MICROBIAL ANALYSIS OF RAW MILK AROUND SMALL SCALE FARMERS IN HARRISMITH FREESTATE, SOUTH AFRICA

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BLOEMFONTEIN
2019

DECLARATION OF INDEPENDENT WORK

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I, **KHASAPANE NTELEKWANE GEORGE**, identity number and student number **,** do hereby declare that this research project submitted to the Central University of Technology, Free State for the Degree **MASTER OF HEALTH SCIENCES: ENVIRONMENTAL HEALTH**, is my own independent work; and complies with the Code of Academic Integrity, as well as other relevant policies, procedures, rules and regulations of the Central University of Technology, Free State; and has not been submitted before to any institution by myself of any other person in fulfilment (or partial fulfilment) of the requirements for the attainment of any qualification.

12 August 2019

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DEDICATION

This dissertation was dedicated to dearly loved family, for their understanding, patience, encouragement, support and their love more especially my mother, D.A Simon and my one and only sister, O. Simon for being the pillar of my strength.

“You are capable of more than you know. Choose a goal that seems right for you and strive to be the best, however hard the path. Aim high, behave honourably, prepare to be alone at times and endure failure. Persist! The world needs all you can give”

By

E.O Wilson

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LIST OF ABBREVIATIONS

Blast : Basic Local Alignment Search Tool

Bp : Base pair

CDC : Centre for disease control and prevention

CM : Clinical Mastitis

CMT : California Mastitis Test

CoNS	: Coagulase negative *Staphylococcus Aureus*
DNA	: Deoxyribonucleic acid
DoH	: Department of Health
EFSA	: European Food Safety Authority
EHP	: Environmental Health Practitioner
Et al	: et alia
Etc.	: et cetera
FAO	: Food and agriculture organisation
gDNA	: Genomic deoxyribonucleic acid
IDF	: International dairy federation
Mega x	: Molecular Evolutionary Genetics Analysis
mPCR	: Multiplex Polymerase Chain Reaction
PCA	: Plate count agar
PCR	: Polymerase Chain Reactions
rRNA	: Ribosomal ribonucleic acid
SCC	: Somatic Cell Counts
SCM	: Subclinical Mastitis
SLST	: Sodium Lauryl Sulphate
TVC	: Total viable counts
VRBM	: Violet red bile agar with mug

SUMMARY

Food manufacturing and agricultural industry have an ancient history of been observed to provide the most favourable conditions for the multiplication and distribution of microorganisms. These microorganisms may be found in the air (airborne), food (milk) and at the surrounding environment. Food handlers have also been found to harbour some of these microorganisms on their hands or skin surfaces. Dairy environment have been receiving quite an extensive attention especially on the composition of microbes in milk. However, studies that have been conducted mostly utilized conventional/traditional microbiological techniques. Hence, there is still lack of research in South Africa that is focusing on molecular techniques to quantify these microorganisms in raw milk.

The overall aim of this dissertation was to assess different microorganisms confined in raw milk from small-scale farmers in the Eastern part of the Free State Province, South Africa. With reference to the main aim of this study, the objectives of the present study were to use molecular techniques to quantify five mostly isolated microorganisms causing subclinical mastitis in bovine .i.e. *E. coli, S. aureus, S. agalactiae, S. dysgalactiae* and *S. uberis*. Then lastly, a metagenomic analysis of raw milk was conducted by targeting the 16S rRNA gene using high throughput sequencing.

The findings of this study in relation to microbial composition as per Chapter 2 showed a high microbial contamination of raw milk and has clearly indicated the need for training of all employees and the enforcement of health and hygiene measures within the dairy environment. While investigating the prevalence of subclinical mastitis around these

farms, the author came across some interesting findings. The author isolated streptococcal species that are more prevalent/isolated in the hospital environment especially on samples derived from human subjects after/during streptococcal infections such as meningitis. Instead of isolating streptococcal species more prevalent on the dairy environment such as*, S. dysgalactiae* or *S. uberis*, the author identified *S. mutans, S. Salivarius, S. pneumonia* and *S. sanguis* which may entail that the employees around the farms are/were the carriers of these species.

The isolation of microorganisms associated with food spoilage and foodborne disease outbreaks, which are known as indicator organisms such as *Escherichia coli, Staphylococcus* and *Bacillus* from both air and surface samples, signified possible faecal contamination and could be attributed to poor health and hygiene practices at the dairy farm plant. Despite the isolation of microorganisms associated with food spoilage and foodborne disease outbreaks, the isolation of microorganisms not usually associated with the food processing industry (usually associated with hospital environments) was an enormous and serious concern which suggested a need for further investigations at dairy farm plants as the implications of these pathogenic microorganisms in food is not known.

CHAPTER ONE

GENERAL INRODUCTION AND BACKGROUND

1.1 General Introduction: Microbial Challenges Associated with Raw Milk

A number of studies have been conducted worldwide on the quality of bovine (cow's) milk and the hygiene requirements that guide milk processing industries. These studies have concluded that microbial proliferation in milk might be both beneficial and detrimental to human health. Furthermore, studies have also alluded to the fact that human beings may derive benefits from bovine milk such as growth and the strengthening of bones, but it is a fact that the development of chronic diseases may also be associated with milk consumption (Elwood *et al*., 2008). Bonnier (2004) argues that humans also keep dairy cows not just for their milk, but also for the benefits of meat production and investment.

The differences between the biological and chemical components of raw milk have been demonstrated as major qualities that attract microorganisms that cause spoilage of milk (Fernandes, 2008; Strohbehn *et al.,* 2008; Mokoena, 2013). It is therefore very important to ensure that milk and products derived from it are stored and treated safely because it is vital that humans consume milk of high quality and that hazards associated with milk are avoided at all costs (Lues *et al.*, 2003). Milk is a precious commodity, yet the consumption of raw milk and its related products is associated with foodborne microorganisms that may be hazardous when ingested by humans (Asaminew and Eyassu, 2011). The introduction of microorganisms in milk occurs mainly through unhygienic sources, contamination on the farm or in the production parlour, and sometimes through contact with the udder of an infected animal (Oliver *et al.,* 2005).

In the dairy industry, the shelf-life of milk and milk products is prolonged by the processing and maintenance of cold storage conditions (generally referred to as the cold chain). The milk processing industry is one of the leading food industries as it processes various dairy products and beverages such as milk, yoghurt, cheese and dairy juice products (Belova *et al.,* 1999). However, an emerging concern in this industry is that milk and its products are associated with foodborne diseases, more especially in developing countries where the production of milk occurs under working conditions that are not always hygienic (Mutaleb, 2012). Factors that need to be considered in milk production are: the standards that guide food and food hygiene safety practices, the transportation of milk and milk products, and the temperature at which milk and milk products are transported and stored (Salman and Hamad, 2011). The fact that milk is composed of nutrients such as proteins, vitamins and minerals makes it prone not only to microbial contamination due to unhygienic practices, but also exposes it to airborne contaminants (Salustiano *et al.,* 2003; Nádia *et al.,* 2012). Contagious microorganisms may also come from the skin of a bovine, soil, water, or bedding, and these contaminants may in turn cause mastitis which might be contagious (Oliver *et al.,* 2004). Microorganisms that are often found in raw milk include Mycobacterium bovis, Brucella, Streptococcus, and other Gram-negative/positive bacteria (Anderson *et al.,* 2011). Raw milk can therefore be contaminated by a large number of somatic cells that may affect its quality (Mokoena, 2013).

Abebe *et al.* (2013) argue that, particularly in small-scale farming enterprises, the microbial composition of milk is influenced by hot and humid conditions, lack of access to cold storage facilities, and inadequate infrastructure. This is why it is important to regulate

this industry and enforce regulations that stipulate how raw milk should be handled and pasteurized to protect the health of the public, especially in developing countries where the outbreak of milk-borne infections is rife (Donkor *et al.,* 2007).

1.2 Rationale for the Study

It is important that good microbiological quality of milk is maintained at the production sites where dairy products are manufactured and stored. Research has revealed that poor monitoring of these industries, particularly in terms of hygiene and sanitation requirements, animal health programmes and transport resources, has resulted in poor and often hazardous milk quality (Tassew *et al,* 2011). Once raw milk has been extracted from bovines, it generally contains several microorganisms and viruses that are detrimental to the health of the public − more especially of children and those who are immune-compromised. The health hazards associated with the ingestion of raw milk and milk products include the increased risk of contracting listeriosis, stillbirths and other neonatal ill effects and diarrheal disease that are caused by *E. coli* O157:H7 (Maldonado *et al.,* 2014). Many health promoters, sponsors and consumers believe that raw milk should be used more often for its great taste and health benefits. However, raw milk has been shown to be a public health risk, especially in developing countries because of its association with pathogens. It is thus imperative that more emphasis be placed on the microbiological quality of raw milk that is derived from local small-scale farmers and informal markets that provide milk for the consumption of local citizens.

The regulations that guide the operation of milking sheds and the transportation of milk (R1256) define an unapproved milking parlour as “a place or structure where milk is produced for human consumption and that does not have a certificate of acceptability or the provisional certificate of acceptability (R1256, 1986). The certificate that is referred to should be issued by a local authority (municipalities) as stipulated by Section 2 of Regulation 1256 of 27 June 1986 (Department of Health, South Africa, 1986). These unapproved farmers can either be producing milk for commercial use or for personal use and they are usually found in small holdings around towns. These farmers will then sell milk to small traders such as ‘spaza’ shop owners (who usually operate from residential homes), street vendors, and small business owners who run cafés (Agenbag, 2008). In South Africa, which is still considered to be a developing country, major problems regarding the registration of informal traders of raw, unpasteurized milk exist, and this presents a barrier in the communication lines between producers and environmental health practitioners (Lues *et al.,* 2010). For example, it has been observed that some of these small-scale farmers transport milk in bulk containers that do not have temperature monitoring facilities. Some also use their private vehicles that are not equipped for this purpose. The milk they transport in this unregulated manner is often purchased by managers of day-care centres and schools, and by community members for family usage. It is no wonder that the Centre for Disease Control and Prevention (CDC) recorded more than 148 disease outbreaks globally in the period 1998 to 2011 (CDC, 2012). It has been surmised that the probability of an individual falling ill after the consumption of unpasteurized milk is 150 times higher than for those who consume pasteurized milk. Moreover, 82% of all cases reported were children (Hueston *et al*., 2014). Oliver *et al.*

(2005) outline a number of reasons why more research should be focused on raw milk than on pasteurized milk, two of which are: (1) illness or disease outbreaks have been attributed to raw milk rather than to pasteurized milk; and (2) raw milk is directly consumed by consumers. Omore *et al.* (2000) also emphasise the need to focus more on the health risks associated with the ingestion of raw milk rather than focusing on the hygiene of milking sheds or parlours.

1.3 Aim of the Study

The study aimed to characterise microorganisms associated with raw bovine milk and to evaluate the quality of raw, unpasteurized milk that was derived directly from small-scale farmers in the Harrismith area in the Free State, South Africa.

1.4 Specific objectives

To ensure that the aim was achieved, the study endeavoured to:

- Enumerate and identify microbiota isolated from raw milk; and
- Identify and enumerate subclinical mastitis-causing pathogens in raw milk.

1.5 Hypothesis

It was hypothesised that raw milk contains a high load of microbial pathogens.

1.6 Significance of the Study

When this study was conceptualised, it was understood that it had the potential to support or challenge the existing body of knowledge regarding the quality of raw milk. Based on the findings, it is envisaged that it will be invaluable in enhancing scholars' knowledge regarding microorganisms associated with raw milk. Both unsuspecting members of the public and dairy farmers, particularly small-scale farmers, will be informed via various platforms of the risks associated with the consumption of raw milk. It is also my intention to sensitise nurturing mothers and the care-givers of infants and small children of the threats associated with the consumption of raw milk.

1.7 Chapter Layout

This dissertation contains the following chapters:

Chapter One: This chapter focuses on the general background of raw milk and its associated microbiome. The threats associated with the consumption of raw milk are illuminated, and the study's aims and objectives are presented.

Chapter Two: This chapter contains the literature review, with particular focus on microorganisms that are of importance in dairy products. Health risks that are associated

with the consumption of milk, especially raw milk, are also discussed in detail.

Chapter Three: This chapter focuses in general on the microbial hazards that small-scale farmers in the study area faced, with particular attention given to *Enterobacteriaceae*, *Streptococci* and total viable count (TBC).

