
113.	10^{-5}	156	3.12
114.	10^{-5}	170	3.4
115.	10^{-5}	184	3.68
116.	10^{-5}	150	3
117.	10^{-5}	201	4.02
118.	10^{-5}	181	3.62
119.	10^{-5}	164	3.28
120.	10^{-5}	130	2.6
121.	10^{-5}	195	3.9
122.	10^{-5}	190	3.8
123.	10^{-5}	144	2.88
124.	10^{-5}	180	3.6

125.	10^{-5}	174	3.48
126.	10^{-5}	182	3.64
127.	10^{-5}	199	3.98
128.	10^{-5}	125	2.5
129.	10^{-5}	160	3.2
130.	10^{-5}	152	3.04
131.	10^{-5}	120	2.4
132.	10^{-5}	154	3.08
133.	10^{-5}	170	3.4
134.	10^{-5}	155	3.1
135.	10^{-5}	162	3.24
136.	10^{-5}	218	4.36
137.	10^{-5}	192	3.84
138.	10^{-5}	148	2.96
139.	10^{-5}	122	2.44
140.	10^{-5}	194	2.8
141.	10^{-5}	164	3.28
142.	10^{-5}	130	2.6
143.	10^{-5}	184	3.68
144.	10^{-5}	130	2.6
145.	10^{-5}	166	3.32
146.	10^{-5}	180	3.6
147.	10^{-5}	138	2.76
148.	10^{-5}	182	3.64
149.	10^{-5}	172	3.44
150.	10^{-5}	163	3.26
151.	10^{-5}	196	3.92
152.	10^{-5}	128	2.56
153.	10^{-5}	152	3.04
154.	10^{-5}	192	3.84
155.	10^{-5}	136	2.72
156.	10^{-5}	191	3.82
157.	10^{-5}	170	3.4
158.	10^{-5}	155	3.1
159.	10^{-5}	144	2.88
160.	10^{-5}	177	3.54
161.	10^{-5}	154	3.08
162.	10^{-5}	172	3.44
163.	10^{-5}	180	3.6
164.	10^{-5}	142	2.84
165.	10^{-5}	163	3.26
166.	10^{-5}	168	3.36
167.	10^{-5}	195	3.9
168.	10^{-5}	172	3.44
Mean:3.1×10^{7} Min: 1.94×10^{7}			

Max:
4.36×10^{7}

Appendix 4: Biochemical Tests Results Table

Sample ID	**Biochemical Tests**					**Possible organism**
	Gram Staining	**Catalase**	**Oxidase**	**Indole**	**Coagulase**	
Ma 1	+ cocci, clusters	+	—	—	+	*Staphylococcus aureus*
Ma 2	+ cocci, pairs	—	—	—	—	*Enterococcus faecalis*
Ts 1	— straight rods	+	—	+	—	*Escherichia coli*
Mi 1	+ cocci, pairs	—	—	—	—	*Enterococcus faecalis*
Ts 2	+ cocci, pairs	—	—	—	—	*Enterococcus faecalis*
Ik 1	— straight rods	+	—	—	—	*Enterobacter* sp.
Ik 2	— straight rods	+	—	+	—	*Escherichia coli*
Mi 2	— straight rods	+	—	—	—	*Shigella* sp.
Mi 3	+ cocci, clusters	+	—	—	+	*Staphylococcus aureus*
Ts 3	+ cocci, pairs	—	—	—	—	*Enterococcus faecalis*
Ik 3	+ cocci, clusters	+	—	—	+	*Staphylococcus aureus*
Di 1	+ cocci, pairs	—	—	—	—	*Enterococcus faecalis*
Ik 4	+ cocci, pairs	—	—	—	—	*Enterococcus faecalis*
Di 2	+ cocci, pairs	—	—	—	—	*Enterococcus faecalis*
Mi 4	+ cocci, pairs	—	—	—	—	*Enterococcus faecalis*
Mi 5	+ cocci, clusters	+	—	—	+	*Staphylococcus aureus*
Ts 4	— straight rods	+	—	—	—	*Enterobacter* sp.
Ik 5	+ cocci, pairs	—	—	—	—	*Enterococcus faecalis*
Di 3	— straight rods	+	—	—	—	*Enterobacter* sp.
Ma 3	— straight rods	+	—	—	—	*Enterobacter* sp.
Di 4	+ rods, straight and slightly curved	—	—	+	—	*Clostridium* sp.

