ANTIMICROBIAL PROPERTIES OF ESSENTIAL OILS AGAINGST BULK-TANK MILK ISOLATED BACTERIA

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DECLARATION OF INDEPENDENT WORK

I, Tshegofatso Nhabe, hereby declare that this research project submitted to the Central University of Technology, Free State for the degree MASTER OF HEALTH SCIENCE: ENVIRONMENTAL HEALTH is my own work and has not been submitted before to any institution by myself or any other person in fulfillment of the requirements for the attainment of any qualification.

Signature Date

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CHAPTER ONE

LITERATURE REVIEW

ANTIMICROBIAL PROPERTIES OF ESSENTIAL OILS AGAINGST BULK-TANK MILK ISOLATED BACTERIA

LITERATURE REVIEW

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SUMMARY

Markets for and consumers of bulk-tank milk exist in many parts of the world; bulk-tank milk is consumed by a large number of people, especially in developing countries. Due to the nutritious nature of milk spoilage and pathogenic bacteria, can grow and multiply in it. Generally, microbial contamination of milk can occur through the development of a bacterial infection on a teat canal or an infected udder (clinical or subclinical mastitis) from the animal, milker (manual as well as automated), cow environment (bedding, housing) or unclean process water. Collectively, the presence of foodborne pathogens in bulk-tank milk either directly or indirectly increase the risk of ingestion and transmission of antibiotic resistant strains from animal origin, these transmitted strains that can persist in the human intestinal tract (Jamet *et al*., 2012).

Antibiotic resistant bacterial strains have been increasingly recognized as a worldwide clinical and public health problem (Levy, 2002). The increased use of antibiotics, both in human or animal medicine, is considered a prime factor in spreading the antibiotic resistance (Hawkey and Jones 2009). However, the contribution of agricultural use of antibiotics on the emergence of antibiotic-resistant bacteria cannot be underestimated (Levy and Marshall, 2004). Silbergeld *et al*., (2008) asserted that the use of antimicrobial drugs in agriculture is the major driving force in spreading antimicrobial resistance worldwide citing four arguments as evidence: the agriculture sector is the largest consumer of antimicrobials worldwide, antimicrobial drugs in agriculture are used mostly at sub-therapeutic levels; every clinical class of drugs has been utilized in agriculture, and antimicrobial-resistant pathogens are exposed to humans through the ingestion of animal food products and passed on to the environment.

Due to the growing concern of antibiotic resistance in food-related bacteria, many studies were undertaken previously to assess the antibiotic resistance of bacteria in agricultural products such as milk (Citak *et al*., 2005; Straley *et al*., 2006). These studies reported that a significant proportion of isolates recovered from the food products demonstrated extensive resistance to antibiotics.

Antibiotic resistant bacteria in food products can transfer resistance genes to the intestinal and commensal flora of humans and these can be a reservoir of resistance genes for pathogenic bacteria (Aarestrup *et al*., 2008). Antibiotic resistance in bulk-tank milk bacteria is of significant importance as the milk is still consumed directly in many farm families, their employees and nearby families in many parts of the world (Oliver *et al*., 2009). The main aim of this study was to evaluate antimicrobial properties of essential oils against antibiotic resistant bacteria isolated from bulk-tank milk in a predetermined area of Mangaung. The objectives of the study were to: quantify and identify bacteria isolated from bulk-tank milk, evaluate antibiotic profiles of the isolated bacteria and assess antimicrobial properties of essential oils against isolated bacteria. The first part of the thesis; scientific literature on isolated bulk-tank milk bacteria and the antimicrobial resistance in food-associated bacteria is reviewed. The second part of the thesis, was the identification and quantification of isolated bulk-tank milk bacteria and in the final sections; antibiotic resistance profiles of isolated bacteria present are tested and alternative methods, particularly the use of essential oils are discussed.

1. INTRODUCTION

Milk is an excellent growth medium for microorganism growth due to its high nutritional composition; such as carbohydrates, proteins, fats, vitamins and minerals required for the growth of microorganisms, combined with higher water activity. These physicochemical characteristics enable bacteria to grow in a wide range of milk-based foodstuffs. Microbial contamination of milk happens in a variety of ways; such as the environment (water, soil and bedding materials), the udder and the milking and storage environments. Similarly, mastitis causing pathogens like *Staphylococcus* spp., and *E. coli* contaminate the milk from within the udder. The milking system, including milking machines, pipelines, bulk tanks and bulk tankers could also be potential sources of contamination of the milk (Hayes and Boor, 2001). Thus the initial microbial population in bulk-tank milk can be highly variable and largely influenced by the type and level of contamination during milking. For example, bedding material, untreated water, soil and vegetation have been reported as major sources of psychrotrophs; soil for coliforms and the bedding material for spore formers. Generally, there is a challenge that bulk-tank milk might be contaminated and some of the sources of contamination may include psychrotrophs, coliforms and spore-forming bacteria.

1.1. Psychrotrophic bacteria

Psychrotrophic bacteria are ubiquitous in nature and are common contaminants of milk. The bacteria originate from equipment, milk-stone deposits, water and people working with milk and give an indication of the potential shelf-life of the milk as they are able to grow under refrigeration conditions. Psychrotrophic bacteria in milk are studied worldwide because of the difficulties associated with controlling their growth during cold storage and the consequent negative effects upon dairy products. Psychrotrophs grow at low temperatures but have optimal and maximal growth temperatures of above 15 and 20^{o} C (Moyer and Morita, 2007). This characteristic makes these bacteria especially significant with regard to food spoilage and safety. The storage of many foods at cold temperatures is a routine practice during production, transportation, processing and post-

purchase (Beales, 2004; Russell, 2002). Psychrotrophs are not part of the normal udder microflora, so their presence suggests compromised sanitary conditions during production including length and temperature of storage (Beales, 2004). Milk thus provides a physicochemical environment which is favourable for the multiplication of a broad spectrum of microorganisms, including a range of psychrotrophic bacterial species (predominantly members of the genus *Pseudomonas*) that contaminate milk during collection and processing (Mcphee and Griffiths, 2011).

The cooling preservation of bulk-tank milk has enabled large scale collection, storage and processing of milk days after milking. Despite the variable initial bacterial population in bulk-tank milk, the refrigeration of milk has a significant effect on the type and number of bacteria in the cold-stored raw milk. Refrigeration can largely reduce the growth of mesophilic bacteria and support the growth of psychrotrophic bacteria. These bacterial species become the main source of spoilage of bulk-tank milk during low temperature storage (Bramley and McKinnon, 1990; Hayes and Boor, 2001; Kumaresan *et al*., 2007).

1.2. Coliforms

Coliforms are aerobic or facultative anaerobic, gram-negative, non-spore forming rods that are capable of fermenting lactose, resulting in gas and acid production within 48 hours at 35^{o}C (Imhoff, 2005). These characteristics allow selective detection and counting of these types of bacteria in milk and dairy products. Traditionally, coliforms were classified into four genera: *Escherichia*, *Klebsiella*, *Citrobacter* and *Enterobacter*. Today, over 20 bacterial genera include strains that have phenotypic characteristics which classify them as coliforms (Imhoff, 2005). Detection of coliforms plays an important role in the dairy industry because coliforms are frequently used as hygiene indicators, and there are clear regulatory limits for the presence of coliforms in finished dairy products. Their presence in food and water indicate contamination; e.g. the presence of faecal coliforms (*E. coli*) suggests the possibility of faecal contamination. Sources of coliform bacteria include flora of mastitic animals, exterior of the animal, dairy barn environment, milk contact

surfaces like dirty environments and inappropriate milk storage time or temperature. Regulatory limits in South Africa states that anything above the legislative standard of 5 x 10^4 cfu/ml for bulk tank milk intended for consumption from guideline set by the R1555 of 1997 as amended by R489 of 2001 (Regulations Relating to Milk and Milk Products) should be condemned.

1.3. Spore forming bacteria

As milk is highly nutritious and has a near neutral pH and high water activity, it provides an ideal environment for the proliferation of microorganisms (Quigley *et al*., 2013). Of the microorganisms that can proliferate in the milk chain on farms or through dairy processing lines, spore-forming bacteria are of a particular concern as they have the ability to withstand harsh environmental conditions (Postollect *et al*., 2012). Spore forming bacteria are ubiquitous bacteria commonly found in the soil as well as being natural colonizers of the gastrointestinal tract of warm-blooded animals (Postollect *et al*., 2012). They are gram-positive organisms consisting of more than 200 species that are capable of forming endospores, which make them resistant to extreme heat or cold, drought, starvation, biocides and UV irradiation (Moeller *et al*., 2008). Bacteria found to contaminate the bulk-tank milk usually come from sources from the environment, within the udder or the equipment itself.

2. Possible sources of contamination

Cows can be exposed to environmental sources of microbes on the farm, which can cause mastitis, an infection of the udder that can spread pathogens during milking (Olivier *et al*., 2005). Faecal contamination from the cows during milking can also allow high amounts of pathogenic microbes to enter the milk. During large scale milk production, unprocessed milk is sent from dairy farms to shops with bulk tanks where large quantities of milk are stored (Olivier *et al*., 2005). Bacteria can grow in these tanks and spread to previously uncontaminated milk and this is usually due through microbial biofilms in the distribution pipes, unhygienic practices of employees, or the use of unsterilized containers. The risk of microbial contamination also occurs due to practices of dairy

workers at all points at the farm such as milk processing, improperly cleaned equipment and on the farm transportation (Straley *et al*., 2006; Munsch-Alatossava and Alatossava, 2007). After milk is distributed, failure to maintain milk at refrigeration temperatures can allow pathogenic microbes to multiply, and this can increase the possibility of illness from consuming the milk (Blowey and Edmondson, 2010). Improper storage can be the fault of the dairy distributors, but also retail workers and milk consumers (Gould *et al*., 2014).

Although refrigeration is recommended as a storage practice, it is not enough to control the growth of psychrotrophic bacteria which are able to grow well between 4^{o}C and 7^{o}C. It is well recognized that the growth of many Gram-negative psychrotrophic bacteria (such as *Pseudomonas*, *Acinetobacter* or *Aeromonas*) cause significant defects in product quality such as flavour defects, emulsion degradation and gelation in the products by their heat resistant extracellular enzymes proteases (Zall, 1990; Barbano *et al*., 2006; Hantsis-Zacharov and Halpern, 2007). In addition, few studies have reported that a significant proportion of psychrotrophic bacteria show extensive resistance to many functional classes of antimicrobials and the level of multiple antimicrobial resistant traits was shown to increase along the cold chain of milk storage and transportation (Straley *et al*., 2006; Munsch-Alatossava and Alatossava, 2007). Antimicrobial resistance in bacteria has been increasingly recognized as a worldwide clinical and public health problem (Levy, 2002). Increased use of antimicrobials, both in human or animal medicine, is considered a prime factor in spreading antimicrobial resistance (Hawkey and Jones, 2009). However, the contribution of agricultural use of antimicrobial on the emergence of antibiotic-resistant bacteria should not be underestimated (Levy and Marshall, 2004).

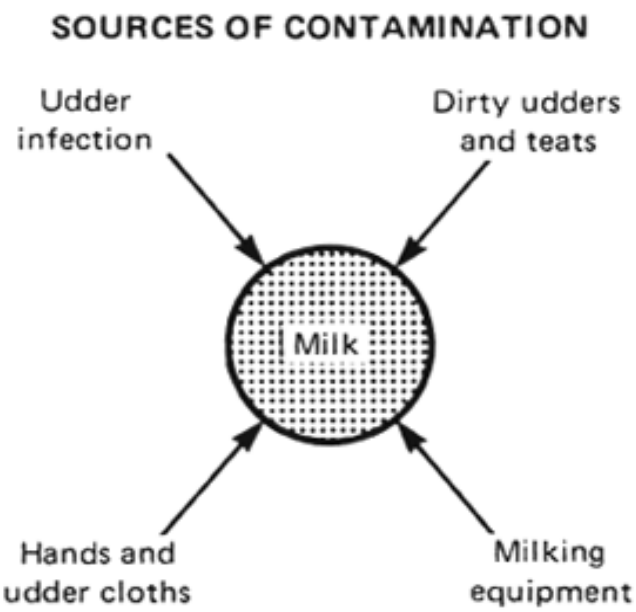

Figure 1: Possible sources of contamination

(Food and agriculture organization of the United Nations, Rome, © FAO 1989)

2.1. Microbial Contamination from within the Udder

The farm environment itself and incoming (animal feed) and outgoing (manure) material may act as a source of contamination and a reservoir of antibiotic-resistant strains. Dirty udders and teats are considered important sources of environmental bacteria in milk (Wallace, 2009) **Figure 1** in this chapter. As the proportion of cows with dirty udders and teats increases, the time required for pre-milking udder preparation also increases (Reneau and Bey, 2007), which may have an influence on milking efficiency and may lead to inadequate preparation of udders and teats. Previous studies have reported a positive association between the degree of udder contamination and the level of mastitis as measured by individual cow linear score (Ellis *et al*., 2007).

Reservoirs for environmental pathogens are materials in the cow's surrounding which include; manure, soil, feed and bedding (Barrett *et al*., 2005). Bedding material is an important reservoir for bacterial growth and therefore creates a heightened concern for teat exposure to environmental pathogens (Godden *et al*., 2008). A common characteristic of major environmental pathogens is the

ability to survive in the gastrointestinal tract of cows and replicate in manure. These bacteria pass from cow to cow during udder preparation and the milking process. Defective milking machines, inappropriate hygiene practices, and presence of carrier cows in the herd can all lead up to this event (Barrett *et al*., 2005). Therefore, managing manure contaminating the environment of the cow is crucial (Godden *et al*., 2008).

2.2. Equipment

Equipment used for milking, processing and storage likewise determines the hygiene level of bulk-tank milk. Milk produced in udder cells is sterile but due to its high nutrient content, it can be a good growth medium for contaminating bacteria (Michaelidou *et al*., 2008). Bacterial contaminants can cause disease in animals and consumers; spoilage of milk thus compromising quality through production of secondary by-products. Bacteria such as spore forming bacteria are of particular concern in this regard as they are able to survive adverse conditions and form biofilms within pipes and stainless steel equipment. These single or multiple-species biofilms become a reservoir of spoilage microorganisms and a cycle of contamination can be initiated. Previous studies have highlighted that these microorganisms are highly prevalent in corners, cracks, valves and the joints of stainless steel equipment used in the dairy manufacturing plants (**Figure 1** in this chapter). Therefore, adequate monitoring and control measures are essential to prevent spoilage and ensure customer safety (Godden *et al*., 2008). Based on the above, milk thus easily favors the growth and multiplication of many bacteria. This is observed even in cases where the milk has been pasteurized or refrigerated. If milk safety and quality standards are not in place, the high nutritional composition and neutral pH of milk may convey many food-borne pathogens and thereby constitute a public health challenge to consumers. In order to protect consumers from foodborne illnesses, antibiotics are used to prevent and treat bacterial diseases in humans; while in animals they are not only used to treat bacterial diseases but are also introduced as part of animal feed (Angulo *et al*., 2009).

3. Antibiotics

Walsh, 2003 defines antibiotics as a group of natural microbial products or synthetic chemical compounds that inhibit the growth of and eliminate bacteria **Table 1** in this chapter (Major antibiotics grouped according to their mechanisms of action and their chemical structures). Natural antibiotics are produced by both bacteria or fungi and act by blocking essential cell processes in other bacteria. Man-made antibiotics, available commercially, also target vital cell mechanisms in bacteria (Walsh, 2003). The first natural antibiotic isolated in pure form was penicillin and it came into clinical practice in the 1940s. Their mode of action include inhibition of bacterial cell wall synthesis, inhibition of protein synthesis, disruption of cell membranes and inhibition of nucleic acid synthesis (Walsh, 2003). Antimicrobial usage in humans has not only been linked to the elevated antibiotic resistance in bacteria, but the use of antimicrobials in veterinary medicine, agriculture, aquaculture and horticulture have been identified as the other sources in escalating the problem (Aminov, 2009). When antibiotics were first introduced in clinical practices in the 1940s, they were virtually effective against pathogenic bacteria. However, the effectiveness of the antibiotics was greatly reduced due to the emergence of the antimicrobial-resistant bacteria soon after their introduction (Walsh, 2003; Aminov, 2009).