Chapter Four: This chapter focuses on the screening and diagnosis of subclinical mastitis using various techniques.

Chapter Five: The chapter focuses on the composition of microbial communities in raw milk using the noble technique of next generation sequencing.

Chapter Six: The general conclusions are presented in this chapter. Recommendations are offered and strategies to improve raw milk quality and ensure its safety are elucidated.

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CHAPTER TWO

LITERATURE REVIEW:

MICROBIAL ANALYSIS OF RAW MILK DERIVED FROM SMALL-SCALE FARMERS

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2.1 Abstract

Milk is an opaque white fluid that is rich in fat and protein and is secreted by female bovine mammals for the nourishment of their young. Regulation R1555 that relates to milk and its products defines milk as “liquid foodstuff secreted from the mammary gland of mammals for their young”. As a foodstuff for humans, it is defined as “the normal, clean and fresh secretion from the udder of a healthy cow, excluding the first 14 days before calving and six days after calving”. The Regulation defines raw milk as “milk that has not undergone any pasteurization, sterilization or ultra-high temperature treatment” (www.health.gov.za). Milk and the products derived from it are part of an important human diet and daily nutrition and is a source of nutrients such as proteins, fats, vitamins and minerals (Elwood *et al.,* 2008). Milk has been regarded as one of the most nutrient-rich foodstuff produced worldwide and it also contributes to the economy of South Africa through exportation (Grimaud *et al*., 2009). It is for these reasons that the milk industry has been classified as one of the most important sectors as it provides a key ingredient to several industries that produce milk fermented products (Britz and Robinson, 2008). Small-scale farmers in South Africa have recently been encouraged to produce milk on rural farms for the purpose of supplying it to urban areas for further processing (Mokoena, 2013). Modern technologies such as milking machines have also been employed and dairy farmers have utilized opportunities to process milk in the vicinity of their farms using cost saving measures (Jansen, 2003).

2.2 Microorganisms of Concern in Dairy Processing

Foodborne illnesses have been problematic in Africa and have claimed the lives of as high as 30% − 90% of children on this continent (Flint *et al.,* 2006; Assob *et al.,* 2017). Although bovine milk is one of the most nutritionally balanced foodstuffs, it may harbour many hazardous microorganisms (Ryser, 1998; Oliver *et al*., 2008). The fact that milk is generally composed of different nutrients and has a neutral pH makes it favourable for microbial survival. When milk is properly stored under appropriate temperatures (<5°C), the multiplication rate of many bacteria can be slowed down; however, psychotropic bacteria (*Pseudomonas* spp., *Listeria* spp. *or Yersinia* spp.*)* can still grow in low temperature conditions (EFSA, 2015). In the period 1973−2009, around 82% of cases reported to the Center for Disease Control and Prevention was associated with raw milk and its products, especially cheese (Dhanashekar *et al.*, 2012), and many pathogens, such as *E coli* 0157:H7, have been isolated from milk. These pathogens pose severe health risks if consumed (Sivapalasingams *et al*., 2004).

Microorganisms that lurk in contaminated raw milk at production sites may result in the formation of biofilms which, in turn, will result in the contamination of processed products that will expose consumers to harmful pathogens (Latorre *et al.*, 2010). The level of microbial load in raw milk is important in identifying risk factors that may impact humans, and issues such as the cleanliness of the production area and utensils, and conditions of storage need to be closely monitored (Gandiya, 2001). It is a known fact that milk that is derived from healthy animals contains fewer bacterial counts than milk from an infected animal with an infected udder; however, even milk from a healthy animal can become

contaminated by a variety of microorganisms during its processing route (O'Connor, 1994; Yilma, 2012).

When ingested through the consumption of raw milk, microbiota (that are usually confined in raw milk) such as Coryneforms, *Micrococci, Lactococci, Pseudomonas* sp., *Brucella* sp., *Escherichia* sp., *Salmonella* sp., *Shigella* sp., *Bacillus* sp. and *Clostridium* sp., usually cause diseases such as brucellosis and mastitis-related enterotoxaemia (Lues *et al.,* 2003). These microbiota are found naturally on the human skin and hair as well as in the intestinal and respiratory tract of humans. However, they may also be found in milk during the processing stage and may cause inevitable contamination (Mokoena, 2013). The latter author also states that processing activities, ventilation systems and employees all contribute to the existence of airborne microorganisms. Another hurdle caused by the prevalence of Gram-negative bacteria such as *E. coli, Salmonella* sp., *Shigella* sp., *Pseudomonas* sp., *Neisseria* sp. and *Haemophilus* sp. in milk is the production of endotoxins that are highly toxic substances commonly found on the outer membrane of the cell wall. Unlike spores, endotoxins can easily be killed by heat during pasteurisation (Todar, 2002).

Table 2.1: Common microorganisms and diseases associated with milk

No.	Type of milk-borne disease	Causative agent	Disease/Disorder
1.	**Food infection**	*Salmonella typhi*, *Shigella dysenteriae, Streptococcus sp.* (enterococci)	Typhoid, Salmonellosis (food poisoning), Shigellosis, Septic sore throat, Scarlet fever, food poisoning
2.	**Food intoxication**		
	Bacterial	*Staphylococcus aureus*	Food poisoning
		Clostridium botulinum	Botulism (food poisoning)
		Escherichia coli	Summer diarrhoea
		Vibrio cholerae	Cholerae
	Fungal	*Aspergillus flavus*	Aflatoxicosis
		Other toxigenic mold sp.	Mycotoxicosis
3.	**Toxic-infection**	*Bacillus cereus*	Food poisoning
		Clostridium perfringens	Gas gangrene
4.	**Other milk-borne disorders (uncertain pathogenesis)**	*Aeromonas sp.* *Proteus sp.*	Food poisoning
5.	**New emerging pathogens**	*Yersinia enterocolitica*	Diarrhoeal diseases
		Campylobacter jejuni	Diarrhoeal diseases
		Vibrio parahaemoyticus	Diarrhoeal diseases
		Listeria monocytogenes	Listeriosis
6.	**Other milk-borne diseases:**		
	Bacterial	*Mycobacterium tuberculosis*	Tuberculosis

Source: Mokoena (2013)

It is therefore important to assess microbial loads at different stages in the milk production line because this will help to identify areas that need improvement. However, a disturbing trend has emerged as many local farmers seem to produce and store milk at incorrect temperatures under unhygienic conditions with the purpose of selling it to local consumers such as tuck shops, day-care centres or schools (Chye *et al*., 2004). Based on its investigations into public health risks associated with raw milk, the European Food Safety Authority (2015) outlines that microorganisms grow in lower temperatures and present a public health risk to consumers. The same report urges that, to control or decrease the number of pathogens such as *Campylobacter* spp*., Salmonella* spp*.* and *STEC* O157 in milk, producers of raw milk need to improve their on-site hygiene programme.

2.3 Legislative Framework

The Regulation relating to Milk and Dairy Products which is derived from the Foodstuffs, Cosmetics and Disinfectant Act No. 54 of 1972 (South Africa. National Department of Health, 1972) stipulates the standards for microbiological determinants in milk as are reflected in Table 2.2 below.

Table 2.2: National Standards applicable to milk in South Africa

Analysis	Raw milk before further processing	Raw milk directly to consumers (public) without Processing	Pasteurized milk
Total count	< $2x10^5$cfu.ml-1	< $5x10^4$cfu.ml-1	< $5x10^4$cfu.ml-1
Coliforms	20 cfu.ml-1	< 20 cfu.ml-1	< 10 cfu.ml-1
E. coli	0	0	0
Pathogens	0	0	0

Source: Adapted from: Foodstuffs, Cosmetics and Disinfectant Act No. 54 of 1972 (South Africa. National Department of Health, 1972)

The recognition of coliform bacteria in milk has been mostly associated with unclean udders, unhygienic milking utensils, and/or contaminated water (Bonfoh *et al.,* 2003). According to Lues *et al.* (2010), keeping raw milk in clean containers at a normal refrigeration temperature soon after milking may decrease the chances of having an increased number of microorganisms, and this further reduces the growth of microorganisms in milk from the farm to the processing plants and ultimately to the consumers.

2.4 Indicators of poor animal health

2.4.1 Bovine mastitis infection

Mastitis is defined as the inflammation of the parenchyma of the mammary glands. It is usually identified by physical, chemical and bacteriological modifications in milk and pathological changes in the glandular tissues of the animal. The occurrence of this infection in bovines is due to a number of factors such as the presence of infectious agents, host resistance, and environmental factors (Gera and Guha, 2011). This infection can be identified clinically when the udder of a cow is observed to have an inflammatory response that causes clots and colour changes in her milk, or it can also occur sub-clinically, although the farmer may not see any sign of the infection (Tiwari *et al.,* 2013; Mpatswenumugabo *et al.,* 2017). The frequent occurrence of mastitis in a dairy environment is financially costly due to reduced milk production during and after infection episodes, the costs of the antibiotics used and their withdrawal period, lowered fertility, and early culling (Erskine, Wagnger and DeGraves, 2003). Sharma *et al*. (2012) list several factors that play an important role in causing mastitis in bovine females. These factors include inadequate sanitation of the dairy environment, poor animal health services, and a lack of proper attention to the health of the mammary glands of cows. The latter author also alludes to a lack of basic training, limited awareness, poor disease detection ability, unhygienic milking practices, and delayed treatment that all play a role in the harmfulness of the disease.

Gitau *et al.* (2014) argue that, to counteract the disease, knowledge regarding the occurrence of this infection, its causal agents and its susceptibility to antibiotics could aid

treatment opportunities and the inability to prevent this infection. Various causal agents of different forms of mastitis are depicted in Table 2.3 below.

Table 2.3: Bacterial mastitis in cows with an average prevalence rate (%) per 100 cows in herds worldwide

Contagious Mastitis	Environmental Mastitis	Opportunistic Mastitis
• *Staphylococcus aureus* (40-70%) • *Streptococcus agalactiae* (8-10%) • *Mycoplasma* (12%) • *Corynebacterium bovis* (1-1.7%) • *Streptococcus dysagalactiae* (1.6%) • *Streptococcus uberis* (1.4%)	• *E. coli* (40%) • *Klebsiela Arcocaobacter*	• *Staphylococcus epidermidis* (1.3%) • *Staphylococcus simulans* (1.0%) • *Staphylococcus chromogenes* (0.7%)

Source: Shaheen *et al.* (2016)

Bacterial mastitis can be observed by employing a variety of tools at farm and laboratory levels for diagnosis purposes. This study focused primarily on subclinical bovine mastitis in two phases. The initial phase occurred after raw milk had been collected from selected farms and transported to a laboratory. Here, Somatic cell counts and California mastitis kit were used as screening tools for subclinical mastitis. These processes were supplemented by a second phase when bacteriological and molecular identification of the causal agents was conducted in a laboratory.

2.4.1.1 Subclinical mastitis as an indicator of animal health

The dairy business is fraught with challenges that are related to the prevalence of bovine mastitis. Subclinical mastitis is usually observed by modifications in milk quality, hence the need to perform specialised tests for diagnosis purposes (Fragkou *et al.,* 2014).The fact that subclinical mastitis negatively affects the freshness of raw milk and its quantity remains a matter of great concern among producers of raw milk due to the accompanying financial losses (Swinkels *et al*., 2005; Halasa *et al*., 2009). Moreover, additional to the financial losses caused by subclinical mastitis, this infection has the potential to transmit zoonotic diseases such as tuberculosis, brucellosis and other streptococcal-related infections such as a sore throat to people (Radostits *et al*., 2000).

Several studies have been conducted globally to determine and assess the prevalence of subclinical mastitis in bovines. A cross-sectional study that was conducted by Katsande *et al.* (2013) determined the prevalence of both clinical and subclinical bovine mastitis on smallholder farms in Zimbabwe. It was found that 95 of 584 samples tested positive for subclinical mastitis and the isolated organisms included coagulase-negative staphylococci (27.6%), *Escherichia coli* (25.2%), *Staphylococcus aureus* (16.3%), *Klebsiella* spp. (15.5%), and *Streptococcus* spp. (1.6%).

Another study that was conducted by Abrahmsén *et al.* (2014), which focused on smallholder farms in a peri-urban area near Kampala, revealed that of 195 cows that were screened for subclinical mastitis, 186 (86.2%) tested positive for subclinical mastitis. Furthermore, isolated microorganisms in this case were coagulase-negative *staphylococci* (54.7 %), followed by negative growth (24.9 %) and *streptococci* (16.2 %).