Di 5	+ rods, straight and slightly curved	—	—	+	—	*Clostridium* sp.
Ma 4	+ rods, straight and slightly curved	+	—	—	—	*Bacillus* sp.
Ik 6	+ cocci, clusters	+	—	—	+	*Staphylococcus aureus*
Ik 7	+ cocci, pairs	—	—	—	—	*Enterococcus faecalis*
Ts 5	+ rods, straight and slightly curved	—	—	+	—	*Clostridium* sp.
Ts 6	+ rod, straight and slightly curved	—	—	+	—	*Clostridium* sp.
Di 6	+ cocci, clusters	+	—	—	+	*Staphylococcus aureus*
Di 7	+ cocci, pairs	—	—	—	—	*Enterococcus faecalis*
Di 8	+ cocci, pairs	—	—	—	—	*Enterococcus faecalis*
Mi 6	+ cocci, pairs	—	—	—	—	*Enterococcus faecalis*
Mi 7	+ cocci, pairs and chains	—	—	—	—	*Enterococcus* sp.
Mi 8	+ cocci, pairs	—	—	—	—	*Enterococcus faecalis*
Ik 8	— straight rods	+	—	+	—	*Escherichia coli*
Mi 9	— straight rods	+	—	—	—	*Salmonella* sp.
Ik 9	— straight rods	+	+	—	—	*Alcaligenes faecalis*
Ik 10	+ cocci, clusters	+	—	—	—	*Staphylococcus* sp.
Mi 10	+ rods, straight and slightly curved	—	—	+	—	*Clostridium* sp.
Ma 5	+ cocci, pairs	—	—	—	—	*Enterococcus faecalis*
Di 9	+ cocci, pairs	—	—	—	—	*Enterococcus faecalis*
Ik 11	+ rods, straight and slightly curved	—	—	+	—	*Clostridium* sp.
Mi 11	+ cocci, clusters	+	—	—	+	*Staphylococcus aureus*
Ts 7	— straight rods	+	—	+	—	*Escherichia coli*

Ma 6	— straight rods	+	—	+	—	*Escherichia coli*
Ik 12	+ rods, straight and slightly curved	—	—	+	—	*Clostridium* sp.
Ma 7	+ cocci, clusters	+	—	—	+	*Staphylococcus aureus*
Di 10	+ cocci, clusters	+	—	—	+	*Staphylococcus aureus*
Ts 8	— straight rods	+	—	—	—	*Shigella* sp.
Ma 8	+ cocci, pairs	—	—	—	—	*Enterococcus faecalis*
Ts 9	— straight rods	+	—	—	—	*Enterobacter* sp.
Ma 9	+ rods, straight and slightly curved	—	—	+	—	*Clostridium* sp.
Ts 10	+ ovoid, pairs and short chains	—	—	—	—	*Lactococcus* sp.
Di 11	+ cocci, pairs	—	—	—	—	*Enterococcus faecalis*
Di 12	+ rods, straight and slightly curved	—	—	+	—	*Clostridium* sp.
Ts 11	+ ovoid, pairs and short chains	—	—	—	—	*Lactococcus* sp.
Ma 10	+ rods, straight and slightly curved	—	—	+	—	*Clostridium* sp.

Positive = (+); Negative = (¯)

Appendix 5: GenBank Obtained Sequences

Isolate	**Assession number**
Citrobacter braakii	**HQ288930.1**
Enterobacter hormaechei	**KF015738.1**
Providencia stuartii	**KF951533.1**
Proteus hauseri	**KY072916.1**
Alcaligenes faecalis	**KY357285.1**
Clostridium bifermentans	**NR 113323.1**
Clostridium sp.	**LC194786.1**
Staphylococcus haemolyticus	**KU977133.1**
Lactococcus lactis	**JQ973595.1**
Lactococcus lactis	**GU936712.1**
Salmonella enterica	**MH356696.1**
Weissella cibaria	**KU302758.1**
Bacillus sp.	**MH455253.1**
Bacillus sp.	**KF870418.1**
Weissella sp.	**MF424649.1**
Escherichia coli	**MF372553.1**
Shigella flexneri	**EU009187.1**
Clostridium bifermentans	**NR 113323.1**
Staphylococcus aureus	**KR085871.1**
Enterococcus faecalis	**EU728747.1**
Enterococcus faecalis	**AB669429.1**