Several factors have been implicated in the emergence of the antimicrobial-resistant bacteria and their spreading in the environment. At the forefront is antimicrobial use in foodstuffs of animal origin; here antimicrobials are used in animal production for treatment of infectious diseases, metaphylactics, prophylactis, and growth promotion (Aarestrup, 2005). Of public importance was the observation that due to exposure of animals to antibiotics, milk contained some type of inhibitory substance. These could consist of antibiotics which are administered to control diseases such as mastitis in lactating animals, and antibiotics applied either by infusion, injection or orally, which may enter the milk supply (Aarestrup, 2005). The main source of antibiotics in the milk is through the application of intra-mammary products. Untreated quarters may be contaminated via

the blood circulation or by diffusion. Other ways are through the percutaneous, subcutaneous, intramuscular and intravenous application of antibiotics (Aarestrup, 2005). Antibiotics can enter the milk supply (Fejzic *et al*., 2014): i) If the correct with-holding period is not adhered to by the farmer after administering antibiotics to lactating cows; ii) Through extra-label use of antibiotics (i.e. Increased dose, increased frequency of treatment, unproven route or administration) which is shown to be associated with an increased risk of antibiotic levels in milk persisting beyond the milk-with-holding time period; iii) Through accidental or intentional transfer of a batch of milk that has antibiotics in the bulk tank. Milk equipment which is not rinsed adequately may also contain residues of disinfectants which are used in the cleaning process (Fejzic *et al*., 2014).

In general, the increased use of antibiotics is reported as the prime factor for the increased resistance in pathogenic bacteria (Levy and Marshall, 2004). When antibiotics are used in humans or animals, approximately eighty to ninety percent of the ingested antibiotics are not broken down; instead they pass through the body still intact and enter the environment as waste. Therefore, retaining their ability to affect bacteria and promote antibiotic resistance even after they enter the soil or water as a waste product (Levy and Marshall, 2004). This is of concern considering that foodborne related illnesses and gastrointestinal illnesses are the most common infections associated with food pathogenic bacteria. While in healthy individuals' symptoms are generally mild, complications can lead to more serious diseases such as meningitis, septicemia and even death in immune compromised individuals, the elderly and the very young (Shiota, 2010).

Table 1: Major antibiotics grouped according to their mechanisms of action and their chemical structures (Levy and Marshall, 2004).

Mechanism of action	Antibiotic families
Inhibition of cell wall synthesis	Penicillins; cephalosporins; carbapenems; daptomycin; monobactams; glycopeptides
Inhibition of protein synthesis	Tetracyclines; aminoglyocides; oxazolidonones; streptogramins; ketolides; macrolides; lincosamides
Inhibition of DNA synthesis	Fluoroquinolones
Competitive inhibition of folic acid synthesis	Sulfonamides; trimethoprim
Inhibition of RNA synthesis	Rifampin/ansamycins
Other	Metronidazole

3.1. Antimicrobial resistance

Alekshun and Levy, 2007 state that microorganisms owe their existence to their ancestor ability to adapt and change. An unfortunate consequence of this process is the development of microbial resistance to clinical antibiotics. When a population of microorganisms first encountered antibiotics, cells that are highly susceptible were rapidly killed. However, cells that already possess some degree of resistance or acquired it later through mutation and genetic exchange survived and proliferated. Therefore, a variety of mechanisms for microbial resistance were discovered (Alekshun and Levy, 2007). Antimicrobial resistance refers to either intrinsic or acquired resistance. Intrinsic resistance is caused by structural or functional characteristics inherited by a bacterial group (that includes species, genus or even to a higher level) such as the low affinity of the antibiotics to the target, the inability of the antibiotics to enter the bacterial cell, removal of the antibiotics from the cell by chromosomally encoded active exporters, inactivation of antibiotics by innate enzymes

and other mechanisms (Guardabassi and Courvalin, 2006). Sometimes, antimicrobials are used too often or incorrectly, which can cause resistance to spread faster than it would naturally. In some cases, a type of bacteria will survive antimicrobial treatment and multiply because it is intrinsically resistant. For example, although many types of bacteria have cell walls, some do not. An antibiotic like penicillin that prevents cell-wall building cannot harm a bacterium that does not build a cell wall in the first place (Guardabassi and Courvalin, 2006).

Guardabassi and Courvalin, 2006 further stated that acquired resistance occurs when a microorganism obtains the ability to resist the activity of a particular antimicrobial agent to which it was previously susceptible and this can result from the mutation of genes involved in normal physiological processes and cellular structures, from the acquisition of foreign resistance genes or from a combination of these two mechanisms (Guardabassi and Courvalin, 2006). Furthermore, unlike intrinsic resistance, traits associated with acquired resistance are found only in some strains or subpopulations of each particular bacterial species (Guardabassi and Courvalin, 2006). Laboratory methods are therefore needed to detect acquired resistance in bacterial species that are not intrinsically resistant and these same methods are used for monitoring rates of acquired resistance as a means of combating the emergence and spread of acquired resistance traits in pathogenic and non-pathogenic bacterial species. Acquired resistance results from successful gene change and/or exchange that may involve mutation or horizontal gene transfer via transformation, transduction or conjugation (Alekshun and Levy, 2007).

Another important characteristic of bacterial resistance associated with milk and milk products is their ability to form biofilms (Alekshun and Levy, 2007). The biofilms may be formed by single bacterial species, including spoilage and pathogenic antibiotic resistant organisms. However, the biofilm often consists of the mixture of microorganisms present in the actual environment. Biofilms are generally a complex structural, heterogeneous, genetically divergent community of microorganisms that exist on a solid surface in the form of an extracellular matrix composed of

polymeric compounds (Constantin, 2009). Microorganisms colonizing surfaces as part of a biofilm matrix display more resistance to toxic compounds than their single-celled counterparts in suspension (Holmes and Evans, 1989). Attachment to surfaces can also have an impact on bacterial resistance to disinfection. Theoretically, the topography of a surface affects the ability of a disinfectant to approach the cell. A freely suspended (planktonic) organism is susceptible to a disinfectant from all sides and at all angles, while an organism attached to a surface is susceptible from only one side. Removing adherent cells of the bacteria from a surface increases their susceptibility to sanitizers equivalent to that of planktonic cells (Frank and Koffi, 1990).

3.2. Implications of resistant bacteria in milk

Antibiotics have proven valuable for treating infections in food animals; however, there is adequate data to believe that the sudden emergence of antibiotic resistant bacteria is spreading from farm to animals through the food supply (Bradly *et al*., 1993). Antibiotic residues in milk have been found to contribute to the development acquired resistance of resistant bacteria and exposure to one antibiotic has also been found to increase the resistance of bacteria to other types of antibiotics (Bradly *et al*., 1993). This means that antibiotic levels previously considered safe, may be the source for resistant populations of bacteria in our food supply (Bradly *et al*., 1993).

The occurrence of antibiotic residues in bulk-tank milk is also considered by South African regulations as undesirable and regarded as another source that contributes to the development or transmission of resistant bacteria (R1555 of 1997 as amended by R489 of 2001). Therefore, public health officers are expected to protect people from exposure to antibiotics that are spread through food sources; as this can cause allergies, cancer, and alterations in the intestinal flora (R1555 of 1997 as amended by R489 of 2001). The use of antimicrobials in foodstuffs of animal origin should be stopped as the regulation Regulations Relating to Milk and Dairy Products, R1555 of 1997 as amended by R489 of 2001 prohibits the use of antibiotic use to protect public health. Interventions that reduce the need for antibiotics may have an added benefit of further reducing the risk of

antimicrobial residues. Though the development of antibiotic resistant determinants is associated with the uncontrolled usage of antibiotics in human and veterinary medicine, the incidence of antimicrobial resistant bacteria in bulk-tank milk samples warrants closer monitoring.

4. Strategies for control

Mechanisms for combating the emergence of resistant microorganisms vary and include newly designed synthetic antibiotics whose mode of action cannot be overcome by a mutating bacterium (DSA, SA. 2012). Meanwhile, new food processes such as organic food production limit the use of certain preservatives and sanitizers, putting a greater burden on the allowable ones which may not be as effective against emerging bacterial strains (DSA, SA. 2012). Surveillance programs are a way to monitor the development of antimicrobial resistance and regulatory guidelines for antibiotic use. These are being enacted in countries around the world as different strategies for control are carefully evaluated (DSA, SA. 2012).

The Dairy Standard Agency (DSA) has an on-going project with a primary objective that deals with the promotion of compliance of milk and other dairy products with product composition, food safety and metrology standards. The project is a partnership between the South African organized dairy industry, DSA and municipal health authorities. The project entails monitoring and evaluation of milk and other dairy products, at a retail level to determine compliance with product composition, safety and metrology standards. The focus of this project is supported by sampling protocols that provide for products in packed and bulk form in the formal and informal retails sectors. In co-operation with metropolitan, district and local municipal health authorities in nine provinces, an estimated five hundred samples are taken quarterly for testing by independent laboratories. The test results are conveyed to the relevant environmental health practitioner (EHP), regional offices of the Department of Agriculture, Forestry and Fisheries (Inspection Services) and the National Regulator for Compulsory Specifications (NRCS). The trend analysis based on the processed data obtained from milk analysis is used to determine food safety and quality compliance

as well as food safety risks; including the detection of antibiotics in milk. Selected data indicating serious food safety, product compositional and metrology non-conformances in terms of legal standards are further dealt with.

Remedial action programs are put in place to facilitate awareness of non-conforming results with processors, distributors of the milk. Environmental health practitioners are also tasked to interact with all processors or distributors whose results are in non-conformance with the relevant legal standards (R1555 of 1997 as amended by R489 of 2001). Technical assistance by means of DSA developed literature, guidelines and information brochures are offered. Further sampling, where appropriate, to establish whether non-conformances in terms of legal standards are addressed. If not, the matter is handed over to another project: Communication with authorities and other organizations, for further action with higher level government officials (DSA, SA. 2012). Other methods should be done on this point of strategies for control; newly designed synthetic antibiotics whose mode of action cannot be defeated by a mutating bacterium can be produced. One of these methods could be essential oils; they might be effective in controlling and possibly eliminate the spread of resistant bacteria.

5. Alternative antimicrobials

Therapy based on antibiotic therapy is not always effective; and this may lead to liabilities in the recovery of infected animals, selection of resistant bacteria and the presence of antimicrobial residues in milk (Hyldgaard *et al*., 2012 and Pozzo *et al*., 2011). Therefore, essential oil-based disinfectants can be used to reduce the spread of bacterial contamination in the dairy industry (Hyldgaard *et al*., 2012; Pozzo *et al*., 2011). The use of essential oils can serve as an alternative to control pathogenic bacteria in bulk-tank milk due their antibacterial action against gram-positive and gram-negative bacteria and no adverse effects on human health (Hyldgaard *et al*., 2012; Pozzo *et al*., 2011). Essential oils are volatile secondary metabolites of low molecular weight derived from plants; they are usually obtained by steam or hydro-distillation. Known for their antiseptic, i.e.

bactericidal, virucidal and fungicidal, and medicinal properties and their fragrance, they are used in the preservation of foods and as antimicrobial, analgesic, sedative, anti-inflammatory and locally anesthetic remedies. Up to the present day, these characteristics have not changed much except that more is now known about some of their mechanisms of action, particularly at the antimicrobial level. They have antimicrobial properties with no reports of resistance after prolonged exposure to microorganisms, and no side effects on human health which makes them a potential weapon against bacterial diseases (Pozzo *et al*., 2011). Essential oils are lipid soluble and soluble in organic solvents with a generally lower density than that of water and can be synthesized by all plant organs, for example; flowers, leaves, stems, seeds, fruits, roots, wood, and are stored in secretory cells, cavities, canals, epidermal cells (Pozzo *et al*., 2011). There are several methods for extracting essential oils. These may include use of liquid carbon dioxide or microwaves and mainly low or high pressure distillation employing boiling water or hot steam. Hyldgaard *et al*., 2012 states that due to essential oils bactericidal and fungicidal properties, pharmaceutical and food uses; they are more and more widespread as alternatives to synthetic chemical products to protect the ecological equilibrium and in those cases; extraction by steam distillation or expression is preferred (Hyldgaard *et al*., 2012). Furthermore, studies have shown that essential oil components can inhibit pro-inflammatory cytokine expression while stimulating production of anti-inflammatory cytokines. Thus essential oils can be utilized in a dual manner whereby they control and inhibit mastitis causing bacteria while in animals with mastitis they can be used for biotherapy (Hyldgaard *et al*., 2012).

Despite the wide use of essential oils especially as fragrances in the cosmetic industry, it is important to develop a better understanding of their mode of action for new applications in human health, agriculture and the environment (Carson and Riley, 2003). Some of them constitute effective alternatives to synthetic compounds produced by the chemical industry, without showing the same secondary effects (Carson and Riley, 2003). Properties of essential oils, cytotoxicity and the mode of essential oils will be discussed in deeper detail later in this study.

5.1. Mode of action of Essential Oils

Over the years, antimicrobial effects of essential oils have been screened against a wide range of microorganisms, but their mechanism of action is still not completely understood. Several mechanisms have been proposed to explain the actions of the chemical compounds contained in essential oils (Burt, 2004; Cox *et al*., 2000). The antimicrobial activity of essential oils is directly correlated to the presence of their bioactive volatile components (Mahmoud and Croteau, 2002). Chemically, essential oils consist of terpene compounds (mon-, sesqui- and diterpenes, alcohols, acids, esters, aldehydes, ketones, amine and sulphides (Pichersky, Noel and Dudareva, 2006). Essential oils are composed of several components and their antimicrobial activity cannot be confirmed based on the action of one compound (Bajpai *et al*., 2012). Several researchers have proposed that the antimicrobial action of essential oils may be attributed to their ability to penetrate through bacterial membranes to the interior of the cell and exhibit inhibitory activity on the functional properties of the cell, and to their lipophilic properties. The phenolic nature of essential oils draws an antimicrobial response against foodborne pathogen bacteria. Phenolic compounds disrupt the cell and eventually cause leakage of the internal contents of the cell (Bajpai *et al*., 2012).

Mechanisms of action may be related to the ability of phenolic compounds to alter microbial cell permeability, damage cytoplasmic membranes, interfere with cellular energy (ATP) generation system and disrupt the proton motive force (Bajpai *et al*., 2012; Burt, 2004). The disrupted permeability of the cytoplasmic membrane can result in cell death. Another important characteristic of essential oils and their components is hydrophobicity; this characteristic allows them to separate the lipids of the bacterial cell membrane and mitochondria in eukaryal microorganisms and in the process cause the microbial cell to become permeable (Burt, 2004). The interaction of essential oils with microbial cell membranes results in the growth inhibition of some Gram-positive and Gram-negative bacteria (Calsamiglia *et al*., 2007).

Gram-positive bacteria such as *Staphylococcus aureus, Listeria monocytogenes* and *Bacillus cereus* are more susceptible to essential oils than Gram-negative bacteria such as *Escherichia coli* and *Salmonella enteritidis* (Chorianopoulos *et al*., 2008). It is generally believed that essential oils mechanistically should be more effective against Gram-positive bacteria due to the direct interaction of the cell membrane with hydrophobic components of essential oils (Sokovic *et al*., 2010). The locations or mechanisms in the bacterial cell thought to be sites of action for essential oils components are indicated in **Figure 2** in this chapter. Based on literature; gram-negative cells should be more resistant to plant essential oils because they possess a hydrophilic cell wall. This outer layer helps to prevent the penetration of hydrophobic compounds (Chorianopoulos *et al*., 2008).