Sanotharan *et al.* (2016) also investigated the prevalence of subclinical mastitis in the Batticaloa district in Sri Lanka. Using the California mastitis test (CMT), these researchers found that, of 152 lactating bovines, 66 (43%) cows and 116 (19.1%) individual quarters tested positive for subclinical mastitis. The results also showed that *staphylococcus spp.* (90.5%) was the most prevalent, followed by *Escherichia coli* (6.0%%) and *Streptococcus spp.* (3.5%).

2.4.1.2 Somatic cell counts as an indicator of animal health

The somatic cells in milk contain macrophages (60%), lymphocytes (30%), neutrophils (10%) and epithelial cells (2%) (Sandholm, 1995; Schukken *et al.,* 2003). According to Griffiths (2010), an enhanced number of somatic cell counts is an indicator that animals have poor health status and it also indicates changes in protein quality, fatty acid configurations, the presence of lactose and other minerals, and the pH of milk (Nòbrega and Langoni, 2011). The SCC tests are generally important tools used to monitor intramammary infections. However, they must be complemented with other bacteriological and enumeration tests (Shome *et al.,* 2011).A study by Oliver[a] *et al.,* (2004), showed that there is an association between somatic cell counts and bacterial counts of *S. aureus* and *S. agalactiae* in all mastitis cases.

A regulation relating to milk and milk products sets standards for somatic cell counts. The regulation stipulates that a cell count of $5x10^5$ ml^{-1} is acceptable but a count > $5x10^5$ ml^{-1} is a possible indicator of mastitis (Department of Health R1555, 1997). The California mastitis kit is used to detect subclinical mastitis. This test is based on the viscosity of somatic cells prior to counting the number of somatic cells in raw milk. The application of

this test is based on the lysis of somatic cells by the reagent provided with the kit. This reagent precipitates the DNA and proteins found in milk, and therefore any change in milk viscosity when mixed with the reagent is a possible indication that the cow may suffer from intramammary infections relative to the somatic cells (Kuehn *et al.,* 2013).

A study that was conducted by Dingwell *et al.* (2003) with the aim of evaluating the utilisation of the California mastitis test for diagnosing intramammary infections caused by common mastitis organisms found that this test, together with bacteriological culturing of mastitis-causing pathogens, had a sensitivity of 82.4% and a positivity of 80.6%. These findings were supported by those of Sharma *et al.* (2010), who described the sensitivity of CMT compared to other on-farm diagnostic tests such as sodium lauryl sulphate (SLST), SCC and bacteriological culturing. The latter authors concluded that the sensitivity of CMT was 86.07% and its specificity was 59.70%, whereas the total accuracy of the CMT was 75.52%.

Guha and Guha (2012) argue that it is important to determine the sensitivity, specificity and accuracy of all diagnostic and screening tools prior to the isolation of causal agents of subclinical mastitis. More recently, Kandeel *et al.* (2018) also found that the sensitivity and specificity of CMT in forecasting intra-mammary infection were high, especially where the CMT scores were of traceability; meaning at the score of three.

2.5 Treatment Opportunities

2.5.1 Raw milk

Researchers worldwide have devised strategies to ensure the effective production of healthy milk and its derived products. These strategies include: good animal health, improved milking hygiene, and pasteurization. These strategies also have the potential to reduce certain zoonotic agents in bovines (Angulo *et al.,* 2009). Pasteurization has also been shown to be an effective method in the treatment of viable microorganisms such as *Brucella abortus, Streptococcal* spp., and Enteric pathogens in milk, thereby increasing the shelf-life of milk (Girma *et al*., 2014). Depending on the region and milk treatment technologies in place, the safety risks associated with milk and other milk products may differ. Mosalagae *et al.* (2011) argue that behavioural changes in the practices of dairy farmers have the potential to decrease the chances of zoonotic milk-borne infections. The latter study also highlights that general hygiene, health education, and disease control and prevention all play a major role in the reduction of public health risks from zoonotic milk-borne infections. While the selling of raw milk through vending machines in rural areas of South Africa has not been well established, environmental health practitioners should play a role in informing consumers about the importance of boiling raw milk before consumption. It is also important that the sellers of milk should ensure that the temperature of the milk in vending machines is below 4°C (European Food Safety Authority [EFSA], 2015). The same report by EFSA indicates that differences in temperature throughout the food chain could result in the multiplication of organisms such as *L. monocytogenes, S. Typhimurium and STEC* O157:H7*.* Therefore, the application of

good animal health, good agricultural practices and good hygienic practices is important in curbing opportunities for the contamination of raw milk.

2.5.2 Mastitis treatment

To better understand the treatment of mastitis in bovines, there is a need for the early identification of the mastitis-causing agents and their susceptibility to antibiotics. It is also important to understand the treatment and control regimens of the infection and to utilise existing knowledge regarding the impact of the use of antibiotics in third world countries on public health (Dhakal *et al.* 2007). In this context, Kuehn *et al.* (2013) state that the early identification of the microbes accountable for causing culture negative mastitis and an evaluation of the modifications in microbial communities throughout the mastitis infection stage will enhance our knowledge of the infection progression.

Moreover, Giesecke *et al.* (1994) highlight a few strategies for the control of mastitis infection in a herd. They point out that, to control mastitis, a 'five-point plan' should be employed to control this infection. The plan involves: 1) disinfection of the teats after milking; 2) good hygiene and milking practices plus sufficient equipment for milking; 3) discarding chronic mastitic cattle; 4) antibiotic dry-cow therapy; and 5) treatment of clinical mastitis in a dry and lactic period. Another alternative regarding the treatment of mastitis, or the control thereof, may involve the reduction of antibiotic use. This could be accomplished by good hygiene practices, good farm management, and the implementation and enforcement of applicable legislation (Ekman and Østerås, 2003).

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CHAPTER THREE

THE ENUMERATION OF MICROBIAL HAZARDS IN RAW MILK PRODUCED BY SMALL-SCALE FARMERS NEAR HARRISMITH IN THE FREE STATE

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3.1 Abstract

The production of raw milk is affected globally by a variety of factors such as milking practices and the impact of the environment on the dairy parlour. These factors can also create an environment for the proliferation of pathogens that may be harmful to consumers. It is against this backdrop that technologies such as pasteurization were developed to reduce microorganisms in raw milk and to ultimately enhance milk quality, safety and shelf life. However, small-scale farmers still experience problems with regards to the quality of milk regardless of the availability of modern technologies. The current study was undertaken to assess microbial hazards in raw milk produced by small-scale farmers in the vicinity of Harrismith, which is located in the Free State Province, South Africa. A total of eight milk samples were collected from milk tanks located at selected farms using 50 ml sterile bottles. Samples from this milk were culturally plated on different selective agars for enumeration of *Enterobacteriaceae*, total viable counts and *Streptococci* spp. The results of this study showed that *Enterobacteriaceae* were present in a range of $1.40X10^{6}$–$3.77X10^{10}$ CFU.mL^{-1}, while those of TVC were $1.60X10^{10}$–$1.71X10^{11}$ CFU.mL^{-1}. While the results of *Streptococci* are were in a range of $7.0X10^{9}$ – $2.28X10^{13}$ CFU.mL^{-1} . The results could be attributed to poor pre-milking hygiene practices and other managerial support (infrastructure and technical) that still need to be improved on these farms to reduce microbial load in the raw milk that is produced. The high load counts thus suggest that intensive training and hygiene awareness need to be implemented on the farms that were surveyed.

Key words: raw milk, hygiene practices, milk safety

3.2 Introduction

Milk is an opaque white fluid that is rich in fats and proteins and it is secreted by female mammals for the nourishment of their offspring. Milk and its derived products are part of the diet of many humans and is a source of daily nutrients. However, it is also prone to rapid microbial growth (Elwood *et al.,* 2008; Asaminew and Eyassu*,* 2011; Mohamed *et al.,* 2017). The nutrients that are found in raw milk create an environment that favours microbial growth, and this therefore necessitates the need to ensure milk safety during all stages of milk production (Mokoena, 2013). As a major source of nutrients in the diet of many humans, good milk quality is vital for the health and well-being of consumers. However, milk producers and traders in developing countries, especially those from low-income groups, may inadvertently not adhere to the safe keeping of milk or may not be aware that milk may contain microorganisms that may be harmful to the health of humans, more especially the health of immune-compromised consumers, children, and the elderly (Lues *et al.,* 2003; Melini *et al.,* 2017).

It is common knowledge that milk naturally contains some bacteria and somatic cells that all play a vital role as milk biological components; however, these can be altered by factors such as production conditions, health status of the bovine, and the hygiene practices of employees during production. Inappropriate storage and transportation of milk may also compromise its quality and may promote bacterial growth (Lues *et al.,* 2010). Martins *et al.* (2006) and De Silva *et al.* (2016) highlight that the conditions of keeping and transporting milk in cold-maintained tanks can modify the raw milk microbiota from Gram-positive to Gram-negative with the concomitant increase of Gram-negative

microbes accounting for almost 90% of all microorganisms isolated in raw cold-stored milk.

Microbiota that may cause foodborne illnesses (especially in the young, immune compromised persons and the elderly) include *Listeria monocytogenes*, *Salmonella*, *Campylobacter*, *Staphylococcus aureus*, *Bacillus. cereus, Clostridium botulinum* and coliforms, especially *Escherichia coli* (Maldaner *et al.*, 2012). However, other microorganisms are beneficial when consumed by humans, such as *Lactococcus* and *Lactobacillus* or fungal organisms that are used for fermentation processes, while microorganisms such as *Pseudomonas* can cause the spoilage of food (Quigley *et al.,* 2013; Mohamed *et al.,* 2017). Some of the above-mentioned bacteria are commonly associated with faecal contamination, inadequate cleaning of the milking parlour, and inadequate personal hygiene of the person/s handling the milk and the cows (Lues *et al.,* 2010).

The deficiencies of milk-derived products have been linked to poor microbial quality of raw milk and the heat resistant enzymes found in milk. Hence the quality of yielded milk should be prioritised to ensure that derived products are of good quality and that the health of the public is protected (Murphy *et al.,* 2016). Nwankwo *et al.* (2015) argue that good quality milk that is free from harmful microbiota is generally difficult to achieve in developing countries because of budgetary constraints, a poor infrastructure, and inadequate storage facilities after milking and during transportation. It was also found that the use of untreated water and poor hygiene and sanitation contribute to the unacceptable

quality of milk that is produced by small-scale farmers. It is for this reason that Oliver[b] *et al.* (2005), Davis (2014) and Setlhare (2016) emphasise that research should focus on raw milk because many disease outbreaks have been attributed to untreated raw milk that is immediately used by consumers. Furthermore, Omore *et al.* (2000) and Zeinhom and Abdel-Latef (2014) suggest that isolated pathogenic microorganisms are associated with public health risks.

3.3 Materials and Methods

3.3.1 Sampling site

The study was conducted by procuring raw milk samples from eight selected farms in the vicinity of the Maluti-a-Phofung Local Municipality in the eastern Free State Province of South Africa (see Figure 3.1).

The farms that were selected were not large commercial farms but small-scale farms owned and managed by upcoming farmers in the eastern Free State. The cows were milked by electric milking machines and later hands were used to sufficiently milk the cows. The milk was stored in an automated milk tank with a cooler directly after milking and was transported to a retailer by bulk tanks that collected the milk every day from the farms. The bulk tanks were refrigerated (-4^{0}C).

3.3.2 Study design and sample collection

Representative samples (eight bottles of milk, one per farm) were collected aseptically from the eight selected farms. These samples were collected from bulk tanks using 50 ml sterile bottles. The samples were subsequently transported to the laboratory for analysis

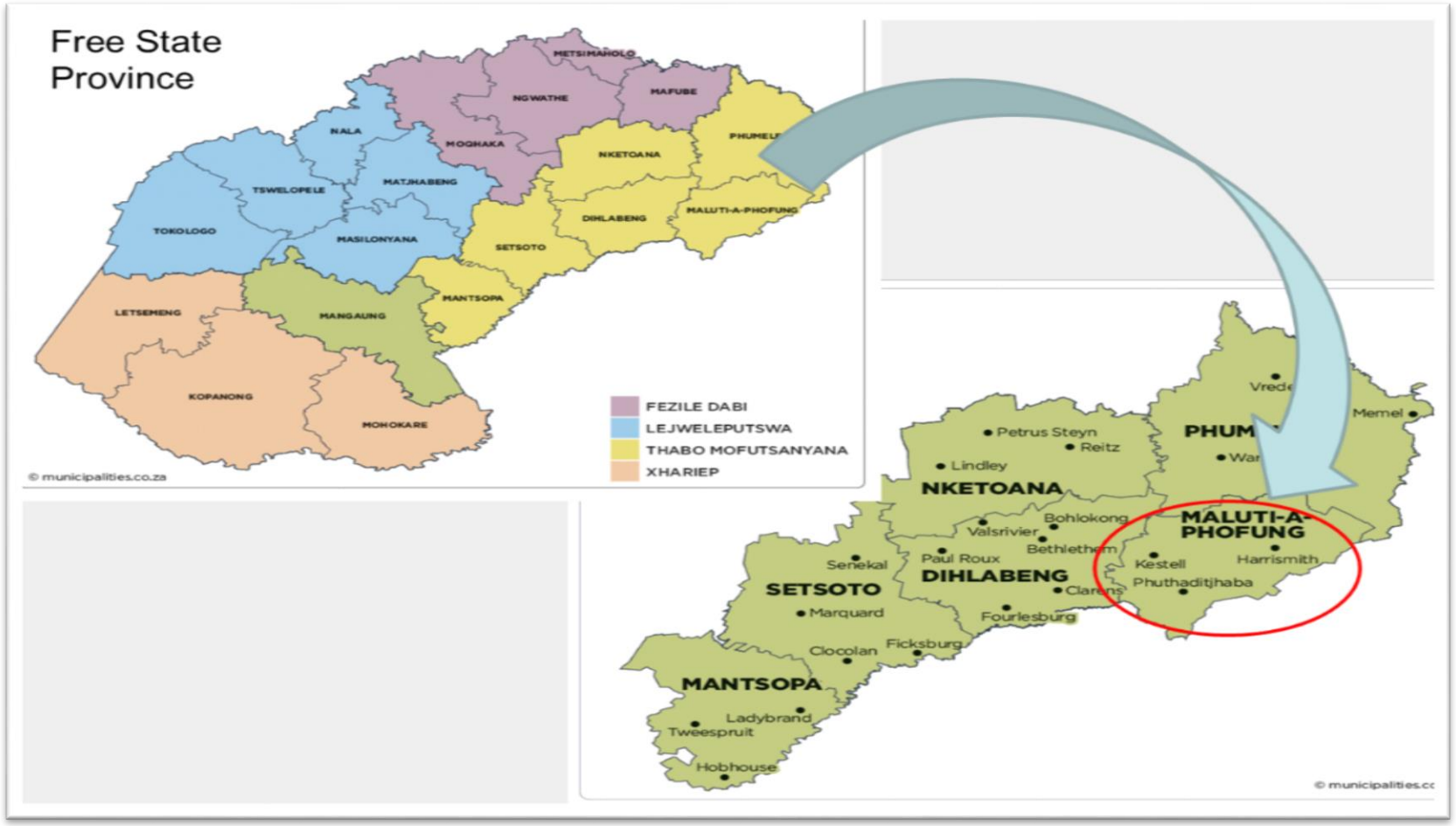
and enumeration in a period of under six hours using a cooler box maintained at 6ºC or lower. Microbiological analyses of the raw milk were conducted to determine the prevalence of microbial pathogens in the raw milk samples.

3.3.3 Microbiological identification of pathogens

For the purpose of this study, both selective and general-purpose media were used. Serial dilutions were prepared with the use of a nutrient broth solution (Merck, SA). The surface spread method (0.1 ml) was applied to quantify the various microbial groups. For the enumeration of members of the family *Enterobacteriaceae*, violet red bile glucose agar (VRBG, Oxoid, SA) was used and incubated at 35ºC for 24 hours. Plate count agar (PCA, Merck, SA) was used to isolate and check the total viable count (TVC) of the raw milk. Lastly, for the enumeration and identification of the family *Streptococci*, Slanetz and Bartley agar was used (ThermoFisher, SA). Further identification of the microorganisms to species level was done by using the RapID identification tool according to the manufacturer's instruction (Oxoid, Thermofisher, Wade Road, Basingstoke, Hants, RG24 8PW, UK.) as described below.

Sufficient culture obtained after 18-24 hours was re-suspended into a 2 ml inoculation fluid. The back lid of the system was then peeled in order to inoculate the contents from the inoculation tube. Subsequently, the entire quantity of the inoculation fluid was transferred to the panel. The system was then inverted, rotated and placed at an angle of 45ºC before it was incubated for 4 hours at 35ºC. The colour reactions were read after four hours (Figure 3.2) as part of the initial microcode and the reagents were added as

indicated and the reaction colours read again for a second part of the microcode prior to using ERIC software.



Source:municipalities.co.za/map/1051/maluti-a-phofung-local-municipality

Figure 3.1: A map showing the eastern part of the Free State Province where the study was conducted.

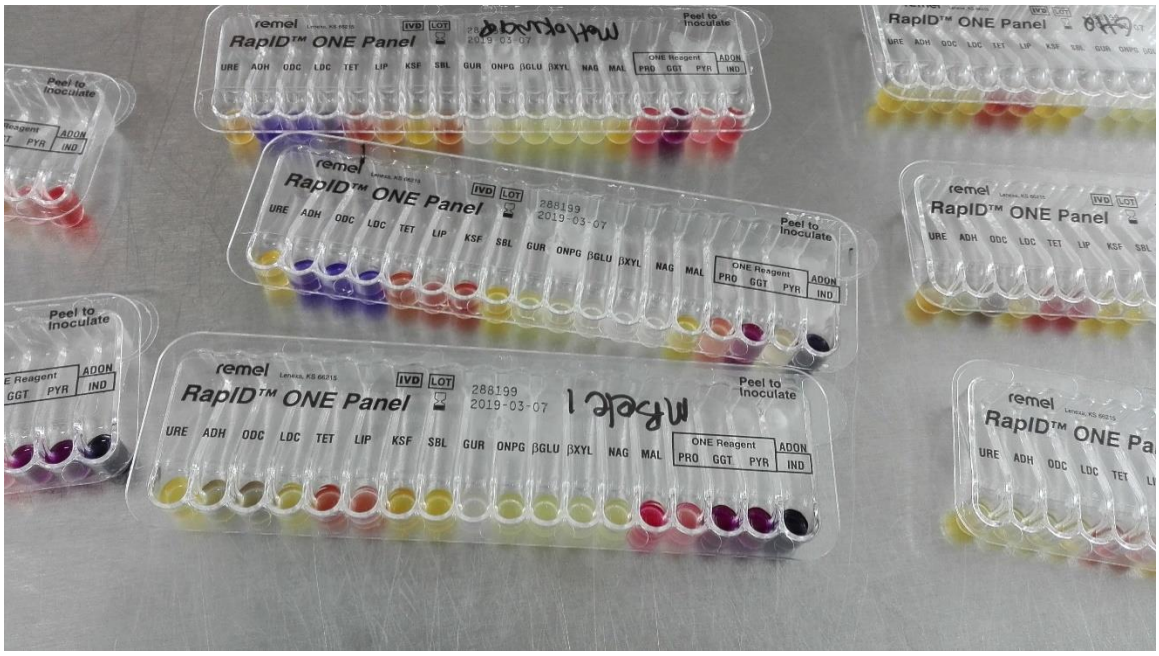


Figure 3.2: RapID panels of the different microorganisms that were identified.

3.4 Results and Discussion

The analyses were done in order to determine whether the sampled raw milk contained microbial hazards. The counts were compared to South African regulations relating to milk and dairy products, R1555 of November 1997 (South Africa, DoH, 1997). Figure 3.3 and Figure 3.4 illustrate that *Enterobacteriaceae* were present in a range of $1.40X10^{6}$ – $3.77X10^{10}$ CFU.ml^{-1}, while the TVC ranged between $1.60X10^{10}$ – $1.71X10^{11}$ CFU.ml^{-1}. The results for the *Streptococci* species (Figure 3.5) were in the range of $7.0X10^{9}$ – $2.28X10^{13}$ CFU.ml^{-1}. The *Streptococci* counts were determined for the purpose of investigating the health and safety risks associated with the raw milk samples, while the TVCs and *Enterobacteriaceae* counts were determined as hygiene indicators.

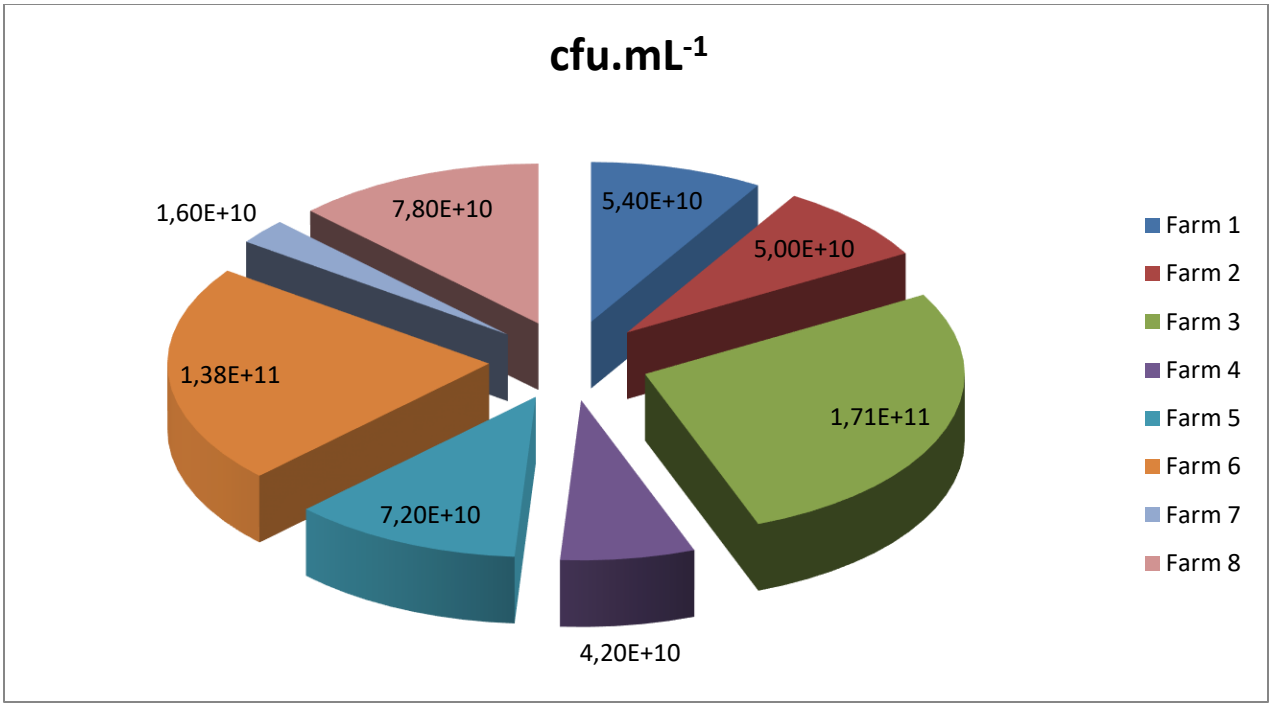


Figure 3.3: Microbial enumeration of the TVCs of raw milk sampled from selected farms

The presence of high loads of TVCs ($1.60X10^{10}$ – $1.71X10^{11}$ CFU.ml^{-1}) as indicated in Figure 3.3 above suggests poor hygiene levels on the farms that were possibly caused by poor milking practices by the milk handlers. The results thus clearly indicate that the raw milk did not comply with the required standards for milk and this suggests that dairy products derived from this milk could have been compromised. A study by Titouche *et al.* (2016), who investigated the hygienic and sanitary quality of raw milk throughout the production chain on selected farms, obtained more or less the same results: 6.73±0.25 log10 CFU ml^{-1} for total bacterial count while samples from the storage tanks and local market were 6.81±0.19 and 7.2±1.05 log10 CFU ml^{-1} respectively. These high bacterial loads in the raw milk samples were indicative of unhygienic conditions during milking and the storage of the milk. Bofoh *et al.* (2006) suggest that a high bacterial load in raw milk

may be an indication that the cleaning of containers for storing milk was inadequate. The latter researchers obtained a total viable count of 4.1 log10 CFU ml^{-1} after the containers had been cleaned.