Small hydrophilic solutes are able to pass through the outer membrane via abundant porin proteins that serve as hydrophilic trans-membrane channels, and this is one reason that gram-negative bacteria are relatively resistant to hydrophobic antibiotics and toxic drugs. The outer membrane is, however, almost but not totally impermeable to hydrophobic molecules, some of which can slowly traverse through porins (Nikaido *et al*., 1996). The mechanisms of action of essential oils and/or their components are dependent on their chemical composition. For instance, thymol and carvacrol have similar antimicrobial effects but have different mechanisms of action against gram-positive and gram-negative bacteria (Nikaido *et al*., 1996). The location of one or more functional groups on these molecules can affect their antimicrobial activity (Nikaido *et al*., 1996). Thymol is structurally analogous to carvacrol, but the locations of the hydroxyl groups differ between the two molecules. However, this difference does not affect the activity of their antimicrobial activity.

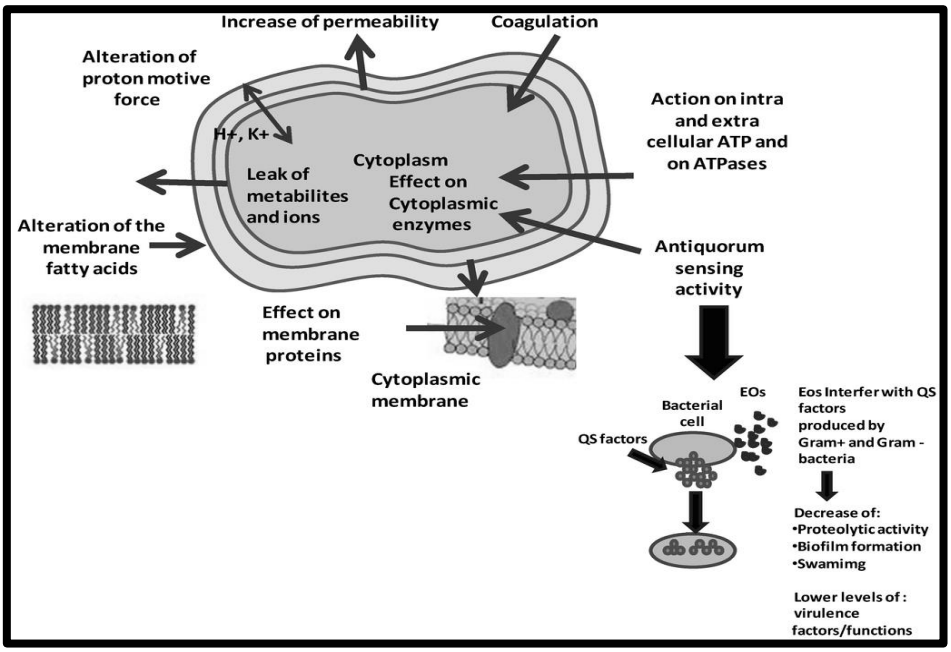

Figure 2: Mechanism of action and target sites of the essential oils on microbial cells (Nazzaro *et al*., 2013)

Generally, essential oils that possess the strongest antibacterial properties against foodborne pathogens contain a high percentage of phenolic compounds such as carvacrol, eugenol (2-methoxy-4-(2-propenyl) phenol) and thymol (Lambert *et al*., 2001). It seems rational that their mechanism of action would therefore be similar to other phenolics and this is generally considered to be the disturbance of the cytoplasmic membrane, disruption of the proton motive force (PMF), electron flow, active transport and coagulation of cell contents (Davidson, 1997). The susceptibility of both gram-positive and gram-negative will be further discussed in the coming paragraphs.

5.2. Susceptibility of gram-negative and gram-positive organisms

Theories describing the antimicrobial mechanism of phenolic compounds exist in large numbers as the pathway of action is unclear and tends to vary by essential oil or microorganism (Tenover, 2006). Most studies investigating the action of essential oils against food spoilage organisms and food borne pathogens agree that essential oils are slightly more active against gram-positive than gram-negative bacteria. It is widely held that gram-positive bacteria experience greater sensitivity to the antimicrobial action of essential oils than gram-negative species based on a technique which detects the thick peptidoglycan cell wall characteristic of gram-positive bacteria (Tenover, 2006).

Gram-negative bacteria are of particular biomedical, technological interest owing to their increasing antibiotic resistance and their utility in many biotechnological processes (Tenover, 2006).

The gram-negative outer membrane resembles most biological membranes; being a lipid layer with embedded membrane proteins, however it is extremely asymmetric (Nikaido, 2003). In lipid terms, the lipopolysaccharide is comprised of saturated fatty acid chains, packing together to form a highly rigid lipid bi-layer in contrast to the more fluid, unsaturated fatty acid chains found in membranes of other species. Each lipopolysaccharide molecule also contains a larger quantity of these hydrocarbon chains than glycerophospholipids, further increasing structural rigidity (Delcour, 2009). Gram-positive bacteria lack this extra, more complex membrane, and instead are surrounded by a thick peptidoglycan wall. This peptidoglycan layer strengthens the wall structurally, but is not dense enough to exclude small antimicrobial particles which can permeate the layer and access the cellular membrane (Nikaido, 2003; Hyldgaard *et al*., 2012).

Gram-negative bacteria are however not immune to the effects of essential oils, despite their additional outer membrane and this is because of the presence of porin proteins embedded along the outer membrane which aid in the acquisition of nutrients by the cell, these proteins allow the transport of macromolecules; and are generally able to exclude toxins and antibiotics from entering the cell (Nikaido, 2003 and Hyldgaard *et al*., 2012). However, some hydrophilic agents and substituted phenolic compounds are able to penetrate the outer membrane by means of these porin proteins, gaining access to the periplasmic space and cytoplasmic membrane where the targets of their antimicrobial activity exist (Nikaido, 2003). The additional outer membrane does not, therefore, grant absolute immunity to gram-negative organisms against essential oils (Nikaido, 2003). A lot of studies done used oils such as thymol, eugenol, carvacrol; this led to the use another type of oil (*Artemisia afra*) to see whether it also has properties that can control and possibly eliminate antibiotic resistant bacteria isolated from bulk-tank milk (Nikaido, 2003).

6. *Artemisia afra* (Lengana)

Many plants from the *Artemisia* genus are used throughout different cultures as traditional medicine (Willcox, 2009). It is considered a future flagship of traditional medicine due to its broad spectrum of activity (Van Wyk *et al*., 2009). The genus' name is derived from the Greek goddess Artemis who gave artemisian plants to Chiron the Centaur. *Artemisia* is one of the largest genera in the family of the Asteraceae and also one of the most widely distributed (El-Sahhar, 2010). There are about 500 species occurring throughout the world, with the majority located in China (150 species), ex-USSR (174 species), and Japan (50 species) (Wright, 2002). Taxonomically, *Artemisia afra* is classified into Kingdom: Plantae, Division: Mannoliphyta, Class: Magnoliopsida, Sub-class: Asteridae, Order: Asterales, Family: Asteraceae, Genus: *Artemisia* and Species: *Artemisia afra* (Van Wyk, 2008). *Artemisia afra* is the only indigenous species from the *Artemisia* genus in the African continent, being prevalent from South Africa up to Ethiopia (Van Der Walt, 2004).

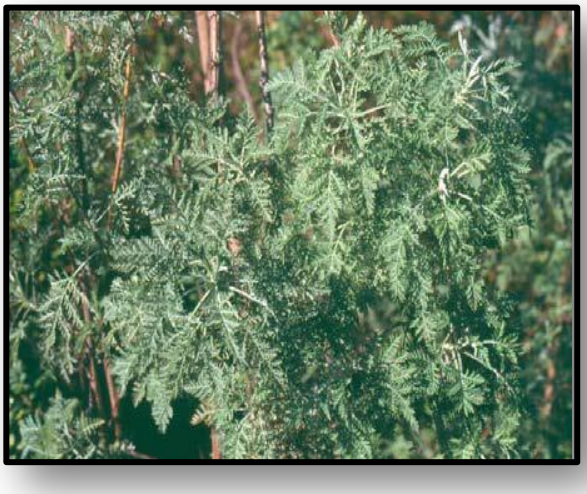

Figure 3: Live plant of *Artemisia afra*

6.1. Morphology and Traditional uses

Artemisia afra is often a woody shrub, which grows up to 2 m tall with a leafy, hairy and ridged stem (Van Wyk *et al*., 1997) **Figure 3** in this chapter. Its leaves are of soft texture, dark green on the adaxial surface and a lighter green on the abaxial surface, reaching a length of 8 cm and a width of 4 cm. *Artemisia afra* blossoms from January to June, producing yellow, butter-coloured flowers with abundant bracts. The plant has an easily identifiable aromatic odour and smells pungent and sweet after bruising. In South Africa, *A. afra* is commonly known as African wormwood, "umhlonyane" (Xhosa, Zulu), "lengana" (Sotho, Tswana) and wildeals" (Afrikaans) (Van Wyk *et al*., 1997). Traditional African medicinal uses of *A. afra* include the treatment of colds, coughs, influenza, sore throat, asthma, pneumonia, blocked nose, stomach ailments, headache, earache, poor appetite, heartburn, parasites, measles, gout, diabetes, colic, flatulence, constipation, malaria, and wounds (Van Wyk, 2008), showing the vast range of diseases and conditions it is applied for and thus its large medicinal potential.

A variety of traditional uses have been tested for significant benefits resulting from the use of *Artemisia afra*, such as for the stomach ailments for which ethanolic extracts of *Artemisia afra* leaves resulted in reductions in spontaneous rhythmic and agonist-induced contractions of isolated mouse duodenum and guinea pig ileum (Mulatu & Mekonnen, 2007), thus confirming traditional practices. Strong antioxidant activity has also been found in *Artemisia afra*, resulting in efficient anti-coccidial action in poultry (Naidoo *et al*., 2008) and use in treating fever, rheumatism, and diabetes. The antioxidant activity is thought to be due to it acting as a non-specific donor for hydrogen atoms (Liu *et al*., 2008) and by being an effective hydroxyl radical scavenging agent.

6.2 Biological activity of Artemisia afra

Antibacterial and antifungal activity has also been tested for in *Artemisia afra*, showing high degrees of growth inhibition of 15 species of bacteria and one species of fungi (Patil *et al*., 2011). In another study, researchers found potent *in vitro* anti-mycobacterial activity and pulmonary inflammation modulation in *Mycobacterium tuberculosis*-infected mice (Ntutela *et al*., 2009). While screening 7500 different plant extracts for anti-cancer properties, *A. afra* was one of 32 plant extracts to have showed significant anti-cancer activity, specifically against melanoma, renal, and breast cancer (Fouche *et al*., 2008). Its anti-cancer properties have also been labelled as 'moderate' when it was tested against 60 cancer cell lines, with its most significant activity being logged for colon, melanoma, and non-small cell lung cancer. Flavonoids found in *A. afra* have exhibited anti-carcinogenic, anti-mutagenic, and anti-tumorigenic properties (Patil *et al*., 2011). Lastly, *A. afra* has also been screened for anti-malarial properties, which showed promising *in-vitro* anti-plasmodial activity when seven flavonoids and sesquiterpene lactones were extracted through guided bio-essay fractionation and tested (Kraft *et al*., 2003). From these findings, this specie has proven to have a scientific foundation and this opened up the possibility of testing its ability to control and possibly eliminate antibiotic resistant bulk-tank isolated bacteria. With current limited information on the antimicrobial properties of *A. afra* essential oils against bacteria this became an opportune moment to assess these properties (Kraft *et al*., 2003).

7. Rationale

Antibiotic/antimicrobial resistance is the ability of microbes to resist the effects of drugs- that is, bacteria are not killed, and their growth is not stopped. The use of antibiotics (Specifically overuse and incorrect use) is the single most important factor leading to antibiotic resistance around the world (Hyldgaard *et al*., 2012). Antibiotics are among the most commonly prescribed drugs used in human and animal medicine (Walsh, 2003). Bacteria that contaminate food can become resistant against antibiotics because of the use of antibiotics in humans and food-producing animals. For

some bacteria, it is primarily the use of antibiotics in food animals that increases resistance because of the link between antibiotic use in food-producing animals and the occurrence of antibiotic-resistant infections in humans. Antibiotics that are medically important to treating infections in humans should be used in food-producing animals only under veterinary oversight and only to manage and treat infectious disease, not to promote growth (Walsh, 2003). The presence of antimicrobial residues in milk has become a threat especially to people with weakened immune systems, pregnant women, and the elderly and very young individuals. Bulk-tank milk contamination can cause foodborne diseases and be regarded as a potential vehicle for the transmission of bacteria to humans (Normanno *et al*., 2007; Huong *et al*., 2010); due to improper sanitary and health care procedures (udder infection; dirty udders and teats; hands and udder cloths and milking equipment) implemented during the production and marketing of these products (Havelaar *et al*., 2010; Newell *et al*., 2010). Although some people are at greater risk than others, no one can completely avoid the risk of antibiotic-resistant bacterial infections. Infections with resistant organisms are difficult to treat, requiring costly and sometimes toxic alternatives. Bacteria will inevitably find ways of resisting the antibiotics developed by humans, which is why aggressive action is needed to keep new resistance from developing and to prevent the resistance that already exists from spreading. The demand for safe foods, coupled with the preference by consumers for foods free of synthetic additives, has increased the interest for natural preservatives. It has been long acknowledged that some essential oils exhibit antimicrobial properties. Studies have shown that essential oils of oregano, thyme, bay and clove are among the most active in this respect against strains of *E. coli* (Burt and Reinders, 2003). The future of naturally occurring antimicrobials seems promising. The aim of this study was to evaluate antimicrobial properties of essential oils against antibiotic resistant bacteria isolated from bulk-tank milk in a predetermined area of Mangaung. The objectives were to quantify and identify bacteria isolated from bulk-tank milk; evaluate antibiotic profiles of the isolated bacteria; and to assess antimicrobial properties of essential oils against isolated bacteria.

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CHAPTER TWO

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QUANTIFICATION AND IDENTIFICATION OF BULK-TANK MILK ISOLATED BACTERIA

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2.1 ABSTRACT

Introduction: Spoilage of bulk-tank milk resulting in gastrointestinal illnesses are due to the outgrowth of psychrotolerant bacteria (e.g. *Pseudomonas* spp., *Enterobacter* spp. etc) which are commonly found in farm environments and bulk-tank milk. Certain strains are capable of growing at refrigeration temperatures, thereby causing milk spoilage.

Purpose: To quantify and identify bulk-tank milk isolated bacteria.

Methods: Samples of bulk-tank milk were obtained randomly from shops in the Mangaung area during August 2015 and November 2015. Milk samples were analysed for total viable counts and coliform bacteria on the initial day of sampling using Nutrient and selective media (Chromocult and Blood agar) within 24 hours. The bacteria were isolated and positively identified using selective media, colony morphology and protein profile analysis (MALDI-TOF mass spectrometry).

Results: Evaluation of the results were carried out in accordance with standards set in R1555 of 2001 as amended by R489 of 2001 which states that standard plate counts may not exceed 5 x 10^4 cfu/ml. Standard plate counts from samples taken winter were high and this might be due to the slow sales in winter and in addition, not separating left over milk and then mixing it with a new batch of milk. Bulk tank milk samples from most shops around the Mangaung area were contaminated. Identified microorganisms included mostly gram-negative bacteria from the genus *Pseudomonas* and the Enterobacteriaceae family.

Conclusions: Results in this study have indicated that contamination might be both from the farm and the milk shops. There is therefore still a need for more stringent control over milk shops by the relevant authorities.

Keywords: Bulk tank milk, R1555 of 1997, Psychrotrophic bacteria

2.2 INTRODUCTION

In South Africa, the extent of microbiological contamination of informal or deregulated dairy products is not always very clear. The main cause is that food-borne pathogens have been isolated from milk and dairy products worldwide (Akineden *et al*., 2008) and despite the fact that outbreaks and even sporadic cases of food poisoning have rarely been reported to date in South Africa, foodborne pathogens have been isolated from bulk-tank milk in the Mangaung area. This therefore indicates that the possibility of ingesting contaminated milk containing pathogenic bacteria is prevalent increasing possibilities of contracting gastrointestinal illnesses. Various studies focused on the quality of milk in bulk-tanks, microbial composition of milk, the associated milk practices amongst small-scale farmers in informal sectors (Prinsloo, 2001; O'Ferrall-Berndt, 2003; Jansen, 2003) and on milk and food quality in developing urban areas in South Africa (Lues *et al*., 2003). And there has been an agreement that, contamination of milk is usually from the cows' environment, equipment, improper sanitary and health care practices.

Milk quality can be affected through contamination at the various points within the value chain and it is essential to identify the Critical Control Points (CCP), and develop standard operating procedures to minimize contamination arising from these points (Agenbag and Lues, 2009). Moreover, Hazard Analysis and Critical Control Point (HACCP) system is necessary during milk production to ensure high quality milk. All milk handlers as well as institutions dealing with milk need public health license (Certificate of Acceptability in terms of R962 of 2012) with constant monitoring and evaluation of the process (Lues *et al*., 2003). In addition, continued training on safe milk production should be enforced. When these controls are ensured throughout the entire milk value chain, quality milk and safe milk, free from any contaminants will be produced for increased income and better health of the population (Agenbag and Lues, 2009).

There is still a great lack of implementation of proper measures by local authorities towards improving hygienic production of milk practices, more stringent control and public education are

required to strengthen the legislation as it cannot survive on its own (O'Ferrall-Brendt, 2003). Although inspections are conducted, there is still a need to manage and increase manpower to control the informal milk selling sector in order to improve service delivery (Agenbag and Lues 2009). Moreover, qualified registered Environmental Health Practitioners in municipalities need to be able to manage and control all activities relating to environmental health. The greatest and most widespread concern of overall milk safety is microbial contamination: the presence of infectious bacteria and this has led to the aim of this study where the microbial quality of bulk tank milk sold in the Mangaung area was assessed. To confirm the identity of microorganisms, Matrix Assisted Laser Desorption Ionization- Time of Flight Mass Spectrometry (MALDI TOF MS) was used. Bacteria are important causes of several infections in humans and animals. However, their isolation in culture and identification is difficult and time consuming (Jousimies-Somer *et al*., 2002). Phenotypic and biochemical methods need time, and commitment for several days, and sometimes they do not distinguish closely related species or many give incorrect or inconclusive results, especially with uncommon or fastidious organisms. Researchers have considered the use of Matrix Laser Desorption Ionization- Time of Flight Mass Spectrometry as a possible solution to the above mentioned challenges.

MALDI TOF MS is used and implemented in some laboratories for efficient, rapid and cost-effective identification of different classes of bacteria, including anaerobes (Jousimies-Somer *et al*., 2002). Furthermore, the correct identification of an organism is dependent on the presence of the reference strains in the database because the species of the reference strain will give the closest match for the identification of the tested strain. In this study, we evaluated a modified protein extraction method of identification performed on a target plate (on-plate extraction method) with MALDI TOF MS (Bruker Microflex LT with Biotyper version 3.1) and direct colony method. The identification of anaerobes by MS offers several advantages in comparison with the conventional routine method. It shortens the time period required to identify an organism, from days to a few minutes with improved outcomes (Cherkaoui *et al*., 2010). There is a great and significant impact

on time to identification of biochemically inert, fastidious and slow-growing anaerobic bacteria (Tan *et al*., 2012). MALDI TOF MS has shown to be a useful method for identification of different microorganisms. Several studies have reported the advantage and performance of MALDI TOF MS systems compared with commercially available systems (Fedorko *et al*., 2012; Seng *et al*., 2009). There are several commercially available MALDI TOF MS systems with software and databases for identification of microorganisms isolated from clinical specimens, eg. Bruker MS (Microflex; Bruker Daltoniks), VITEK MS (bioMerieux) and Shimadzu MS (AXIMA; Shimadzu Corporation). They are used to identify aerobic and anaerobic bacteria (Fedorko *et al*., 2012; Van Veen *et al*., 2010), mycobacterial (Saleeb *et al*., 2011), Nocardia (Verroken *et al*., 2010) and yeasts (Van Veen *et al*., 2010) isolated on solid media from clinical specimens. MALDI TOF MS has also been recently used for the identification of bacteria and yeasts directly from positive blood culture bottles (Ferroni *et al*., 2010). The MALDI TOF MS system appears to be associated with rapid turnaround time, low sample volume requirements and modest reagent costs. The use of MALDI-TOF MF (Bazzini *et al*., 2010) is mostly successful in the identification of groups of bacteria; and this is because it is a powerful, rapid, precise and cost effective method for identification of intact bacteria when compared to molecular biology techniques (Van Veen *et al*., 2010).

2.3 MATERIALS AND METHODS

2.3.1 Sample collection

Altogether 8 bulk-tank milk samples were analyzed in this study. The milk samples were received from milk tankers collected from 4 out of 10 shops around the Mangaung area during the period August and in November 2015. Four milk samples were studied at a time; thus the analysis was performed in two separate experiments. After aseptically (through a bulk-tank tap) collecting the milk using the institutions (Central University of Technology, Free State) sterile sample bottles (500 ml), the bulk-tank milk samples were brought to the Central University of Technology, Free State laboratory in a cooler box (4^{o}C). Immediately after receiving the first set at the lab, the

samples were tested promptly so that any bacteria present in the sample cannot multiply prior to testing and analysis was performed in triplicate during August and in duplicate during November, this time a spiral plater was used to plate out the organisms. The storage temperature and the time period were selected such that the normal storage condition of the bulk-tank milk storage (4°C) can be represented in this study.

2.3.2 Isolation and identification of bacteria

Bulk-tank milk sample sources were collected for microbiological analyses to detect the presence of bacteria and subsequent serial dilutions of milk (1 ml) were prepared. Nutrient and selective media (Chromocult and Blood Agar-Selecta-MEDIA from ThermoFisher Scientific) were used to isolate or identify particular organisms at an incubation of 37 degrees Celsius within 24 hours. Nutrient agar was used because it allows growth of all microorganisms and to determine total viable counts in a sample while; selective media allows certain types of organisms to grow and inhibit the growth of other organisms.

2.4 Confirmation of microbial isolates

2.4.1 MATRIX ASSISTED LASER DESORPTION IONIZATION TIME-OF-FLIGHT (MALDI TOF MS)

For identification of colonies, direct placing or placing on a steel target after extraction was performed according to the manufacturer's instructions.

Direct colony method: A single colony from each plate was picked up using a sterile pipette tip and smeared as a thin film directly on a MALDI steel target. Following this, 1µl of matrix solution (20 mg/ml 3, 5-dimethoxy-4-hydroxycinnamic acid in acetonitrile (ACN): purified water: trifluoroacetic acid (TFA) (50:50:0.1) was used to overlay the smeared colony on the steel target. The steel target was air-dried for 10 minutes and placed in the MALDI-TOF MS for analysis.

Extended direct colony method: Each strain was applied and dried on the target plate as in the direct colony method. Following this, 0.5 ul of 70% formic acid was mixed with the sample on the plate by pipetting, followed by 0.5 ul of acetonitrile, and the resultant mixture was dried at room temperature for approximately 10 minutes. Finally, 1.5 ul of the matrix solution was applied onto the spot as in the direct colony method.

Standard extraction method: Bacterial isolates from the bulk tank milk were grown overnight on Nutrient agar. An inoculation loop full of cells was then introduced into 1.2 ml of 95% ethanol (Sigma-Aldrich) and suspended by mixing with a vortex. The sample was centrifuged for 2 minutes and the protein sample was put into a pellet and in order to extract the proteins; 50 ul of 70% formic acid (Sigma-Aldrich) and 50 ul of 100% acetonitrile were added to the pellet and thoroughly mixed using a vortex.

The protein-containing supernatant was spotted onto the MALDI target, allowed to dry and covered with 1 ul of saturated matrix solution containing HCCA (hydroxycinnamic acid) (Bruker Daltonics) at a concentration of 10 mg/ml in acetonitrile-water-trifluoroacetic acid (TFA) (50:47.5:2.5 vol/vol/vol) (Sigma-Aldrich). Sample spectra were collected with a microflex LT MALDI TOF mass spectrometer (Bruker Daltonics) using flexControl software (Version 3.4) (Bruker Daltonics) and applying the measurement parameters suggested by the manufacturer for classical biotyping: laser frequency of 60 Hz in the positive linear mode with acquisition ranging from 2 to 20 kDa. Final spectra consisted of 240 shots per spot (40 shots per raster spot). The laser intensity was chosen so as to obtain spectra with maximal absolute peak intensities ranging from about 5 x 10^3 to 10^4 arbitrary units. The spectra were evaluated using the accompanying MALDI BioTyper OC software (Version 3.1) (Bruker Daltonics).

2.5 RESULTS AND DISCUSSION

2.5.1 Microbiological composition of samples

The primary objective of this study was to isolate, quantify and identify bacteria from bulk-tank milk obtained from shops around the Mangaung area, South Africa. Generally, bulk-tank milk samples analyzed in this study consisted mainly of psychrotrophic; these bacteria are commonly found in bedding, vegetation, milking equipment, teat contamination or pipelines (biofilm contamination) within the dairy environment. This makes us believe that the milk might be contaminated at the farm and not at the retail outlet, however; unclean bulk-tanks have also been reported to be another source of re-contamination of the bulk-tank milk (Godden *et al*., 2008). **Table 1** and **2** in this chapter show standard plate counts of the results obtained in various samples taken in August 2015 and November 2015.

Evaluation of the results were carried out in accordance with standards set in R1555 of 1997 as amended by R489 of 2001 which states that standard plate counts may not exceed 5 x 10^4 cfu/ml and further states that, for both the purpose of direct consumption and further processing, counts must be below 20 cfu/ml of milk intended for direct consumption as well as no colonies must be present on 0.01 ml of milk intended for further processing (R489 of 2001). Anything less than the said limit in the regulation, the environmental health profession uses the 'best before' principle. Bulk tank milk samples were taken at the same sampling points from four (the only shops that were selling at the time) out of ten retail outlets around Mangaung area and were evaluated.

Milk samples were collected from the bulk-tank upon arrival at the shop. Generally, an increase in the standard plate count was observed in the milk samples taken in winter (August 2015) than in summer (November 2015). A distribution of the total viable microorganisms ranged from 8 standard plate count from Sample 4 which showed a formation of purple colonies on selective media (Chromocult agar) to a standard plate count of TMTC (Too Many to Count) from Sample 1

with a formation of a white bio-film on Nutrient agar. Standard plate counts from samples taken winter were high and this might be due slow sales in winter and in addition, not separating left over milk and then mixing it with a new batch of milk. In this case psychrotrophic bacteria would dominate over the mesophiles as they are able to survive refrigeration temperatures. The growth of psychrotrophs may result in off flavors such as stale, bitter, putrid and rancidity. Defects in the milk such as coagulation and thickening may result from heat resistant lipases and proteinases degrading the casein (Frank *et al*., 1992).

The growth of psychrotrophs in farm bulk tank milk is also stimulated when cooling to 4^{o}C is slow or delayed. Another reason for high bacterial counts could also be due to the poor quality water on the farm. Water in the dairy should be potable so that it cannot have an effect on bacterial counts; contaminated water has a negative effect on cleaning agents, binding them so that higher concentrations have to be used in the cleaning equipment. Although 5% of the samples complied, bulk tank milk from the study area was mostly contaminated and the high counts suggested probable contamination via environmental contaminants, infection from within the udder and poor storage practices as observed elsewhere (Godefay and Molla, 2000).

The current study did not focus on somatic cell counts however related studies indicate that a cow with mastitis has the potential to shed large numbers of microorganisms into the milk supply and the influence of mastitis on the total bacterial count of milk depends on the strains of the infecting microorganism, stage of the infection and percentage of the infected herd (Murphy and Boor, 2000). Even though a number of studies have identified bacteria in bulk-tank milk on farms, the microbial content of milk as it reaches the site of retail has remained largely unknown. Other studies indicated that bacterial populations in bulk-tank milk are highly diverse and heterogeneous (Quigley *et al*., 2013) this was also observed in this study. Such heterogeneity might be a result of highly nutritive content of milk and the numerous potential sources of bacteria such as bedding, feed, and

microbiota residents on the milking equipment, on-site bulk-tanks used for storage and tanker trucks used for transport (Quigley *et al*., 2013).

Identification of isolated bacteria was done using morphology, selective media and MALDI TOF-MS. An interesting find was that the bacteria observed on agar media ranged from a flat, pink colour to colourless, yellow and navy blue/dark purple colour. The change in colours were mostly observed in selective media, Blood and Chromocult agar; whereby pink, navy or dark purple colonies were seen. From literature, most gram negative bacteria that ferment lactose (the family of Enterobacteriaceae, *Serratia* spp, *Pseudomonas* spp etc) produce acid which turns colonies dark purple/navy blue. In addition, certain lactose-fermenting bacteria produce flat, dark colonies while non-lactose fermenting organisms grow as colourless or clear colonies (BIO203 Laboratory Media and Biochemical Tests).

For the species identification, the direct colony method, extended method and standard extraction methods were used to identify 25 isolates at a genus and species level (**Table 3** in this chapter). The standard extraction plate method has been used in the construction of a database for BioTyper and has been recommended as a reference method for identification (Alatoom *et al*., 2011). The variations of identification rates among different studies may be explained by differences in growth conditions, sample preparation, number of reference strains, and version of BioTyper software; and the study design (Benagli *et al*., 2011). The standard extraction method consisted of approximately 13 steps, including completion of a round of centrifugation for 2 minutes, requiring 30 for 25 samples. Because it is difficult to automate, the routine use of the standard extraction method may not be suitable for primary preparation (Alatoom *et al*., 2011; Bazzini *et al*., 2010). Standard extraction may be more suitable as a reference method for use when the direct colony method fails to identify the species.

The direct colony method offers the advantage of a simple and easy-to-use procedure. It requires only 4 steps and less than 30 minutes for a complete identification of 25 samples. Bacterial

identification rates achieved with the direct colony method were at least equal to those achieved using the standard extraction method. It was found out that *Pseudomonas* had a higher identification rate than other organisms. Other species showed smaller identification differences between the direct colony and standard extraction method. This indicates that for some species of *Pseudomonas*, the direct colony method may have a better identification rate than the standard extraction method.

The protocol of complete dry-up time of 10 minutes at room temperature may provide an explanation; the total time required for protein extraction is shortened, potentially producing a better identification rate for standard extraction. Critical for the successful use of MALDI TOF MS in clinical laboratories is the demonstration that the method is reproducible and highly discriminatory. The pre-analysis preparation of the sample for MALDI TOF MS is critical for reproducible spectral profiles and test sensitivity (Findeisen *et al*., 2005). Bacteria can be selected from a culture plate and either transferred directly to a target plate or pre-treated with ethanol followed by protein extraction with formic acid or acetonitrile. Pre-treatment is beneficial because it inactivates the organisms, enhances detection of biomarkers above 15 kDa, and improves sample stability (Winkler *et al*., 1999). Before analysis by MALDI TOF MS, optimum sensitivity requires disruption of the cell wall structure by treating the cells with a strong organic acid (e.g. formic acid, trifluoroacetic, and acetic) either before or concurrent with the addition of the matrix solution.

In this study; bacterial colonies identified were mostly gram-negative belonging to the genera; *Enterobacter*, *Hafnia*, *Lelliottia*, *Serratia* and *Pseudomonas* species; while other bacteria species were not identified and these results were inconclusive **Table 3** in this chapter. In biomarker research with MALDI protein profiling, the aim is to identify peak intensities (or peak areas) that are different between case and control samples, and the reproducibility of peak intensities is of highest importance. However, poor reproducibility has been considered one of the major problems in protein profiling with MALDI-TOF MS and it is believed that the same happened in this study while trying to analyze the results. The matrix (co)crystallization and desorption/ionization steps in

MALDI-TOF MS have been derived empirically, and the processes are poorly understood. It is said that different matrix molecules crystallize in different shapes and dimensions; and proteins tend to accumulate at the droplet periphery, and therefore the composition of the matrix solution and the rate of crystal growth influence the spectral output (Dreisewerd, 2003). Studies indicate that these phenomena produce shot-to-shot variation, which is related to sampling different parts of the target surface and progressive sample ablation with repeated sampling (Dreisewerd, 2003). Furthermore, peak signal of other ions in the sample, and peptides with greater hydrophobicity show the greatest suppression effects and the presence of basic residues may favor ionization in MALDI-TOF MS analysis (Hortin, 2006). In summary, the peak intensity in MALDI protein profiling has significant analytical variation and is poorly understood, therefore peak intensity is related to the concentration of the individual protein, to its primary structure, and to the complexity of the sample and it is believed that is what happened in this study.