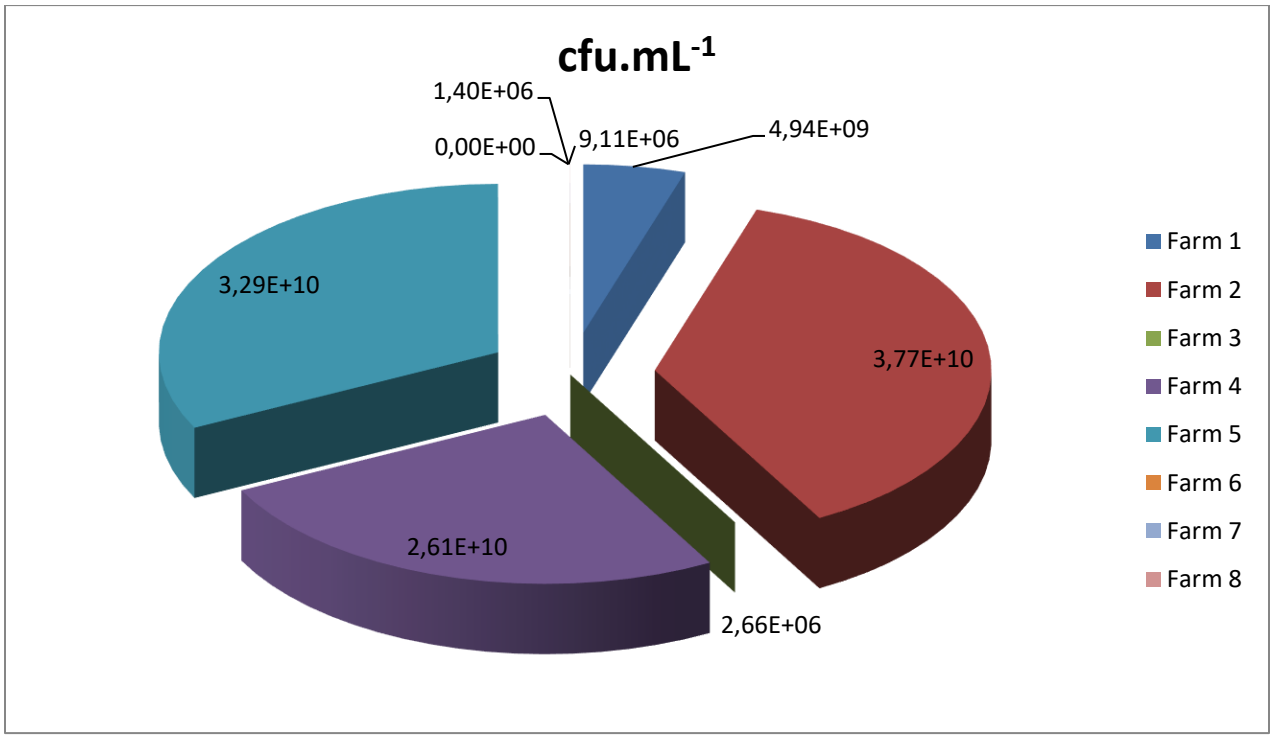


Figure 3.4: Microbial enumeration of *Enterobacteriaceae* detected in the raw milk samples collected from the selected farms

As previously stated, the *Enterobacteriaceae* counts were in a range of $1.40x10^{6}$-$3.77x10^{10}$ CFU ml^{-1}. The prevalence of the identified species was as follows: 25% *Pantoea agglomerans;* 18.75% *Enterobacter sakazakii;* and 12.5% of each of *Escherichia coli* and *Enterobacter cloacae*. Additionally, *Klebsiella oxytoca*, *Yersinia enterocolitica* and *Shigella spp.* each had a prevalence of 6.25%.

These results were similar to those obtained by Junaidu *et al.* (2011), who found that the prevalence of *Enterobacteriaceae* was as follows: *E. coli* (9.78%), *Klebsiella* spp. (4.35%), *Proteus* spp. (8.69%), and *Enterobacter* spp. (1.09%). Salman and Hamad (2011) also found that raw milk sampled from farms in Sudan contained *E. coli* (32%), *Enterobacter* spp. (29.2%), *Klebsiella* spp. (19.4%), *Serratia* spp. (11.1%), and *Citrobacter* spp. (1.0%).

Initially, *Y. enterolitica* was observed in raw pork products such as tongue, chops and ham, but it has more recently been found to be associated with, inter alia, raw milk, pasteurized milk and untreated water (Bernardino-Varo *et al.,* 2013). This organism has been observed to withstand refrigeration temperatures and its presence thus poses a public health threat (Trjkovic-Pavlovic *et al.,* 2007). Recent studies have investigated the prevalence of *Y. enterolitica* and have concluded that its prevalence is mostly associated with the season of the year, location, the size of the stable, and hygiene practices within the dairy plant/parlour (Nesbakken *et al.,* 2006; Poljak *et al.,* 2010).

Enterobacter sakazakii was also isolated from raw milk in the current study, which is not a common finding. It may be surmised that its presence in the raw milk samples could be attributed to external contamination that might have occurred at any point in the milk production process. Hochel *et al.* (2012) also reported the prevalence of *Cronobacter* spp. from 53 of 399 samples in food products, including milk. More than half (53%) of those samples represented species of *E. sakazakii*. This organism was also observed by Fand

et al. (2012) and they discovered that it was resistant to osmotic pressure, extreme temperatures, and drying.

E. coli is one of the most virulent causative agents of gastrointestinal infections and it has mostly been isolated in humans and the food they consume (Zeinhom and Abdel-Latef, 2014). The same authors further investigated public health risks associated with milk-borne pathogens, and they found that a total of 16% of hand swabs had a presumptive *E. coli* presence on milk handlers' hands. They argued that this suggested that high counts of *E. coli* in raw milk might be due to the poor personal hygiene of milk handlers. The latter study also found a prevalence of 16.7% for the *E. coli* serogroup at 0:148 and 83.3% for an uncharacterised group of *E. coli*, thus highlighting the importance of personal hygiene in the milking parlour.

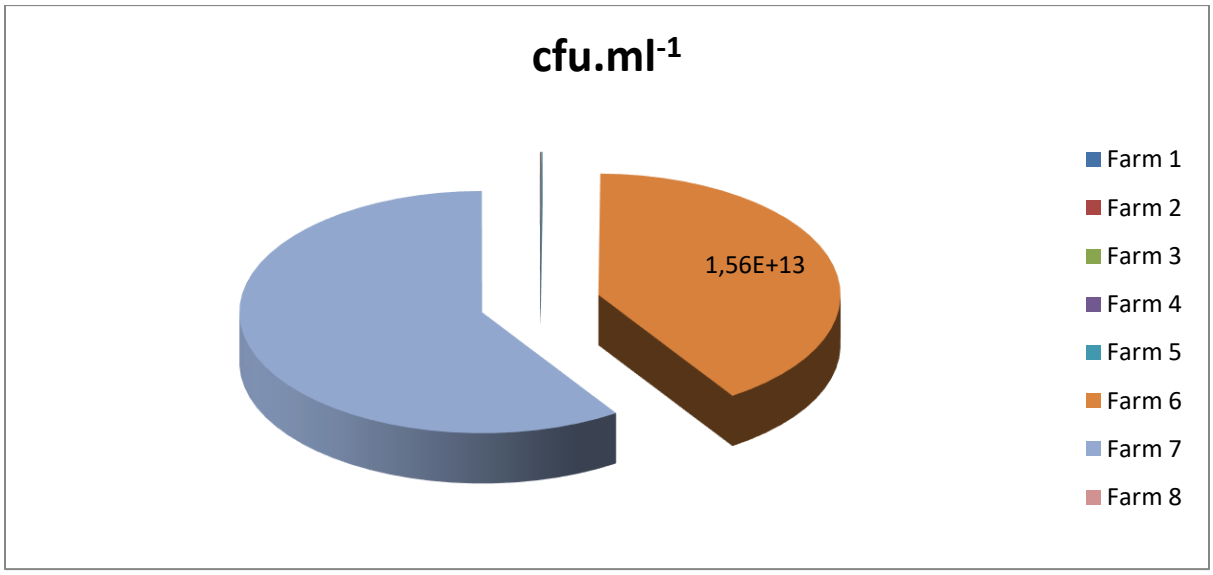


Figure 3.5: Microbial enumeration of *Streptococcus* detected in the raw milk samples collected from the selected farms

The presence of streptococci in raw milk might be due to environmental factors such as contaminated bedding of the livestock and the unhygienic practices of workers. High counts of *Streptococci* in raw milk were also recorded by Seham *et al.* (2016), who observed mean values of $4.5x10^3 \pm 0.7x10^3$ in raw milk. The latter researchers also recorded high counts from derived products such as cheese and yogurt as they obtained mean values of $5.7x10^3 \pm 1.6x10^3$ CFU/ml^{-1} and $7.6x10^4 \pm 0{,}59x10^4$ CFU/ml^{-1} to $5.5x10^3 \pm 0{,}64x10^3$ CFU/ml^{-1} respectively. The *Streptococcus* species has also been found to be responsible for aggressive neonate infections in both human adults and children (Schuchat, 2001).

When measured against the regulations for milk and dairy products (South Africa, DoH of 2001), the bacterial counts obtained in this study did not comply with the set standards. None of the farms complied with the set limits for total viable counts and pathogenic bacteria (this study focused on *Streptococci*) because they exceeded the set limits of < 2x105 cfu.ml^{-1} for TVC and 0 cfu/ml^{-1} for pathogens, while the two farms that had *E.coli* also did not comply with the set standards for *E.coli* (0 cfu.ml^{-1}). The high counts that were obtained may have been due to various factors such as environmental factors (seasonal change, temperature, humidity), poor management, inappropriate hygiene practices in the milking parlours, and poor hygiene practices by the workers. Factors impacting the cows such as infection (mastitis) or other conditions that affect bacterial counts in raw milk may also have contributed to the high counts (Lues *et al.,* 2010). Tassew and Seifu

(2011) suggest that high counts of coliforms may be due to the condition of the containers used for storing milk as well as the milking environment.

All these factors indicate the need to conduct training of farmers and their workers on proper hygiene practices within a milking environment. According to the regulations that guide the production of milk and dairy products, there should not be any *E. coli* present in milk, not only because the presence of *E. coli* in milk is indicative of faecal contamination, but also because it poses health risks to consumers if the milk is consumed without having been treated or pasteurized.

3.5 Conclusion

The present study found that the quality of raw milk that came from some of the eight farms in the Harrismith area in the Free State Province was of poor quality and potentially posed a health threat to consumers. However, some of the farmers complied with the regulations relating to milk and dairy products. A limitation was that the study did not evaluate the knowledge, attitude and behaviour of farmers and their workers with regards to food safety and foodborne illnesses, but it only assessed the quality of the raw milk. The poor microbiological quality of the raw milk could most likely be attributed to limited knowledge, a careless attitude (possibly due to a lack of information and training) and inappropriate milk production and storage practices. The use of unclean storage tanks and environmental factors on the farms, particularly inside the milking parlours, were most probably causative factors that exacerbated the microbial contamination of the milk. For

example, it was observed that all the workers who milked the cows cleaned the udders of cows using their bare hands or cloths that were not washed or sterilised after each cleaning application. Both unwashed hands and untreated cloths are associated with high microbial contamination of milk and improved chances of cows contracting contagious mastitis. It was also observed that aluminium electrified cooling equipment was used for storage to prevent microbial growth in the milk. However, on some farms the cooling tanks were switched off for some time after milking, which means that the appropriate cold storage temperature was not maintained.

The indication that there were *E.coli* and other *Enterobacteriaceae* in all the samples that were collected signifies that personal hygiene was practised inadequately, which was an indication of the possible presence of other pathogens in the milk. Good storage practices of milk is a vital requirement as the bacteria that generally impact raw milk are able to survive despite refrigeration temperatures. Raw milk that is produced by small-scale farmers is generally sold directly to the community and is consumed without having been exposed to appropriate treatment regimens; therefore, the continuous training of these farmers and their workers is paramount to create awareness and knowledge of the risks associated with the production and consumption of untreated raw milk.