The bacteria identified were part of the Enterobacteriaceae group; they were gram-negative bacteria that are commonly found in the environment, e.g. in soil, plants, feed and water and may be accidental mastitis pathogens (Yao *et al*., 2016). *Enterobacter, Hafnia* and *Lelliottia* bacteria are mainly isolated from milking machines, milk pipelines or coolers, dirty udders and teats have also been sources of these bacteria (Samoes *et al*., 2010). *Serratia, Hafnia, Lelliotia* and *Enterobater* have also been isolated from bedding and parlor floors. New infections in cows caused by these bacterial species can occur during the dry period (Simojoki *et al*., 2011) and in lactation. Even though they are considered to be environmental organisms; transmission via the milking machine may occur (Yao *et al*., 2016). *Pseudomonas* is widespread in the environment of dairy cows because it requires few nutrients to grow and multiply. Water supplies of all types (wells, troughs, ponds, parlor wash hoses, and sprinkler pens), contaminated teat dips and infusion equipment are the major sources of *Pseudomonas* on dairy farms. *Pseudomonas* has also been isolated from waste feed, soil, manure, and animal skin (Yao *et al*., 2016). The presence of unsanitary housing and bedding conditions can contribute to occasional outbreaks of *Pseudomonas* infections, while water

and soil are the primary sources of *Pseudomonas* spp. (Jay, 2000). *Pseudomonas* species were found to be dominating in this study and this has been found to be one of the species that has a high impact on milk quality (Jay, 2000). These species can also produce heat stable lipases as well as proteases that cause milk defects such as bitterness and putrefaction due to the production of slime and coagulation of proteins (Samarzija *et al*., 2012). These common environmental bacteria may cause elevated bulk tank milk bacterial counts. These bacteria enter the milking system via dirt, contaminated water or manure and careless rinsing of the milking cluster which can carry contamination into the system.

Dirty milking systems provide a place for bacteria to lodge, grow and develop into large numbers. The bacterial build-up may be transferred to the tank as fresh milk passes over it and becomes inoculated. Dirty pipelines, unwashed zones in the milk handling system, long milking times (8-10 hours), no sanitizing, etc., are all factors that can cause bacterial build-up in the system. When these occur, the tank microbial counts can rise. The current results indicate that bulk-tank milk is contaminated from the farm but, the milk might also be re-contaminated further at the shop if the bulk-tanks are not properly cleaned and sanitized according to manufactures instructions (Scientific report of EFSA and ECDC, 2011). All of these bacterial species discussed in the study have the ability to cause foodborne illnesses and contaminate milk products thereby affecting consumers. These bacteria are the main causative agents of spoilage of milk and dairy products and some are considered to be opportunistic pathogenic bacteria and therefore contribute to significant economic losses for the agricultural and food industries (Brenner and Farmer, 2005). Bacterial species that grow rapidly under warm conditions (32.2°C) produce high Standard Plate Counts. This includes all of the typical mastitis pathogens (including *Pseudomonas*, and the Enterobacteriaceae family – *Enterobacter*, *Hafnia spp*, *Lelliottia*, *Serratia* spp).

The Enterobacteriaceae and coliform bacteria are often used to provide evidence of poor hygiene, inadequate processing or post-process contamination of foods (Brenner and Farmer, 2005). Their

absence in food provides a degree of assurance that the hygiene and food manufacturing process has been carried out appropriately, whereas their presence usually indicates that a potential problem or failure in the process has occurred and it is believed to be the case in this study.

Table 1. Standard plate counts in samples taken in August 2015.

Sample 1	**10^1**	**10^2**	**10^3**	**Colony morphology**
Nutrient Agar	TMTC	TMTC	TMTC	A white biofilm formed
Chromocult	TMTC	TMTC	TMTC	Pink colonies formed
Blood Agar	130	110	99	Big clear colonies formed, together with pink flaky colonies
Sample 2				
Nutrient Agar	126	59	TFTC	Big white colonies formed
Chromocult	189	45	No growth	Yellowish big colonies formed
Blood Agar	152	33	No growth	Pink colonies formed
Sample 3				
Nutrient Agar	194	TFTC	No growth	White colonies formed
Chromocult	156	TFTC	TFCT	Yellow, white and navy blue colonies formed
Blood Agar	TNTC	TFTC	TFTC	Yellow biofilm formed at 10^1 Yellow colonies at 10^2 Flat, dry pink colonies

				formed
Sample 4				
Nutrient Agar	TMTC	TMTC	TMTC	A white biofilm formed
Chromocult	TMTC	160	34	Blue,pink and clear yellow colonies formed
Blood Agar	TMTC	TMTC	TMTC	Clear yellow and flaky colonies formed

Table 2. Standard plate counts in samples taken in November 2015.

Sample 1	**10^1**	**10^2**	**Colony morphology**
Blood Agar	40	19	White and flat, dry pink colonies formed
Chromocult	24	4	Yellow, white and flat, dry pink colonies formed
Nutrient Agar	61	47	White colonies formed
Sample 2			
Blood Agar	93	31	Yellow colonies formed
Chromocult	24	22	Navy blue and

			yellow colonies formed
Nutrient Agar	64	34	White colonies formed
Sample 3			
Blood Agar	24	20	Yellow colonies formed
Chromocult	38	10	Purple colonies
Nutrient Agar	27	10	White colonies formed
Sample 4			
Blood Agar	46	46	White colonies formed
Chromocult	8	5	Purple colonies formed
Nutrient Agar	27	10	White colonies formed

Table 3. MALDI TOF MS direct colony and Extraction method isolates.

Sample ID	Organism (Colony Direct Method)	Organism (Extraction Method)	Genus/Species
1	*Lelliotia amnigena*	*Lelliotia amnigena*	*Lelliota*
2	*Lelliotia amnigena*	*Lelliotia amnigena*	*Lelliotia*
3	*Pseudomonas ludensis*	*Pseudomonas ludensis*	*Pseudomonas*
4	Inconclusive	Inconclusive	Inconclusive
5	*Pseudomonas ludensis*	*Pseudomonas ludensis*	*Pseudomonas*
6	*Pseudomonas ludensis*	*Pseudomonas ludensis*	*Pseudomonas*
7	*Pseudomonas oleovorans*	*Pseudomonas oleovorans*	*Pseudomonas*
8	*Pseudomonas rhodesiae*	*Pseudomonas oleovorans*	*Pseudomonas*
9	*Serratia liquefaciens*	*Serratia liquefaciens*	*Serratia*
10	Inconclusive	Inconclusive	Inconclusive
11	Inconclusive	*Pseudomonas gessardii*	*Pseudomonas*
12	*Pseudomonas ludensis*	*Pseudomonas ludensis*	*Pseudomonas*
13	*Pseudomonas ludensis*	*Pseudomonas ludensis*	*Pseudomonas*
14	Inconclusive	*Pseudomonas corrugata*	*Pseudomonas*
15	*Pseudomonas taetrolens*	*Pseudomonas taetrolens*	*Pseudomonas*
16	*Pseudomonas ludensis*	*Pseudomonas ludensis*	*Pseudomonas*
17	Inconclusive	*Enterobacter caancerogenus*	*Enterobacter*
18	*Pseudomonas fragi*	*Pseudomonas fragi*	*Pseudomonas*

19	*Pseudomonas ludensis*	*Pseudomonas ludensis*	*Pseudomonas*
20	*Lelliotia amnigena*	*Lelliotia amnigena*	*Lelliotia*
21	*Enterobacter cloacae*	*Enterobacter cloacae*	*Enterobacter*
22	Inconclusive	Inconclusive	Inconclusive
23	*Lelliotia amnigena*	*Lelliotia amnigena*	*Lellitia*
24	*Serratia amnigena*	*Serratia liquefaciens*	*Serratia*
25	*Hafnia alvei*	*Hafnia alvei*	*Hafnia*

2.6 CONCLUSION

Milk is rarely but occasionally linked to outbreaks of foodborne disease in humans. Several disease causing pathogens in cows (*Salmonella spp., Listeria monocytogenes and E. coli*) can contaminate milk. However, the threat to human health from ingestion of bulk-tank milk should not be underestimated. In the current study, bacterial counts in bulk-tank were compared to the appropriate standard regulation; R489 of 2001. Standard plate counts should be less than 5 x 104 cfu/ml as stated in R489 of 2001, and it was not the case in this study as there were counts more than the regulatory standard.

Hafnia, Enterobacter, Lelliottia, Pseudomonas and *Serratia* species observed in bulk-tank milk cultures and might be due to milking wet, dirty cows as these are usually environmental organisms; they may also be found in the milking machine, colonise pipelines and this is regarded as an indication of milking system cleaning problem. Some organisms can also be found from within the udder later causing mastitis in the herd. People might become ill with foodborne illnesses from the bulk tank milk if the bacteria present in the milk are very high in numbers. However, simple exposure to bacteria does not necessarily lead to human infection or disease but the health impact of exposure is influenced by the volume of milk consumed, the concentration of the pathogens within that milk, the total number of organisms to which a person is exposed through various sources and

dose-response of an individual to such exposure. These factors will vary between situations and individuals. High risk people are those who are immune-compromised; including pregnant women, the elderly and young, HIV and cancer patients (Brenner and Farmer, 2005). Not only can unsafe milk affect the health of the consumer, but it may also have economic implications such as medical and hospitalization costs, productivity losses, and the long term reduction in quality of life. The results of this study have shown that contamination might be both from the farm and the milk shops; based on possible sources of contamination. There is a need for more stringent control over milk shops by the relevant authorities.

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CHAPTER THREE

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ANTIBACTERIAL RESISTANCE PROFILES OF BULK-TANK MILK ISOLATED BACTERIA

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3.1 ABSTRACT

Introduction: In the agricultural industry antimicrobials are administered for therapeutic purposes but some antimicrobials are used to prevent diseases during periods of increased susceptibility. The use of antimicrobials in foodstuffs of animal origin has the potential to affect human health by increasing the risk of antimicrobial residues and influence the development of antimicrobial resistant foodborne pathogens.

Purpose: To investigate the antibiotic susceptibility profiles of bacteria isolated from bulk-tank milk obtained from retail outlets in the Mangaung area, South Africa.

Methods: The antimicrobial resistance profiles of the isolates were determined using the Epsilometer test method and MALDI-TOF MS.

Results: Among all the samples examined, all strains were found to be resistant to all three antibiotics tested; ampicillin, Ceftazidime and Cefotaxime. The findings of this study showed that bulk-tank milk in the Mangaung area is contaminated with potentially resistant bacterial strains.

Conclusions: Therefore, there is a need to implement appropriate control measures to reduce contamination as well as the possible spread of antibiotic resistant strains as this can increase the burden of disease in humans.

Keywords: Antibiotic susceptibility, Bulk-tank milk, MALDI TOF, API, Resistant bacteria

3.2 INTRODUCTION

Bulk tank milk plays an important role in assisting individuals meet their nutrient requirements. Due to its high nutrient composition, it is often associated with food-borne pathogens if proper sanitary and health care procedures are not implemented during the production and marketing of these products (Havelaar *et al*., 2010). It is also regarded as a potential vehicle for the transmission of antibiotic resistant bacteria; because it serves as an excellent medium for the growth and survival of many different types of pathogenic bacteria (Havelaar *et al*., 2010). According to Cosgrove (2006) antimicrobial resistance is an important health problem worldwide and the development of resistance, both in human and animal bacterial pathogens has been ascribed to the extensive use of antimicrobials or with their use as growth promoters in animal feed production (Silbergeld *et al*., 2008).

Antimicrobial agents are classified based on their principal mechanisms of action. These mechanisms include interference with cell wall biosynthesis (B-lactams and glycopeptides agents), inhibition of bacterial protein synthesis (marcolides and tetracyclines), interference with nucleic acid synthesis (fluroquinolones and rifampin), inhibition of a metabolic pathway (trimethoprim-sulfamethoxazole, and disruption of bacterial membrane structure (polymyxins and daptomycin) (Tenover *et al*., 2006). Three main targets of antibiotics are the cell wall, protein and nucleic acids biosynthesis. Throughout the years since antibiotics were introduced, bacteria have acquired various resistance mechanisms to survive antibiotics. The mechanisms vary and make the work of mitigating the spread of resistance more challenging (Tenover *et al*., 2006).

Resistance is often seen in B-lactam antimicrobials, this antibiotic class consists of four major groups: penicillins, cephalosporins, monobactams and carbapenems. Hydrolysis of B-lactam compounds by b-lactamases is the most widespread mechanism of bacterial resistance against this class of antibiotic (Tenover *et al*., 2006). Numerous methods have been used to detect antibiotic

resistance in bacteria but new and sensitive methods such as MALDI-TOF MS are currently used to detect antibiotic resistance. One example is where B-lactam hydrolysis is used in MALDI-TOF MS analysis to detect if bacterial species have to the ability to produce the B-lactamase enzyme. The most common mode of microbial resistance to beta-lactams, the largest class of antibiotics is their enzymatic hydrolysis by beta-lactamases. The production of beta-lactamases is detected by MALDI TOF MS employing a 'mass spectrometric B-lactamase (MSBL) assay. In this essay, buffered solution of the antibiotic is mixed with bacterial culture and incubated. The reaction mixture is centrifuged and supernatant subjected to MALDI TOF MS analysis. The B-lactamase producers inactivate the B-lactamase ring of the antibiotic by addition of a residue of water. The mass shift in the non-hydrolyzed and the hydrolyzed forms of the antibiotic confirms the presence or absence of B-lactamase producing bacteria. The MSBL assay has been applied for detection of resistance to B-lactam antibiotics like penicillin, ampicillin, piperacillin, cetazidime, cefotaxime, ertapenem, meropenem and imipenem (Hrabak *et al*., 2011).

Using MSBL assay researchers have successfully detected B-lactamase producing organisms like *Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa, Acinetobacter baumanni, Citrobacter freundii, Enterobacter cloaceae, Salmonella* spp etc. (Hrabak *et al*., 2011; Hooff *et al*., 2012; Sparbier *et al*., 2012; Kostrzewa *et al*., 2013). Capability of *Staphylococcus aureus* in producing penicillinase (a form of b-lactamase) to destroy penicillin G was reported within two years after the antibiotic was first introduced (Kirby, 1944). The occurrence of MRSA is a classic example of the redundancy of new antimicrobials with regards to only one species. This resistance might be brought on by the intrinsic and extrinsic resistance in which bacteria exhibit. The aim of this study was to investigate antibiotic susceptibility profiles of bacteria isolated from bulk-tank milk obtained from retail outlets in the Mangaung area, South Africa.

3.3 MATERIALS AND METHODS

An epsilometer test was done to analyze the susceptibility of bacteria to antibiotics using phenotypic techniques. The phenotypic methods are based on the ability of an antibiotic to inhibit the growth of bacteria under specified growth conditions. Some of the phenotypic methods of antimicrobial susceptibility testing are agar dilution, broth dilution and disk diffusion (Schwarz *et al*., 2010). The antimicrobial diffusion methods based on discs containing antibiotics or strips impregnated with antibiotics (E-test) are widely used *in vitro* antibiotic susceptibility testing (Schwarz *et al*., 2010). The size of the diameter of the zone of inhibition when an antibiotic disc is placed on the surface of inoculated (test organism) Mueller-Hinton agar plate and incubated at optimum temperature for 24 hours classifies the test bacterium as susceptible, intermediate or resistant based on the criteria set by the Clinical and Laboratory Standards Institute or CLSI (Jorgensen and Ferraro, 2009). The E-test strips contain antibiotics in a continuous gradient which when placed on the culture smeared surface of a Mueller-Hinton agar plate and incubated gives the MIC value of the antibiotic for the test culture. The MIC value is the corresponding concentration in the strip at the intersection point of the lowest part of the elliptical zone of inhibition and the test strip (Schwarz *et al*., 2010).