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CHAPTER FOUR

DETECTION OF SUBCLINICAL MASTITIS-CAUSING AGENTS IN THE CATTLE OF SMALL-SCALE FARMERS IN THE VICINITY OF HARRISMITH IN THE FREE STATE USING CULTURE AND MULTIPLEX PCR

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4.1 Abstract

Subclinical mastitis infection (SCM) represents a huge burden of mastitis in cows because there are no visible changes in milk or udder appearance, thus making it difficult to detect infection. The aim of this phase of the study was to detect subclinical mastitis-causing pathogens in the cattle of small-scale farmers using somatic cell count (SCC), the California mastitis test (CMT), culturing techniques, and multiplex PCR (mPCR) to characterise and detect five common mastitis-causing agents. A total of 32 milk samples were collected from selected cows of small-scale farmers in the vicinity of Harrismith in the Maluti-a-Phofung Local Municipality, Free State Province, South Africa. The results showed that *S. aureus* (93%) was the most prevalent pathogen, followed by *Streptococci* spp. and *E. coli* at 36.4% and 13.3% respectively. The multiplex PCR (mPCR) test could detect only *E.coli* as the most dominant of the detected species. This study thus confirmed the presence of SCM-causing pathogens in raw milk collected from the cows of small-scale farmers in the Harrismith area, and it urges that large-scale epidemiological studies of SCM be conducted in the area.

Key words: Sub-clinical mastitis, somatic cells, bacteria.

4.2 Introduction

Mastitis is a disease in cows that is characterised by inflammation of the teats. The disease is usually observed by changes in the teat pathology as well as through physical and chemical changes that manifest in raw milk (Shome et al., 2011). Mastitis in bovines has been regarded as a major economic drain in the dairy sector worldwide (Man'ombe, 2014; Gitau *et al.,* 2014; Joanna *et al.,* 2013). Furthermore, the economic burden of this infection manifests in factors such as low milk production during pre- and post-infection, the need to administer medicinal agents, low fertility rates, and the onset of the culling of bovines (Erskine *et al.,* 2003; Abebe *et al.,* 2016). This infection also affects the vital nutrients in milk which leads to reduced nutrient quantities (Girma, 2001; Shitandi and Kihumbu, 2004; dos Reis *et al.,* 2013).

Mastitis can be classified as clinical mastitis or subclinical mastitis, with the former being observed when the inflammatory response is robust and causes visible modifications in the milk (e.g., clots and colour changes), a swollen udder, and symptoms of ill health displayed by the cow (e.g., off-feed, dehydration) (Oliveira *et al.,* 2015; Mpatswenumugabo *et al.,* 2017). Subclinical mastitis on the other hand is characterised by asymptomatic characteristics, which means that there is a need to screen bovines for infection by means of somatic cell counts (Tiwari *et al.,* 2013). The sudden onset of this infection in bovines is due to bacterial, mycotic, algal and, in some instances, viral species attacking the tissue surrounding the udder, which results in the inflammation of the mammary glands (Motaung *et al.,* 2017). Sharma *et al*. (2012) explain that factors such

as inadequate sanitation of the milking shed, poor animal health services, and lack of attention to the health of mammary glands play a role in the development and duration of this infection. So far, about 135 microbial strains have been identified as causal agents of mastitis in bovines, with *Streptococcus* spp. and *Staphylococcus* spp. being the most prevalent (Lim *et al.,* 2007). Over and above the species already mentioned, it has been observed that *Escherichia. coli Mycoplasma bovis* and *Klebsiella pneumonae* also cause mastitis in bovines (Tiwari *et al.,* 2013). This means that milk producers could possibly be linked to outbreaks of diseases relating to the consumption of raw milk that is contaminated with *Staphylococcus aureus* and *Escherichia coli*. It further suggests that raw milk production and consumption pose food safety hazards for the unsuspecting public (Little *et al.,* 2008).

In the previous century, the identification of mastitis-causing pathogens relied on conventional methods that were time-consuming, as a period of at least 48 hours was needed to make a diagnosis, and this prolonged the administration of treatment (Paraguison-Alili *et al.,* 2014). The use of traditional or conventional methods for the identification and diagnosis of mastitis-causing agents was based on morphology due to serotyping, biochemical testing, and enzyme activities. However, these methods could lead to a negative culture if an antibiotic had been administered to the cow during a pre-sampling protocol (Phuektes *et al.,* 2001). The identification and diagnosis of mastitis thus relied on factors such as specificity, sensitivity and the cost of the techniques that were employed. Therefore, to bypass the difficulties related to conventional methods for diagnosis and identification, DNA-based techniques are currently utilised to focus on the

DNA composition of microorganisms instead of the colony phenotypic expression (Hegde, 2011).

The early identification of mastitis-causing pathogens ensures the application of appropriate treatments, allows producers to devise rapid solutions, and provides farmers with the opportunity to promptly heal an ill bovine and return her back to the producing line (Paraguison-Alili *et al.,* 2014).

However, even though molecular methods have been found to be quite efficient and reliable, there is still a need for rapid and accurate molecular identification of mastitis-causing agents; hence the introduction of multiplex PCR (mPCR) (Cremonesi *et al.,* 2009; Zadoks and Watts, 2009). The present study thus set out to detect pathogens that cause SCM in the herds of small-scale farmers. Somatic cell count (SCC), the California mastitis test (CMT), and mPCR were employed for the identification of five common mastitis-causing agents as described by Hegde (2011) and Sarvesha *et al.* (2017).

4.3 Materials and Methods

4.3.1 Sample collection

This study was conducted on eight selected small-scale farms in the vicinity of Harrismith in the Maluti-a-Phofung Local Municipality that is situated in the eastern region of the Free State Province, South Africa. After the farmers had been approached and agreed that

their farms could be included in the study, samples were collected in the period November 2017 to January 2018.

Before taking the samples, the researcher observed the milking process on each farm. Machines were used for milking on the farms and it was noted that the workers washed the teats using clean water and their bare hands. Thereafter, the teats were dried with a cloth in some cases. It was also noted that no handwashing was performed between milking of individual cows and that the cloths that were used were not cleaned or sterilised between cleaning of the teats of the selected cows.

Before collecting, the samples 4 cows per farm were screened for subclinical mastitis using CMT on a farm. Then later, 32 milk samples were subsequently collected from the quarters of all the selected milking cows on all the selected farms (4 samples per farm). The samples were directly collected from asymptomatic teats (i.e., there were no visible indications of mastitis on the teats) using 50 ml sterile bottles. The samples were then transported in a cooler box with ice packs to the laboratory for analyses that were conducted within 6-8 hours after collection.

4.3.2 Microbiological analyses

4.3.2.1 Screening of cows using somatic cell counts and the California mastitis test

Somatic Cell Counts

In order to obtain and somatic cell counts, the milk samples were sent to an outsourced laboratory (Swift Silliker (Pty) Ltd t/a Mérieux NutriSciences, Midrand, South Africa).

The California mastitis test

A California Mastitis Kit (DeLaval, South Africa) was used to assess whether selected individual cows had intramammary infections and thus to determine subclinical mastitis. The functioning of this mastitis kit is based on the condition that the raw milk is mixed with its reagent and this will cause the somatic cell in the milk to break. The DNA in these somatic cells will then coagulate and form a slimy, viscous liquid. The California Mastitis Kit was used according to the manufacturer's instructions (DeLaval, South Africa) in the following manner: The first two to three streams of the foremilk were discarded; thereafter, another two to three streams of raw milk were collected directly from the individual teats and were dispensed to each well of the kit. The paddle was held vertically until all excess milk in the well had been poured in order to visualise the lining in the well. Subsequently, an equal volume of reagent (3 ml) was added to each of the wells containing the milk and the paddle was gently swirled for about 10 seconds. Thereafter, the consistency and viscosity of the gel reaction were recorded. Positive results based on the viscosity of the milk indicated high somatic cell counts. These were classified (or categorised) as

numbers from 1-3, while 0 indicated negative results, meaning there was no jelly-like appearance in the wells**.**

4.3.2.2 Isolation of Streptococcus species, Escherichia coli, and Staphylococcus species

Subsequent to analysing the milk samples for SCC, all samples were further analysed to isolate and characterise disease-causing pathogens. For the isolation of different mastitis-causing pathogens, 0.1 ml of the milk sample was initially enriched in 9 ml Nutrient Broth. This was done to minimise the number of pathogenic cells within the samples in order to obtain colonies between 30-300 counts per ml. The following selective media were used to cultivate different microbial species of interest:

Slanetz and Bartley medium: For the isolation of the *streptococcus* species, 0.1 ml of diluted samples was plated out on Slanetz and Bartley medium petri plates. Thereafter, the plates were inverted and incubated at 35°C for 48 hours. All the colonies that were pink or dark red with a narrow, whitish border were enumerated (Oxoid, ThermoFisher, UK).

Violet red bile agar: For the isolation of *Escherichia coli*, 0.1 ml of diluted samples was spread on violet red bile agar plates. This process was followed by incubating the plates at 35ºC for 24-48 hours. All the colonies that were pink to reddish in colour were presumed to be *E. coli* (Oxoid, ThermoFisher, UK).

Baired-Parker agar: For the isolation of *Staphylococcus* species, 0.1 ml of diluted samples was spread on Baird-Parker agar plates and supplemented with egg yolk and 3.5% potassium tellurite solution. The plates were then incubated at 35ºC for a period of 24 hours. Grey-black shiny and convex colonies ranging to entire narrow white colonies with margins surrounded by a zone of clearing were presumed to be *Staphylococcus aureus* (Merck, SA).

Thereafter, all the isolated colonies were subjected to Gram staining and catalase testing prior to the use of the RapID identification kit and the staphylase test (Oxoid, Thermofisher, Wade Road, Basingstoke, Hants, RG24 8PW, UK) for final confirmation of *E. coli*, *Streptococcus* spp., and *Staphylococcus aureus.* The manufacturers' instructions were followed rigorously.

4.3.3 Molecular characterisation

4.3.3.1 Extraction of genomic DNA

The QIAamp DNA mini kit was used for deoxyribonucleic acid extractions according to the manufacturer's instructions (QIAGEN, Hilden, Germany). Genomic DNA (gDNA) was extracted from the pellets of centrifuged milk samples. Thereafter, the pellets were resuspended in 200 µl of phosphate buffered saline (PBS) and 20 µl of proteinase K was then added to the mixture. Subsequently, 200 µl of Buffer AL was added to the mixture, vortexed thoroughly, and incubated at 56ºC for 10 minutes. After incubating the mixture, 200 µl of 96-100% ethanol was added and the mixture was vortexed thoroughly and pipetted into the QIAamp mini spin column, placed in 2 ml collection tubes, and then

centrifuged at 8000 rpm for 1 minute. Thereafter, the flow-through and collection tubes were discarded. The spin column was then placed in a new 2 ml collection tube and 500 µl of Buffer AW1 was added and centrifuged at 8000 rpm for 1 minute. After centrifugation, the flow-through and collection tube were again discarded and the spin column was again placed in a new 2 ml collection tube and 500 µl of Buffer AW2 was added into the spin column, followed by centrifugation at 14000 rpm for 3 minutes. The flow-through and collection tubes were discarded once again. Thereafter, the spin columns were transferred to a new 2 ml micro centrifuge tube and 200 µl of Buffer AE was added to elute the DNA in the spin column and incubated for 1 minute at room temperature. Subsequently, they were again centrifuged for 1 minute at 8000 rpm. The yielded DNA was determined using NanoDrop TM Spectrometer (Thermofisher, Wade Road, Basingstoke, Hants, RG24 8PW, UK).

All the equipment that was used was meticulously cleaned and sterilized before use to avoid cross-contamination.

4.3.3.2 Primer selection

The primers that were used for the amplification of different pathogens by means of mPCR were published by Hegde (2011) and were synthesised at Inqaba Biotechnical company (Pretoria, South Africa) as outlined in Table 4.1 below.

Table 4.1: Species-specific primers of targeted genes and their product sizes

Gene	Primer name	Sequence	Targeted species	Product size
16S rRNA	Sdys F	GGA GTG GAA AAT CCA CCA T	*S. dysgalactiae*	549
	Sdys R	CGG TCA GGA GGA TGT CAA GAC		
sip	Strep sip I-F	ACTATTGACATCGACAATGGCAGC	*S. agalactiae*	266
	Strep sip I-R	GTTACTGTCAGTGTTGTCTCAGGA		
pau	Strep pau I-F	TGCTACTCAACCATCAAAGGTTGC	*S. uberis*	439
	Strep pau I-R	TAGCAGTCTCAGTAGGATGAGTA		
nuc	SAU-nuc- I F	GTGCTGGCATATGTATGGCAATTGT	*S. aureus*	181
	SAU-nuc- I R	TACGCCGTTATCTGTTTGTGATGC		
alr	EC-alr-F	CTGGAAGAGGCTAGCCTGGACGAG	*E. coli*	366
	EC-alr-R	AAAATCGCCACCGGTGGAGCGATC		

4.3.3.3 Protocol: standard multiplex PCR (mPCR)

Standard mPCR was conducted using NEB OneTaq 2X MasterMix with Standard Buffer (10 µl). The reaction mix contained gDNA (10-30ng/µl) (1 µl), forward primer (10µM) (1 µl), reverse primer (10µM) (1 µl) and nuclease-free water (Catalogue No. E476) (7 µl). The reaction mix was then mixed thoroughly by pipetting the mixture up and down a few times. Appropriate volumes were then dispensed into the PCR tubes. The PCR tubes were subsequently placed in the thermal cycler for 35 cycles as follows: for the initial activation step, the tubes were subjected to 94°C for 5 min, denaturing occurred for 30 s at 94ºC, annealing occurred for 30 s at 50ºC, and extension occurred for 60 s at 68ºC. The final extension was at 68ºC for 10 min and holding was at 4ºC. Thereafter, the samples were analysed using agarose gel.

4.3.3.4 Agarose gel analysis

The integrity of the PCR amplicons was visualized on a 1% agarose gel (CSL-AG500, Cleaver Scientific [Ltd]) and stained with EZ-vision® Bluelight DNA dye.

4.3.3.5 PCR products clean-up and sequencing

The PCR products were cleaned using the ExoSAP protocol and their sequences were determined using the Applied Biosystems™ BigDye™ Terminator v3.1 Cycle Sequencing Kit (Catalogue No. 4337455) according to the manufacturer's instructions at an outsourced company (Inqaba Biotechnical, Pretoria, South Africa).

4.4. Results and Discussion

This phase of the study was undertaken with the objective of assessing the prevalence of subclinical mastitis and isolating its common causal bacteria; i.e., *S. agalactiae, S. dysgalactiae, S. uberis*, *S. aureus,* and *E. coli.* The intention was to detect these pathogens by application of mPCR and culture methods.

4.4.1 Screening of the milk samples

A total of 32 individual cows from eight small-scale farms in the Maluti-a-Phofung municipality in the Harrismith area were screened for subclinical mastitis using CMT. Raw milk samples were collected from lactating bovines without taking into account their age and the lactation stage. Prior to sample collection, four (4) individual cows from each farm were randomly screened for subclinical mastitis using the California Mastitis Test (CMT). Upon testing positive/negative, these cows' milk was then screened for SCC by a

contracted company. Based on the findings of these tests, the milk samples were classified into four groups; i.e. (i) 0-1.5x10^5 cells.ml^{-1}; (ii) 1-2x10^5 cells.ml^{-1}; (iii) 2-5x10^5cells.ml^{-1}; and (iv) >5x10^5 cells.ml^{-1}. All the samples that manifested somatic cells of 1x10^5-5x10^5 cells.ml^{-1} were regarded as positive and thus as infected.

4.4.1.1 The California mastitis test (CMT)

Subclinical mastitis is an intramammary infection arising from either underlying infections that are not resolved in time or it can result from new infections that arise during dry climatic periods (Dingwell *et al.,* 2003). The presence of subclinical mastitis in lactating cows can also be correlated with the introduction and development of clinical mastitis. The CMT remains the diagnostic tool of choice and is used to detect clinical mastitis on farms globally.

The CMT was used to diagnose the first four cows from the selected eight farms in the Maluti-a-Phofung area. Figure 4.1 below illustrates that, of the 32 cows that were screened, only 21.87% tested positive.

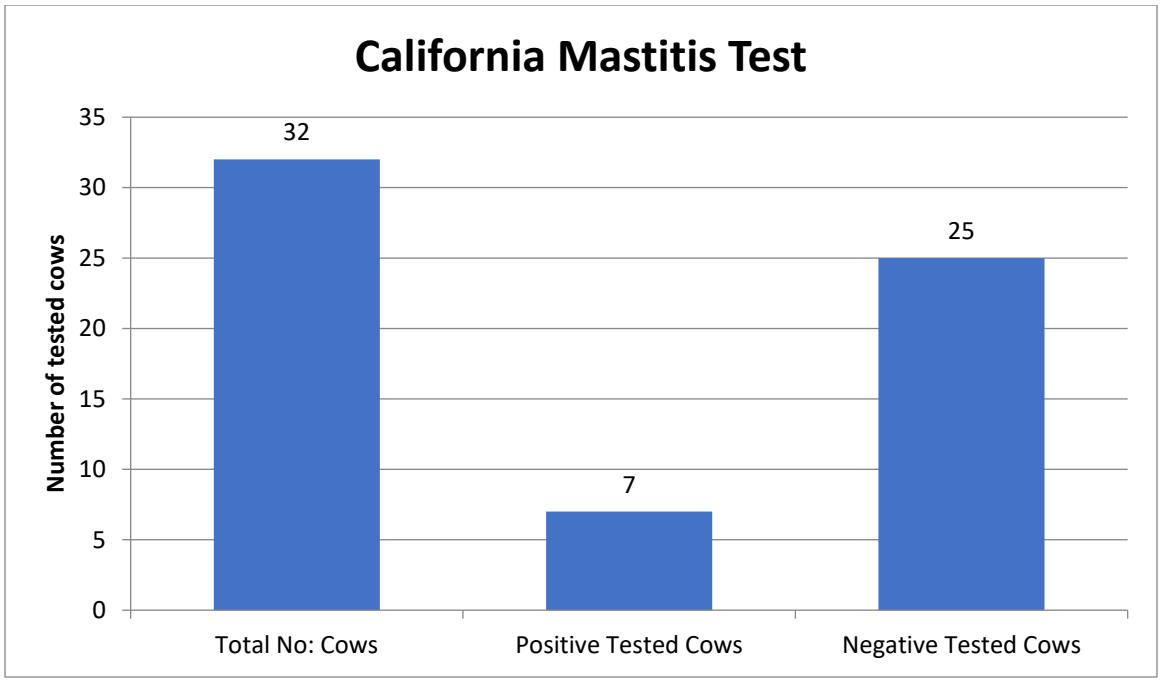

Figure 4.1: Number of cows that tested positive / Number of cows that tested negative

A study by Saidi *et al.* (2013), which evaluated mastitis in bovines in Algeria, found that, of the 107 cows that were tested, only 29.62% tested positive. A study by Godden *et al.* *(*2017), which evaluated automated milk leukocyte using a differential test and CMT for detecting intramammary infections, found that, of the 306 cows that were lactating early and late, only 25.2% and 25.8% were infected on either one or more quarters respectively. According to Birhanu *et al.* (2017), such high percentages of subclinical mastitis could be attributed to potential risks such as age, condition of the udder, milk yield, and parity of the cows. Guha and Guha (2012) emphasise that farmers cannot only rely on the use of CMT to screen mastitis in a dairy herd, but they also need to test the milk *in vitro* for the identification of etiological agents. It was observed that CMT did not provide an adequate test sensitivity for the identification of infected quarters and cows, therefore all lactating cows should be treated as suspects for IMI and routine biosecurity measures should be

taken. Such measures include the use of disposable hand towels or gloves when handling the teat, using buckets when stripping, using disinfected hands when milking cows with low productivity, milking only twice a day, and hand washing after handling teats or milking each cow (Kandeel *et al.,* 2018).

The differences in the results that were cited could be attributed to varying management practices when handling the cows, or they may have been due to the lack of knowledge of the farmers and their employees concerning mastitis and its treatment. However, other factors such as climatic conditions could also have played a role in the results.

4.4.1.2 Somatic cell counts

Somatic cells are an important milk constituent and their condition is a vital indicator of teat health and the quality of the produced milk. To better understand the role of somatic cells in dairy manufacturing processes, we need to consider factors such as the physiochemical changes that occur in milk, bacterial counts, and the health status of the cow (Li *et al.,* 2014). Somatic cell counts are commonly used indicators of subclinical mastitis in bovines as they usually increase during intramammary infections caused by bacteria. Other environmental factors as well as cow-specific factors such as age, stage of lactation, season of the year, stress, and management of the farm also play a role in subclinical mastitis infections (Hegde, 2011). The latter author argues that standards/limits of somatic cell counts differ among countries globally. For example, the European Union regulations and New Zealand, Canada and United States set these standards at $4x10^5$ cells.ml^{-1}, $5x10^5$ cells.ml^{-1} and $7.5x10^5$ cells.ml^{-1} respectively. The

International Dairy Federation (IDF) requires a limit of $5x10^5$ cells.ml^{-1} for SCC which, according to R1555, is what South Africa regards as the standard somatic cell count for milk and milk products (South Africa. Department of Health, 1997).

A number of studies have investigated the correlation between different mastitis diagnostic tests and the number of somatic cells in milk, and they have established different thresholds for diagnosing subclinical mastitis. For the purpose of this study, the three thresholds to diagnose whether the cow or the teat was infected or not were considered: SCC of $1x10^5$ cells.ml^{-1} or less indicated an uninfected cow; SCC of $1x10^5$ cells.ml^{-1} - $2x10^5$ cells.ml^{-1} would indicate that a cow had intramammary infection in at least one or more teats; and SCC of $2x10^5$ cells.ml^{-1} - $5x10^5$ cells.ml^{-1} or greater indicated that the cow was infected significantly and probably had high bacterial counts.

The thresholds referred to above were established according to the National Mastitis Council's guidelines on normal and abnormal raw milk based on SCCs and signs of clinical mastitis (Petzer *et al.,* 2018). The findings (see Figure 4.2 below) revealed that, of the 16 samples that were analysed for SCCs, 10 (62.5%) had SCCs ranging from $1x10^5$ cells.ml^{-1} - $5x10^5$ cells.ml^{-1}; 5 samples (31.25%) had SCCs of more than $5x10^5$ cells.ml^{-1}; and 1 sample (6.25%) had a SCC above the designated thresholds. Moreover, of all the samples, only four (25%) had SCCs ranging from $1x10^5$ to $2x10^5$ cells/ml, therefore it was concluded that the prevalence of subclinical mastitis in the cows of small-scale farmers in the study area was 25%.

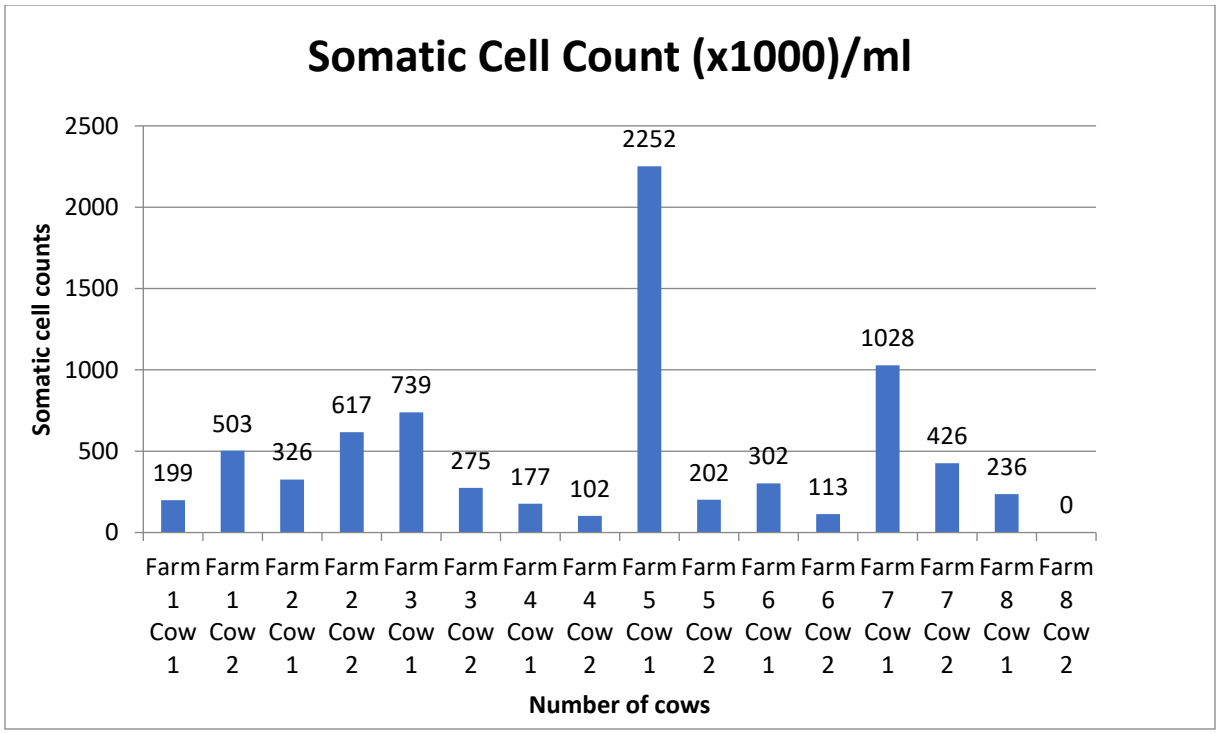

Figure 4.2: Number of cows that were tested and their somatic cell counts

Much higher counts were revealed by Tripura *et al.* (2014), who found that the overall prevalence of subclinical mastitis, regardless of the number of infected teats, was 51.8%. A study by Björk (2013) found an even higher prevalence of clinical mastitis in Kampala as he found that the count was 63% at teat/quarter level with staphylococci being the most predominant organism. An investigation of the prevalence of subclinical mastitis in lactating cows by Sanotharan *et al.* (2016) in Batticaloa District in Sri Lanka, found that the pervasiveness of the infection was as high as 60.7% in all lactating cows. This high percentage of infection was attributed to age, parity and housing systems. It is also alluded by Tilahun and Aylate (2015) that age, parity and housing systems play a role in the prevalence of both subclinical and clinical mastitis. They found that the prevalence of mastitis was 68.0%, with subclinical mastitis accounting for the highest infections in the

bovines of commercial farmers in Addis Ababa. The latter authors also highlight that factors such as breed, age, parity and period of lactation contribute to significant differences in the prevalence of mastitis among bovines. Islam *et al.* (2011) suggest that findings pertaining to the prevalence of subclinical mastitis may differ among areas depending on the diagnostic tool used.