Following epsilometer tests was the ‘mass spectrometric B-lactamase (MSBL) assay was conducted (Bizzini *et al*., 2010); this was set up to analyze the hydrolysis reactions of different beta-lactam antibiotics and rapid detection of resistance against beta-lactam antibiotics. The method as introduced is technically simple and rapid; bacterial colonies were exposed to the antibiotics first and incubated for 24 hours and removed from agar culture plates, mixed with an excess of matrix, and air dried on steel target plates to monitor hydrolysis of the antibiotics by bacteria. This is because beta-lactam antibiotic resistance can be monitored by mass spectrometry since hydrolysis of the beta-lactam ring by beta-lactamases results in disappearance of the original mass peak through a molecular mass shift of +18 Da of the antibiotic agent. The materials and methods of this

experiment included a hydrolysis assay with plated microorganisms. Ampicillin, Ceftazidime and Cefotaxime were tested with the different *Pseudomonas* species and Enterobacteriaceae group of microorganisms. For identification of beta-lactam hydrolysis the tests were performed in the presence of clavulanic acid and the amount of bacteria filling an inoculation loop was suspended in 10 ul of the antibiotic solution and incubated at 37^{o}C under agitation for 24 hours. Subsequently the tubes were centrifuged for 2 minutes at 15000 g at room temperature and the cell-free supernatant was analyzed by MALDI TOF MS to determine the presence of absence of the +18Da peak.

For MALDI TOF MS analysis; one microliter of the supernatant from the hydrolysis assay was directly spotted onto a polished steel MALDI target plate. Dried spots were overlaid with MALDI matrix (10 mg/ml of hydro-cinnamic acid (HCCA) in 50% of acetonitrile and 2.5% of trifluoroacetic acid). After drying of the matrix, MALDI TOF MS measurements were performed with a MicroflexLTbench-top mass spectrometer equipped with a 60-Hz nitrogen laser. Data analysis with MALDI TOF MS spectra were analyzed with software Flexianalysis 3.1. Spectra were smoothed and the baseline subtracted. Peaks were manually selected with the following parameter settings: peak detection algorithm, centroid, signal-to-noise threshold, 2; relative intensity threshold, 0%, minimum intensity threshold, 100; peak width, 0.2 m/z; height, 80%; baseline subtraction, TopHat. Only peaks belonging to the corresponding antibiotic drug and the respective degradation products were labelled.

3.4 RESULTS AND DISCUSSION

Twenty-five presumptive isolates were screened for antimicrobial resistance against three antimicrobials using an Epsilometer test and MALDI TOF MS method in accordance with the manufacturers' instructions. The antimicrobials tested comprised of Ampicillin (AM), Ceftazidime (TZ) and Cefotaxime (CT) in 10 mg. Five genera were positively identified from bulk tank milk samples; majority being *Pseudomonas* spp, and the remainder *Hafnia* spp, *Enterobacter* spp, *Lelliottia* spp and *Serratia* spp which all belong to the Enterobacteriaceae family. An epsilometer

test analysis of the antimicrobial susceptibility of the bacterial strains revealed that all genera were resistant to all antibiotics tested. The study revealed the potential prevalence of inhibitory substances in bulk tank milk, and the presence of these residues is illegal in terms of the Foodstuffs, Cosmetics and Disinfectants Act, 54 of 1972, this should be further investigated. The potential presence of antibiotics in the bulk tank milk may mean that the milk originated from cows which were previously treated and could pose a threat to public health. There may also be biologically active metabolites or unchanged antibiotics in the milk which may result in problems such as allergies in humans and may lead to increased resistance of microorganisms (Owlia *et al*., 2009).

The MALDI TOF MS spectrum corresponding to the supernatant derived from the bacteria after incubation with all three antibiotics for 24 hours were inconclusive and although the experiments results were inconclusive, MALDI TOF MS-based analysis of bacteria according to their presumptive resistance/susceptibility against beta-lactam antibiotics is a novel approach which has been applied for the analysis of the hydrolysis of Ampicillin, ertapenem and meropenem by different bacteria strains (Burckhardt and Zimmermann, 2011; Hrabak *et al*., 2011 and Mellmann *et al*., 2009).

We believe the results of the MALDI TOF MS antibiotic susceptibility tests in this study were inconclusive; because the cells spotted onto the steel plate were not allowed to concentrate on the anchor plate spot during solvent evaporation, which results in a more homogenous crystallization of the matrix and excellent biomarker spectra reproducibility. Or there might have been cross-contamination of samples due to the spreading of a sample into an adjacent spot; another common reason for an isolate is not identified is because it is not included in the data base. Although only limited work has been reported on the use of mass spectrometry to determine antibiotic susceptibility results, these are areas of promising research and as experience is gained with MALDI TOF MS, it is expected that the database will be expanded to resolve many of the current inadequate identifications and algorithms for potential misidentification will be developed.

The Epsilometer test results observed in this study revealed that there is a presence of antibiotic resistant bacteria in foodstuffs of animal origin; and this generally indicates the overuse of antimicrobials; particularly at the agricultural and/or veterinary industry level and also show that the dairy environment harbors resistant psychrotrophic bacteria. Literature also indicates that it is because bacteria from foodstuffs of animal origin have the ability to produce determinants of resistance such as Extended-spectrum Beta-lactamases. Production of extended-spectrum beta-lactamases (ESBL) is a significant resistance–mechanism that impedes the antimicrobial treatment of infections caused by Enterobacteriaceae and is a serious threat to the currently available antibiotic therapy and further implications to the health of the public (Meeta *et al*., 2013).

Implications of resistant bacteria in milk

The spread of antibiotic resistant microbial pathogens is one of the most serious threats to successful treatments of infectious diseases (Owlia *et al*., 2009). Bacterial resistance to antimicrobials is usually brought on by Pseudomonadaceae and Enterobacteriaceae and it is a serious problem. These families of bacteria are two opportunistic pathogens that cause severe and life-threatening infections in immune-compromised patients (Lestari, 2004). Gram negative Pseudomonadaceae is mainly responsible for food poisoning illnesses and Enterobacteriaceae bacteria present in the human intestine cause lower urinary tract infections, coleocystis or septicaemia (Jose and Reddy, 2010). These resistant bacteria have the potential to contribute to the development and transmission of antibiotic resistant bacteria (Bradly *et al*., 1993). Most genera of psychrotrophic communities are gram-negative and are considered human opportunistic pathogens, which may have antibiotic resistant traits, and infections due to those species may eventually be untreatable (McGowan, 2006; Gray *et al*., 2006). Studies have also concluded that large antibiotic resistant gene pools are present in commensal bacteria in many ready-to-eat products and implicated food production and processing environments in the evolution and dissemination of antibiotic resistance (Gray *et al*., 2006). The demonstration of the presence of transmissible

antibiotic resistant genes in the human food supply chain has been made; thus food can be considered to be a direct source of antibiotic resistant genes that is consumed daily (McGowan, 2006). Many studies have determined the prevalence of antibiotic resistance among mastitis pathogens (McGowan, 2006; Davies and Davies, 2010), several compared the antibiotic resistance of conventional and organic dairy farming practices (Wilhelm *et al*., 2010; Davies and Davies, 2010; McGowan, 2006) and others have determined the prevalence of antibiotic resistance among gram negative enteric bacteria in bulk tanks (Straley *et al*., 2006), or mastitis milk samples (Nam *et al*., 2010).

All bulk-tank milk isolated bacteria were investigated and this lead to identification of the presence of antibiotics, Munsch-Alatossava *et al*., (2007) indicated that the presence of antibiotics increases during the cold chain of milk storage and transportation. Moreover a constant increase in antibiotic resistance for isolates originating from farms, lorries, or silos was observed for the following antibiotics: ticarcillin, aztreonam, cefepine, ofloxacin, and trimethoprim-sulfamethoxazole; bacterial isolates from milk stored in silos more frequently exhibited resistance to clavulonic ticarcillin-clavulanic acid, ceftazidim, imipenem, colistin, gentamicin, and ofloxacin compared with farm isolates; unfortunately this study considered independent samples (Munsch-Alatossava *et al*., 2007).

3.5 CONCLUSION

From the experiments done; all bulk tank milk isolated bacteria showed resistance against the tested antibiotics (Ampicillin, Cefotaxime and Ceftazidime). From the results observed; it was revealed that antibiotic use in bulk-tank milk has the potential to lead to the presence antibiotics residues in the milk that might have caused bacterial resistance. The findings of antibiotic-resistant bacteria also imply that an urgent need for alternatives that achieve results for treating bacterial infections and further studies are required to get a clear picture of the antibiotic resistance in bulk tank milk associated bacteria in the farm and the processing plant environment. The emergence of antibiotic resistance among bulk-tank milk isolated bacteria may have potentially negative implications and the characterization of resistance mechanisms provides additional information about the epidemiology of the resistance seeing that the source of antimicrobial-resistant bacteria can spread to humans through the food supply chain. All identified bacteria in this study belong to the gram-negative group and are presented in **Table 3** of Chapter 2; and the results reveal that all bacteria (*Hafnia*, *Enterobacter*, *Lelliottia*, *Serratia* and *Pseudomonas* species) that were tested against Ampicillin, Ceftazidime and Cefotaxime are resistant to it; therefore, the high bacterial resistant level to Ampicillin, Ceftazidime and Cefotaxime in our study indicates the possibility of resistance/or cross-resistance to other drugs in previously studied literature (Beceiro *et al*., 2013).

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CHAPTER FOUR

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ANTIMICROBIAL PROPERTIES OF *ARTEMISIA AFRA* AGAINST BULK-TANK MILK ISOLATED BACTERIA

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4.1 ABSTRACT

Introduction: Plants produce secondary metabolites as natural protection against microbial pathogens. Essential oils, known as volatile oils are products of the secondary metabolism. Known for their antibacterial, anti-inflammatory, anti-carcinogenic and anti-fungal activity; they may present as potential antimicrobials against controlling and eliminating antibiotic resistant bacteria in foodstuffs of animal origin.

Purpose: To investigate the antimicrobial properties of *Artemisia afra* against antibiotic resistant bulk-tank milk isolated bacteria.

Methods: The efficacy of plant essential oils against antibiotic resistant bulk-tank isolated bacteria was examined using the agar diffusion bio-assay. The study included the incorporation of the essential oils, or their major constituents, into agar to allow uniform dispersion of the substance throughout an agar surface. Scanning electron microscopy and transmission electron microscopy were used to assess the effect of the oils on bacterial morphology.

Results: Antimicrobial properties of *Artemisia afra* (Lengana) were observed against nine antibiotic resistant psychotropic bacterial species (*E. cloacae, H. alvei, L. amnigena, P. alveovorans, P. fragi, P. ludensis, P. taetrolens, P. rhodesiae and S. liquefaciens*). Scanning and transmission electron microscopy results observed showed that the oils have antibacterial activity against bacteria tested against the *A. afra* oil. Furthermore, SEM and TEM micrographs showed that no influence on untreated (Control) bacterial samples; well-separated, rod-shaped, firm and intact were observed. This was different in a case of the treated organisms (organisms exposed to essential oils), the essential oil affected both the external envelope of the cell wall and the cytoplasm leading to cell lysis/leakage and the presence of holes on the cell wall, an indication of activity of essential oils against antibiotic resistant bacteria.

Conclusions: The results observed for the SEM and TEM images; prove that *Artemisia afra* oil has an antimicrobial effect on the gram negative bacteria isolated in the bulk tank milk in this study. The compounds of this oil have been shown to exercise their antibacterial action/activity through membrane perturbations.

Keywords: Psychotropic bacteria, Bulk-tank milk, Essential oils, *Artemisia afra*

4.2 INTRODUCTION

The use of antimicrobial agents is reportedly the driving force for the emergence and spread of microbial resistance (Abad *et al*., 2007). Studies have reported the presence of multi-resistant bacteria in populations exposed to antibiotics due to over-prescription of antibiotics and increased use in human and animal medicine (Enne, 2010). Therapeutic failures associated to antimicrobial resistance increases morbidity and mortality, with serious implications at individual, social and economic levels (Enne, 2010). Furthermore, antimicrobial resistance limits the choice of therapeutic agents and increases the potential for treatment failures and adverse clinical outcomes (Travers and Barza, 2002). An emergence of multi-resistant strains created an atmosphere of anxiety that called for immediate action leading to the aim of this study.

Since ancient times, plants and their derivatives, such as essential oils (EOs), have been used in folk medicine. Essential oils play an important role in the protection of plants and they are concentrated natural products with strong smells that are produced by aromatic plants as secondary metabolites (Van de Braak and Leijten, 1999). *Artemisia afra* essential oils are usually extracted from aromatic plants that are generally found in temperate or warm countries, where they often represent an important part of the traditional pharmacopoeia (Van de Braak and Leijten, 1999). Known for their antiseptic, bactericidal, virucidal, fungicidal, and medicinal properties as well as their fragrance, they are used in embalmment, preservation of foods and as antimicrobial, analgesic, sedative, anti-inflammatory and locally anesthetic remedies (Van de Braak and Leijten, 1999). Till today; these characteristics have not changed much except that more is now known about some of their mechanisms of action, particularly at the antimicrobial level (Davidson *et al*., 2005). The understanding of these oils antimicrobial mechanisms of action has led to increased interest in the specific compounds responsible for this activity, specifically those phenolic in nature (Davidson *et al*., 2005).

These oils also have antibacterial properties, with no reports of resistance after prolonged exposure to gram-positive and gram-negative bacteria, and no side effects on human or animal health which makes them potential alternative antimicrobials against bacterial diseases (Pozzo *et al*., 2011). Due to the antibacterial and antifungal characteristics of essential oils and their main components, they are increasingly studied for the control of microorganisms (Andrade *et al*., 2012). It was demonstrated (Aiemsaard *et al*., 2011) that the lipophilic components of lemon grass essential oil play an important role on the lipid layer of the bacterial cell membrane, causing loss of its structural organization and integrity. The secondary metabolites that essential oils have can inhibit or slow the growth of bacteria, yeasts and moulds (Burt and Reinders, 2003; De Martino *et al*., 2009). Essential oils and their components have a variety of targets, particularly the membrane and cytoplasm, and in certain situations, they completely alter the morphology of the cells. These oils antimicrobial mechanisms of action have led to increased interest in the specific compounds responsible for this activity, specifically those phenolic in nature and this research chapter will describe the activity of essential oils against bulk tank milk isolated bacteria. Therefore, the study's main purpose was to examine antimicrobial activities of *Artemisia afra* oil on bulk tank milk isolated resistant bacteria by means of agar diffusion method.

4.3 MATERIALS AND METHODS

Media and culture preparation

When undertaking microbial susceptibility testing, the cultures used i.e. *E. cloacae, H. alvei, L. amnigena, P. alveovorans, P. fragi, P. ludensis, P. taetrolens, P. rhodesiae* and *S. liquefaciens* as well as the media used play an important role in the accuracy of results obtained. The culture medium selected for *in vitro* susceptibility testing must be able to support good growth of the organism to be tested. In addition, it should not have any effect on the action of the antimicrobial agent being tested (McGinnis and Rinaldi, 1996).

4.3.1 Essential Oil Extraction and Characterization

This study's essential oil was extracted from the *Artesimia afra* plant. The oil was extracted using a steam distillation method. Following extraction, the oil was characterized by gas chromatography mass spectrometry using the method of Kirbaslar *et al*., (2009).

4.3.2 Bio-assay Preparation

Bio-assays were separately prepared as described by Kock *et al.,* (2009). Cells were scrapped from PCA (0.5 % m/v agar) grown cultures and suspended in sterilized distilled water (dH_2O) from where 0.2 ml were streaked out on PCA agar (0.5 % m/v) to produce a uniform lawn completely covering the agar surface. Next, a well 0.5 cm in diameter and depth was constructed at the centre of the Petri dish and 46 µl of essential oils were added. In addition, controls were constructed by the addition of similar amounts of only 96% ethanol to wells. All plates were incubated at 37 °C until growth was observed; usually after 24 h.

4.3.3 Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM)

Bacterial cells were assessed for morphological changes using scanning and transmission electron microscopy according to the method of Van Wyk and Wingfield, 1991. Bacterial cells were fixed for SEM and TEM analysis. Consequently, the fixed cells were dehydrated via a graded ethanol series for SEM analysis and acetone series for TEM, dried using a critical point dryer for (SEM) or embedded in epoxy and allowed to polymerize in an oven for TEM. The material was sputter-coated with 200 nm gold (Biorad, London, United Kingdom) before being examined in the scanning electron microscope (Shimadzu SX500 SEM, Tokyo, Japan). Following polymerization of TEM samples, thin sections were made using a LKB III Ultramicrotome (Stockholm, Sweden) and stained with uranyl acetate for 5 min and lead citrate for 1 minute. Finally, these sections were viewed using a Phillips EM 100 transmission electron microscope (Eindhoven, the Netherlands). These procedures were performed in triplicate.

4.4 RESULTS AND DISCUSSION

Characterization of the oil was by gas chromatography mass spectrometry using the method of Kirbaslar *et al*., (2009). The chemical compositions of the *Artemisia afra* oil are seen in **Table 1** below.

Table 1. Chemical composition of *Artemisia afra* oil

Artemisia afra	
Pentane-3-methyl	6.4%
Octane	0.5%
Cyclopropane,1,dimethyl-2-(2methyl-2-propenyl)	3.6%
Camphene	1.3%
Eucalyptol	4.9%
Thujone	17.3%
Bicyclo(3,1,0)hexa-3-ol,4-methyl-1-(1-methylethyl)	19.7%
1,5 Heptadiene-4-ol,3,6,6trimethyl	10.8%
Benzyldehyde-4-(1-methylethyl)	3.9%
p-Cymen-7-ol	1.0%
4-Methyl-5-penta-1,3-dienyltetrahydrofuran-2-one	0.7%
Benzene,1-(1,5-dimethyl-4-hexenyl)-4-	2.7%

methyl	
(-)Spathulenon	4.8%
Isoaromadendrane expoxide	0.3%

The antibacterial activity of *Artemisia afra* oil is due to the components such as those mentioned in the above **Table 1** in this chapter; with the highest component being 4-methyl-1-(1-methylethyl) at 19.7% and the lowest being Isoaromadendrane expoxide at 0.3%, the differences could be ascribed to the solubility differences or instability of compounds during the different methods of extraction (Steam distillation, which determine extractive property both in terms of yield and chemical composition from the plant). These components are called *terpenes* and their general chemical structure is $C^{10}H^{16}$ and some examples of common terpenoids include methanol and *Camphor* (*monoterpes*), *farsenol* and *Artemisin* (*sesquiterpenoids*) and the results (*Artemisia afra* experiment) observed in this study show reports that the terpenoids are active against bacteria isolated from this study (Turina, 2006). It is speculated that the mode of action of the terpenes revolves around the membrane disruption by the lipophilic compounds. Generally, the major components are found to reflect quite well the biophysical and biological features of the essential oils from which they are isolated (Ipek *et al*., 2005), the amplitude of their effects being just dependent on their concentration when they are tested alone or comprised in essential oils. Thus, synergistic functions of the various molecules contained in an essential oil, in comparison to the action of one or two main components of the oil, seems questionable. However, it is possible that the activity of the main components is modulated by other minor molecules (Hoet *et al*., 2006).

From literature review; it was indicated that gram-negative bacteria were more resistant to essential oils but from this study's bio-assay results, it can be inferred that the *Artemisia afra* oil suggest significant growth inhibiting effects of the gram negative bacteria isolated from the bulk tank milk

and to prove this theory; an inhibition zone was observed in the bio-assay experiment, **Figure 1** in this chapter. This efficacy of the *Artemisia afra* oil against these microorganisms may provide a scientific ground for the application of the oil in the prevention and treatment of bacterial infections caused by various pathogenic bacteria, which have developed resistance to antibiotics. Observing both the SEM and TEM pictures (**Table 2 and 3** in this chapter); the control pictures of the bacterium tested (*E. cloacae, H. alvei, L. amnigena, P. alveovorans, P. fragi, P. ludensis, P. taetrolens, P. rhodesiae* and *S. liquefaciens*) with the oil also show that before the bacteria was treated; the bacteria cells were well-separated, rod-shaped, firm and intact. While bacterial cells treated with the *Artemisia afra* oil had holes in them, were not firm and cells were not separated from one another (Turina *et al*., 2006).

The activity of an essential oil can affect both the external envelope of the cell wall and the cytoplasm. The oils hydrophobicity is responsible for the disruption of bacterial structures that leads to increased permeability because of the cells inability to separate the oil from the bacterial cell membrane. The permeability barrier provided by the cell membrane is indispensable to many cellular functions, including maintaining the energy status of the cell, membrane-coupled energy-transducing process, solute transport and metabolic regulation. The cell membrane is also essential for controlling the turgor pressure (Ouwehand *et al*., 2010). Toxic effects on the membrane structure and function are generally used to explain the antimicrobial activity of essential oils (Hyldgaard *et al*., 2012). Literature indicates that, the mechanisms of action of essential oils includes; the degradation of the cell wall (Ultee *et al*., 2002) damaging the cytoplasmic membrane, cytoplasm coagulation, damaging membrane proteins, increased permeability leading to leakage of the cell contents, reducing the proton motive force (Ultee and Smid, 2001), reducing the intracellular ATP pool via decreased ATP synthesis and augmented hydrolysis that is separate from the increased membrane permeability and reducing the membrane potential via increased membrane permeability (Burt, 2004).

In this study, *Artemisia afra* induced damage to the cell membrane structures that was accompanied by decreased viability for all nine microorganisms in this study, and the membrane damage was confirmed as the most likely cause to cell death, see the **Table 3** in this chapter. The hydrophobic nature of essential oils allows them to penetrate microbial cells and cause alterations in their structure and functionality. Although the external capsule of some gram negative bacteria limits or prevents the penetration of essential oils into the microbial cell; in this study it was not the case as the oil penetrated the microbial cell possibly through membrane porins and caused cell interference. Possibly inhibiting the electron transport for energy production and disrupting the proton motive force, protein translocation including synthesis of cellular components. These are physiological changes that can result in cell lysis and death (Turina *et al*., 2006). The integrity of the cell membrane is essential for the survival of bacteria because it is a key element for the fundamental biological activities taking place within the cells, therefore the membrane represents as an effective barrier between the cytoplasm and the external environment of the cell.

The results of this study are not consistent with the available literature reporting that psychrotrophic bacteria are inherently more resistant to essential oils and in this case we can report that the antimicrobial properties of *Artemisia afra* essential oil affected the growth of psychrotrophic bacteria negatively. Similar results were observed with other organisms but the tables below (**Table 2 and 3** in this chapter) show only three organisms (*Lelliotia amnigena, Pseudomonas rhodesiae* and *Pseudomonas ludensis*) under the SEM and TEM.

Moreover, it is likely that several components of the essential oils play a role in defining the density, the texture, the colour and above all, cell penetration (Cal, 2006), lipophilic or hydrophilic attraction and fixation on cell walls and membranes, and cellular distribution. This last feature is very important because the distribution of the oil in the cell determines the different types of radical reactions produced, depending on their compartmentation in the cell. In that sense, for biological

purposes, it is more informative to study the entire oil rather than some of its components because the concept of synergism appears to be more meaningful.

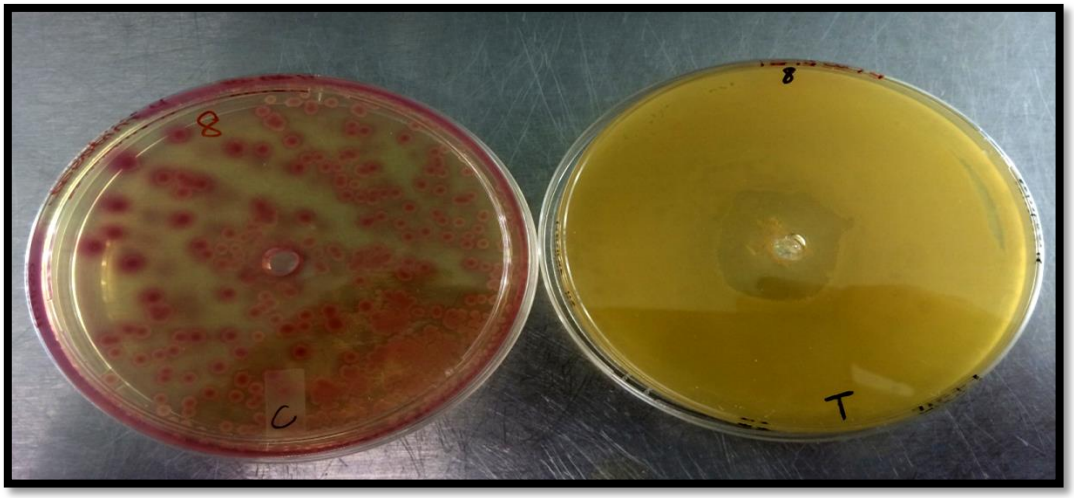


Figure 4. *Pseudomonas rhodesiae* bio-assay (C-control; T-Treated)

Table 2. Scanning Electron Micrographs showing bacterial cells untreated and treated with *Artemisia afra* oil.

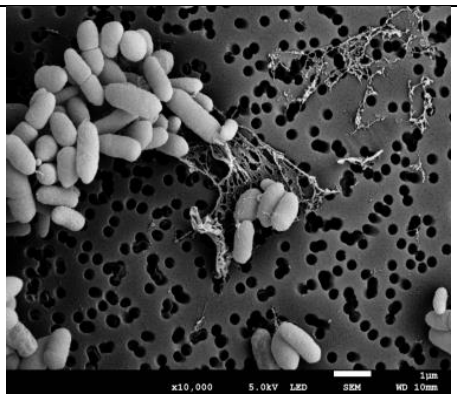
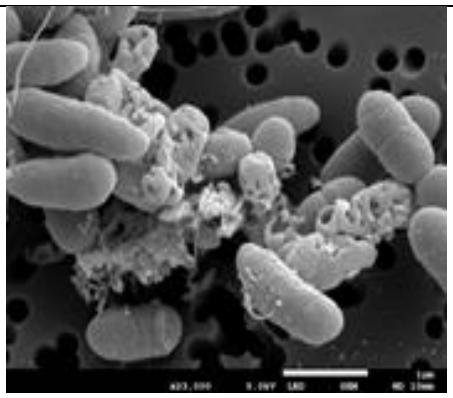
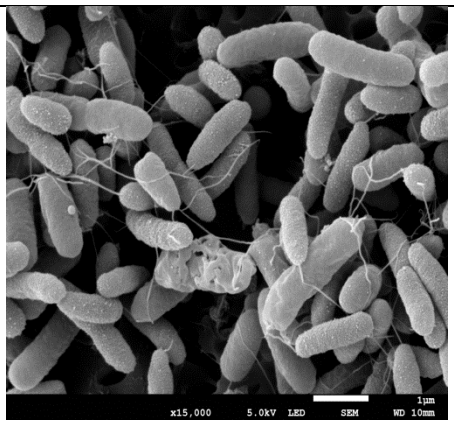
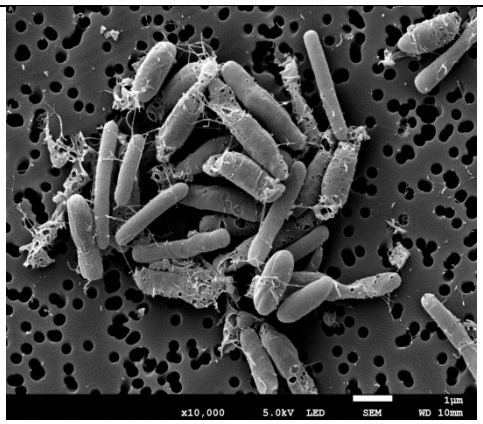
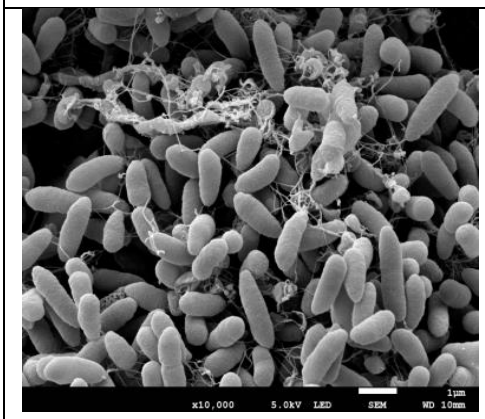
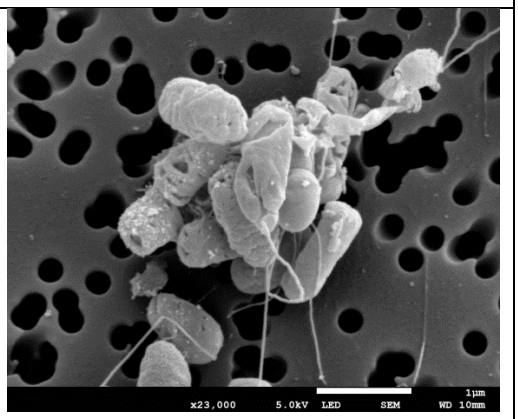
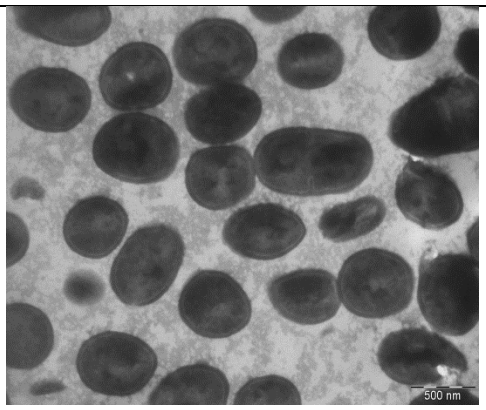
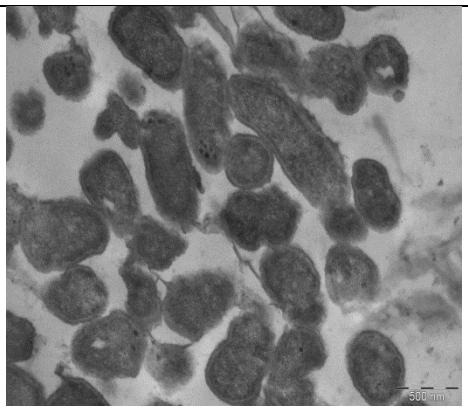
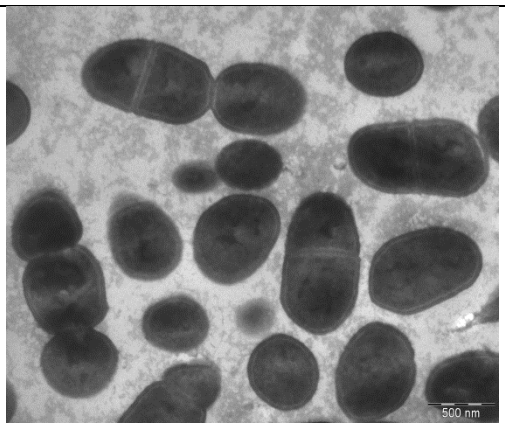
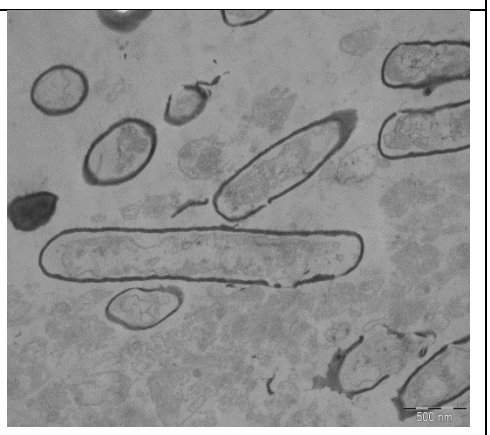
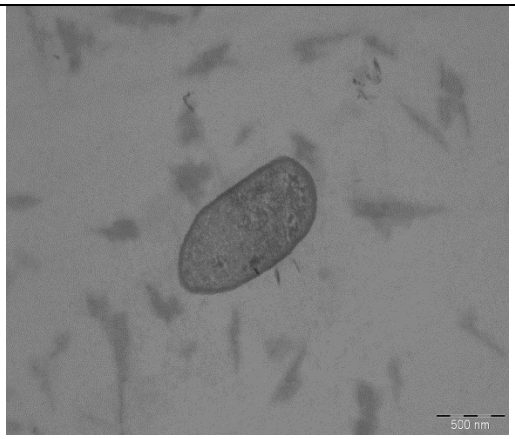
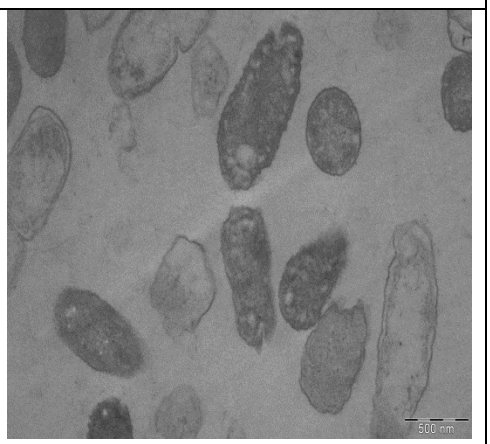
CONTROL	TREATED
	
Lelliotia amnigena	*Lelliotia amnigena*
	
Pseudomonas rhodesiae	*Pseudomonas rhodesiae*
	
Pseudomonas ludensis	*Pseudomonas ludensis*

Table 3. Transmission Electron Micrographs showing bacterial cells untreated and treated with *Artemisia afra* oil.

CONTROL	**TREATED**
 *Lelliotia amnigena*	 *Lelliotia amnigena*
 *Pseudomonas rhodesiae*	 *Pseudomonas rhodesiae*
 *Pseudomonas ludensis*	 *Pseudomonas ludensis*

4.5 CONCLUSION

The objective of this study was to assess antimicrobial properties of essential oils against bulk tank milk isolated bacteria. From the results observed in this study, it is evident that the essential oil used in this study possesses antibacterial activity and that the bacteria showed susceptibility to the plant extract of *Artemisia afra*. Medicinal plants have been considered a healthy source of life for people. Therapeutic properties of medicinal plants are very useful in healing various diseases. The results observed for the SEM and TEM images; prove that *Artemisia afra* oil has an antimicrobial effect on the gram negative bacteria isolated in the bulk tank milk in this study. The compounds of this oil have been shown to exercise their antibacterial action/activity through membrane perturbations. It showed that; *Artemisia afra* induced damage to the cell membrane structures that was accompanied by decreased viability for all nine gram negative bacteria in this study, and the membrane damage was confirmed as the most likely cause to cell death. It has also been revealed that some plant derived compounds can improve *in vitro* activities of some peptidoglycan inhibiting antibiotics by directly attacking the same site (i.e. Peptidoglycan) (Pozzo *et al*., 2011). This study has provided another basis that *Artemisia afra* oil can be a prospective source of natural multi-resistant drug inhibitors that can modulate the performance of antibiotics against resistant bacterial strains. The oil has potential for application within a food system as it has antibacterial activity against all bulk-tank isolated milk bacteria examined in this study. The most promising information taken from this study is the efficacy of the essential oil components of *Artemisia afra*.

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CHAPTER FIVE

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CONCLUSIONS AND RECOMMENDATIONS

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5.1 INTRODUCTION

Microbial contamination of bulk tank milk might be the cause of diseases and bacteria are commonly listed as organisms responsible for infection occurrence. Microbial contamination of milk can occur through the development of a bacterial infection on a teat canal or an infected udder (clinical or subclinical mastitis) from the animal, milker (manual as well as automated), cow environment (bedding, housing) or unclean process water (Jamet *et al*., 2012). Collectively, the presence of foodborne pathogens in bulk-tank milk either directly or indirectly increases the risk of ingestion and transmission of resistance strains from animal origin that can persist in the human intestinal tract (Jamet *et al*., 2012). The aim of this study was to evaluate antimicrobial properties of essential oils against bacteria isolated from bulk tank milk in a predetermined area of Mangaung. In order to attend to this aim, the following objectives were considered: quantification and identification of bacteria isolated from bulk tank milk, evaluating antibiotic profiles of the isolated bacteria and the assessment of antimicrobial properties of essential oils against isolated bacteria.

The study was arranged in four parts:

Chapter 1: Gathering information from a review of the literature relating to bulk tank milk bacteria; for example, the occurrence of foodborne illnesses by bacteria from within the udder; the environment; milking equipment and storage environments.

Chapter 2: Quantification and identification of bulk-tank milk isolated.

Chapter 3: To investigate the antibiotic susceptibility profiles of bacteria isolated from bulk-tank milk obtained from retail outlets in the Mangaung area, South Africa.

Chapter 4: To investigate the antimicrobial properties of *Artemisia afra* against antibiotic resistant bulk-tank milk isolated bacteria.

5.2 GENERAL DISCUSSION

Standard plate counts in the bulk tank milk samples analyzed were above the legal limits. It is not known how long the milk was stored in the bulk tank prior to sampling, but the results showed that the milk had high bacterial counts. During observations as discussed in Chapter 2, milk samples were collected from the bulk-tank upon arrival at the shop and evaluation of the results were carried out in accordance with standards set in R1555 of 1997 as amended by R489 of 2001 which states that standard plate counts may not exceed 5 x 10^4 cfu/ml. An increase in the mean total aerobic plate count was observed in the milk samples taken in summer (November 2015) than in the winter (August 2015). Standard plate counts from samples taken winter were high and this might be due to the slow sales in winter and in addition, not separating left over milk and then mixing it with a new batch of milk might also be another source of contamination. Another reason could be that bulk tank milk, which was often contaminated by spoilage organisms, was not discarded but kept until it was sold. Farm conditions also play a role in the hygiene and safety of the milk.

Farm bulk tank milk produced under hygienic conditions can be safely held at 4^{o}C or less for two to three days before processing but the storage at 7^{o}C or higher encourages the multiplication of psychrotrophic bacteria and the development of slightly unclean or rancid flavors within 48 hours. Milk is often transported in small bulk tanks on the back of the trailers or bakkies (trucks). These trailers or bakkies are not usually equipped with refrigerators and therefore the cold chain is broken, especially when the weather is hot. Another source of bulk tank milk contamination could be pipes which are used to pump the milk from the bulk tank on the farm into the tank on vehicles are often not cleaned in between farms and the milk remaining in these pipes during transport to the next farm become an ideal medium for bacteria to multiply in it at ambient temperatures. Another reason for high bacterial counts could also be due to the poor quality water on the farm as poor quality water has a negative effect on cleaning agents, binding them so that higher concentrations have to be used in the cleaning equipment. In our study; ninety-five percent of the bulk tank milk was

mostly contaminated with *Hafnia, Enterobacter, Lelliottia, Pseudomonas and Serratia* species as identified by the MALDI TOF MS. The high counts in the study area suggest that it might be due to the contamination via environmental contaminants, infection from within the udder, milking equipment and poor storage practices.

In Chapter 3, the plate counts were quantified and identified; and 25 presumptive isolates as seen in **Table 3** of Chapter 2 which belonged to 5 genera of bacteria; *Pseudomonas* spp, *Hafnia* spp, *Enterobacter* spp, *Lelliottia* spp and *Serratia* spp. The isolates were screened for antimicrobial resistance against three most commonly used antimicrobials Ampicillin, Ceftazidime and Cefotaxime; using an epsilometer test and for further identification MALDI TOF MS (Bizzini *et al*., 2010) test was used. An epsilometer test analysis of the antimicrobial susceptibility of the bacterial strains revealed that all genera were resistant to all three antimicrobials tested, while the MALDI TOF MS spectrum corresponding to the supernatant derived from the bacteria after incubation with all three antimicrobials for 24 hours were inconclusive, and this might be because the cells spotted onto the steel plate were not allowed to concentrate on the anchor plate spot during solvent evaporation, which results in a more homogenous crystallization of the matrix and excellent biomarker spectra reproducibility, or there might have been cross-contamination of samples due to the spreading of a sample into an adjacent spot; and another common reason for an isolate to not be identified is because it is not included in the data base. And although the MS spectrum results were inconclusive, an epsilometer test proved otherwise and as seen in literature, bacteria have the ability to become resistant to antibiotics; therefore, possibilities of the spread of resistant bacteria through the ingestion of bulk-tank milk to the consumer is high which leads to alterations of the gut flora and people developing resistance against antimicrobials.

Van de Braak and Leijten, 1999 stated that therapy of bacterial disease is a frequent problem due to the emergence of bacterial strains that are resistant to numerous antibiotics; however, alternatives were found which might be the new potential ‘antimicrobial’ that might reduce the spread of

antimicrobial-resistant bacteria. Since ancient times, plants and their derivatives, such as essential oils, have been used in folk medicine. Essential oils play an important role in the protection of plants and are concentrated natural products with strong smells that are produced by aromatic plants as secondary metabolites. Literature states that aromatic plants are frequently used in traditional medicine as antibacterial agents and their essential oils. Volatile compounds isolated have been known to possess antibacterial and antifungal properties (Van de Braak and Leijten, 1999).

Artemisia afra as discussed in Chapter 4 is one of the known aromatic plants that have awakened interest in investigating the antimicrobial activity of essential oils (Suliman *et al*., 2010). *Artemisia afra* was the essential oil of choice in this study; this is because of the oils' antibacterial and antifungal activity and the oil has shown high degrees of growth inhibition of 15 species of bacteria and one species of fungi (Liu and Van der Kooy, 2009). *Artemisia afra* has anti-oxidant, anti-fungal, anti-bacterial, and immuno-modulatory activities which are all potential areas for future research and the *in vitro* antimicrobial activity of the oil extract of *Artemisia afra* was evaluated using the agar diffusion method and the results of the oil tested against gram negative bacteria showed antimicrobial/antibacterial activity against the bacteria in Chapter 4 study. As seen on the SEM and TEM images; the control differed from the treated organism; before the bacteria was treated; well-separated, rod-shaped, firm and intact were observed. While bacterial cells treated with the *Artemisia afra* oil had holes in them, were not firm and cells were not separated from one another (Turina *et al*., 2006). This study has demonstrated the importance of this plant genus and the untapped potential it still holds; research is on-going, and hopefully in the future this genus plant species will be explored further as an alternative in eliminating bacterial diseases.

5.3 CONCLUSIONS AND RECOMMENDATIONS

The results showed that bulk tank milk samples obtained for the four shops in the Mangaung area were not fit for human consumption on the basis of the Foodstuffs, Cosmetics and Disinfectants Act 54 of 1972, as discussed in Chapter 2. They further showed that the bulk tank milk sampled from the milk shops is of poor bacteriological quality and that many samples contained pathogens and possibly residues of inhibitory substances which may affect the health of the consumer. Consumers might therefore be exposed to unnecessary health risks, by drinking unsafe milk. From the results obtained in this study; it can be concluded that microorganisms from within the udder, the cow environment, milking equipment and storage environments played a role in the presence of microbial contamination. From the observations made within the bulk tank milk analysis; it is recommended that dairy farm owners together with shop owners develop cleaning and sanitizing programs that will help minimize the multiplication of resistant bacteria. Environmental Health Practitioners should also provide educational training programs that will help in equipping the dairy industry people on how to take care of dairy products and milk shop owners should know that when milk is no longer fresh, it should be discarded and not kept until it is ultimately purchased. Hygienically processed milk ought to be kept for at least 7 days before it can be discarded. Monthly milk sampling tests will also provide evidence to Environmental Health Practitioners with regards to milk and contamination, and doing this helps to identify the milk contamination problem and their sources. Cleaning and sanitizing the milking equipment is of the utmost importance in removing milk soils, organic and mineral solids that form on the equipment surfaces as these contribute to the contamination, resulting in elevated bacterial counts in milk.

Inadequately washed and sterilized milking and milk handling equipment constitutes as the main source of bacteria in the farm supplies (Hyldgaard *et al*., 2012). It has been reported that good procedures of cleaning and sterilizing milking equipment resulted in milk with lower numbers of total and of psychrotrophic bacteria. Milk stone deposits are caused by inadequate milking machine

cleaning and poor quality water in the dairy (Hyldgaard *et al*., 2012). These can protect bacteria in mineral and protein deposits on stainless steel and other surfaces. They can also protect bacteria from hot water, detergents and the sanitizers used to clean the milking equipment. They also provide nutrients for the rapid growth of bacteria in the milk. When the milk stone breaks down or is dislodged from the stainless steel surface, large numbers of bacteria can be released into the milk. It is stated that poorly cleansed pipeline milking plants contribute to exceedingly large numbers of bacteria in the milk, especially in the presence of milk stone or milky residues (Hyldgaard *et al*., 2012).

To limit bulk tank milk contamination; dairy owners should properly clean and sanitize the bulk-tanks, so that there will not be any multiplication of psychrotrophic bacteria. Tanks should be cleaned by using essentially the same procedures as recommended for milking equipment. The milk hauler is normally responsible for rinsing the tank immediately after the milk is removed. Rinse water temperature should be 90-120^{o}C. Following this, the tank must be washed, rinsed and sanitized. Allow the mechanical cleaning device to operate until the tank is clean (6-10 minutes). Cleaning solution temperature should be 120^{o}C during the wash cycle and that means starting with hot water. Rinse the tank completely with tepid water, finishing the rinse with acidified solution as it neutralizes and removes detergent residues and inorganic soils. Tank covers and gaskets should be disassembled and the calibration rod removed for manual cleaning. The outlet connection and valve should be cleaned manually. The tank exterior should be washed. Sanitizing should occur just before the next milking. Allow the sanitizer to drain from the outlet to prevent sanitizer residues in milk. Tanks may be cleaned manually or mechanically.

Results in Chapter 3 further identified that bacteria isolated from bulk tanks had the ability to be resistant to the three antibiotics they were tested against and this could be a possible source of transmission of antimicrobial resistant bacteria to humans. All bacterial organisms proved to be resistant to three antibiotics; Ampicillin, Ceftazidime and Cefotaxime. It was stated in literature that

bacteria have the ability to resist antimicrobials; it is advised that alternatives that have an antibacterial action against bacteria, but does not have diverse effects on human or animal health; those that control and possibly eliminate pathogenic bacteria should be used (Hyldgaard *et al*., 2012). Such alternatives are essential oils as they are known for their antiseptic, i.e. bactericidal, virucidal and fungicidal; and medicinal properties and their fragrances, they are used in the preservation of foods and as antimicrobial, analgesic, sedative, anti-inflammatory and locally anesthetic remedies (Pozzo *et al*., 2011). And up to the present day, these characteristics have not changed much except that more is known about some of their mechanisms of action, particularly at the antimicrobial level. Chapter 4 results proved that essential oils have antimicrobial properties with no reports of resistance after prolonged exposure to microorganisms, and no side effects on human health which makes them a potential weapon against bacterial infections (Pozzo *et al*., 2011).

This work can serve as the basis for future developments of antibiotics and disinfectants from traditional plants. The herbs should be tested *in vitro* by means of clinical trials and they should also be tested for their toxicity to cells. Different parts of the plants should also be tested for their antibacterial activity to a wide range of bacteria. Trials should be run with different types of extracts and this study has indicated that there is synergism between plants and it warrants further research as this plant has an antibacterial effect on bulk tank isolated milk bacteria from the genera *Pseudomonas* spp, *Hafnia* spp, *Enterobacter* spp, *Lelliottia* spp and *Serratia* spp analysed in this study. The potential for developing antimicrobials from higher plants appears rewarding as it will lead to the development of a phytomedicine to act against microbes. Plant-based antimicrobials have enormous therapeutic potential as they can serve the purpose with lesser side effects that are often associated with synthetic antimicrobials (Pozzo *et al*., 2011). Continued further exploration of plant-derived antimicrobials is needed today. Further research is necessary to determine the identity of the antibacterial compounds from these plants and also to determine their full spectrum of

efficacy. However, the present *in vitro* antimicrobial evaluation of some plants forms a primary platform for further phytochemical and pharmacological studies.

5.4 REFERENCES

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