4.4.2 Microbial isolation and characterisation

For the isolation and characterisation of microorganisms in the current study, 16 of the milk samples were subjected to a variety of standard phenotypical and biochemical methods. All the isolates were identified at genus level based on the size, shape and colour of the colony in question by using an Interscience plate counter (78860, Saint Nom, France). The results that were obtained are presented in Figures 4.3, 4.4 and 4.5 below. The tests revealed that there were 40 isolates in total: presumptive *Staphylococci* (14); *E. coli* (15); and *Streptococci* (11). A RapID identification kit and a staphylase test were also used to identify organisms at species level. The results showed that *S. aureus* was the most abundant pathogen at 93%, followed by *Streptococci* spp. at 36.4% and *E. coli* at 14.3%. For the purpose of this study, the identified causal agents were used to define the detected subclinical mastitis as either being contagious, environmental, or coliform related.

The results of the current study were similar to those obtained by Balakrishnan *et al.* (2004) who revealed that, of the 40 bacterial isolates that had been recovered, the most predominant was S. aureus (35%), followed by *E. coli* (27.5%), *S. agalactiae* (17.5%),

and *S. dysgalactiae* (2.5%). Furthermore, Mpatswenumugabo *et al.* (2017) investigated the prevalence of subclinical mastitis in dairy farms in areas in Rwanda and it was found that the overall prevalence at cow level was 50.4%. The same author further identified these isolates at species level and revealed that 51.5% was CoNS, followed by *S. aureus* (20.6%), *Streptococci* spp. (10.3%), *Bacilli* spp. (10.3%), and *E. coli* (1.5%). However, the latter study did not take age, lactating stage or seasonal differences around the study area into consideration. The Food and Agriculture Organisation (1990) and Demme and Shimeles (2015) state that a high prevalence of subclinical mastitis may be associated with poor hygiene practices among the farm workers that may be linked to the transmission of etiological agents from infected to uninfected udders by contaminated hands. In the current study, *S. aureus* was the most prevalent species at 93%, which was an indication of contagious mastitis. This finding is clearly a cause for concern, particularly as Hussein *et al.* (2017) caution that contagious mastitis associated with *S. aureus* is a public health risk with the potential hazard of staphylococcal infection of farm workers or consumers should they consume the milk that is produced from these infected cows.

The organisms that were isolated and identified in the current study are discussed below. The figures that are presented indicate their respective number of colonies.

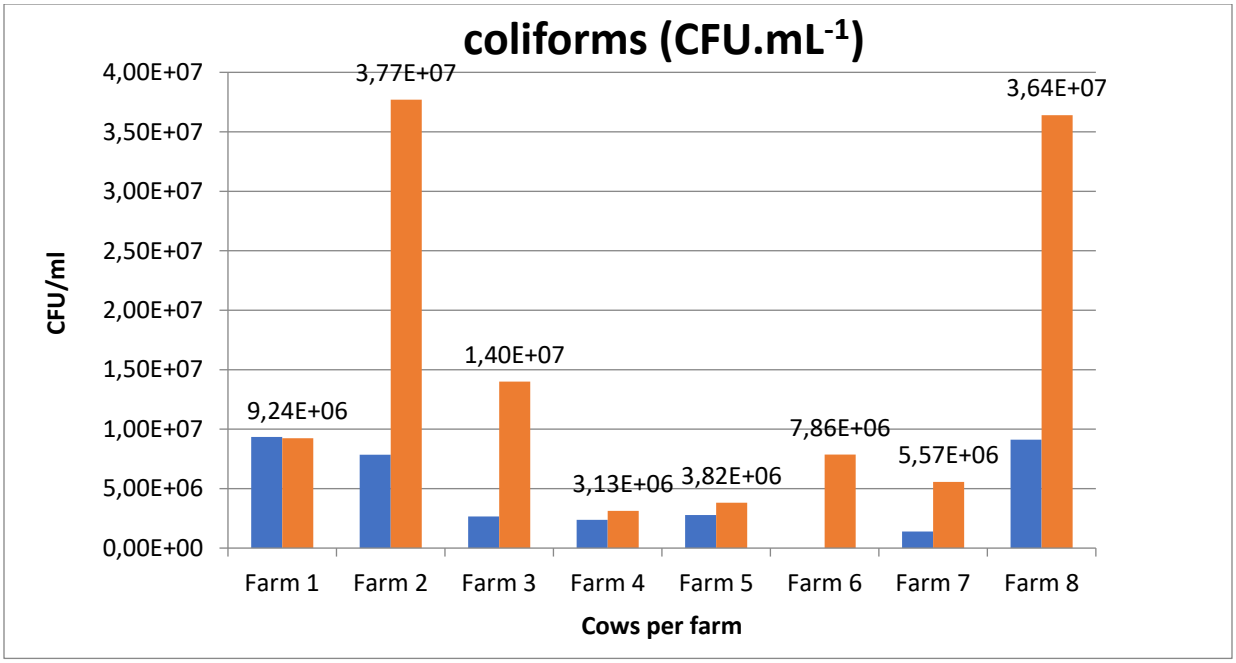

Figure 4.3: Coliform results for individual cows per farm in CFU.ml^{-1}

Bovine mastitis that is caused by a Gram-negative bacteria is classified as coliform mastitis and it may further be classified based on its severity as either clinical or subclinical mastitis (Shome *et al.,* 2011). As a coliform bacterium, *E. coli* is mostly isolated in cases of mastitis. This bacterium is ubiquitous in the environment and is commonly found in the bedding of cattle, their manure, and in water (Hegde, 2011). Mastitis that is caused by *E. coli* is mostly sporadic and mild, but sometimes it may be servere and may even have fatal consequences (Shpigel *et al.,* 2008). *E. coli* has also been found to cause severe damage to the teat (Hogan and Larry-Smith, 2003; Roussel *et al.,* 2017).

The severity of the ability of *E. coli* to cause damage to the teat is based on host susceptibility factors such as health status, lactation period, parity, and genetic make-up of the host (Roussel *et al.,* 2017). Mastitis that is caused by *E. coli* is classified in terms

of the unavailability of virulence genes rather than by their presence (Kempf *et al.,* 2016). Phynotypically, the ability of these bacteria to multiply in milk was observed to be the reason why they colonise the udder. Studies have also indicated that *E. coli* strains that are isolated from mastitis incidences have revealed enhanced adherence to the udder (Dogan *et al.,* 2006; Döpfer *et al.,* 2001).

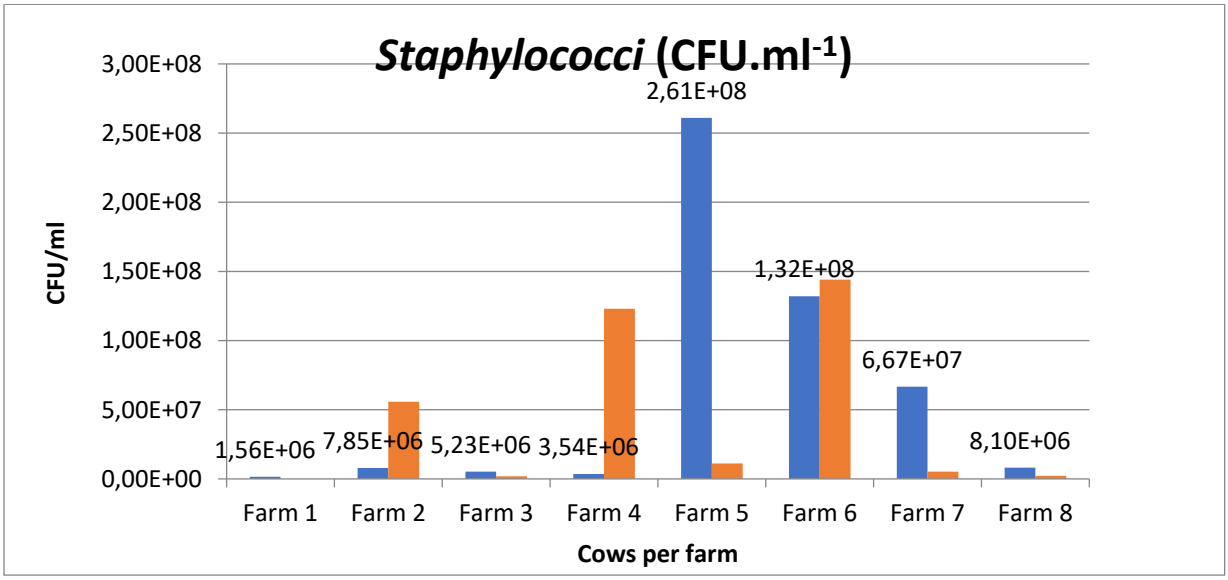

Figure 4.4: *Staphylococci* results per farm in CFU.ml^{-1}

Staphylococcus aureus is a Gram-positive bacterium that, when observed under microscope, appears purple by Gram staining. This bacterium is cocci-shaped and resembles grape-like clusters. It grows aerobically or anaerobically in temperatures between 18°C and 40°C and it can be found in both humans (e.g., on the skin and in the mucous membranes) and on environmental surfaces (Taylor and Unakal, 2017). A study by Schukken *et al.* (2009) has shown that 3% of all mastitic cows are infected with *S. aureus*. Moreover, Tenhagen *et al.* (2009) argue that *S. aureus* is present in only 10% - 12% of all clinically infected cows. Interestingly, both authors who were referred to above

conclude that not all cows that are infected with *S. aureus* generally have increased somatic cell counts. The *S. aureus* abolishes the cell membranes of the teats and directly injures the tissues that produce the milk. Subsequently, the white blood cells become attracted to these damaged tissues in order to counteract the inflammation (Cremonesi, 2012). Thereafter, bacteria will move up through the ducts to form deep-seated pockets within the alveoli. An abscess will start to form in a clinically infected cow to prevent the spread of bacteria; however, for the bacteria to be undetected by the immune system, antibiotics will be prevented from reaching the bacteria (Petersson-Wolfe *et al.,* 2010).

Vlkova *et al.* (2017) argue that *S. aureus* is more consistent when samples are collected more than once from subclinically infected cows. This is why it is important to screen subclinically infected herds for *S. aureus* in order to monitor this pathogen. The reason why this bacterium is consistent and persistent is the ability of *S. aureus* to form biofilms that enhance resistance to antibiotics (Melchior *et al.,* 2006). The incidence of higher isolation for *S. aureus* in mastitis-infected cows has been observed to be more frequent in cows that have an enhanced parity rate; thus *S. aureus* mastitis risk increases with an enhanced parity rate. Cervin-Kova *et al.* (2013) found that that enhanced prevalence of *S. aureus* was only observed at certain farms, and they argue that this suggested that other genetic subpopulations of *S. aureus* could have been present and that mastitis might have been caused by other populations of the *Staphylococcus* family.

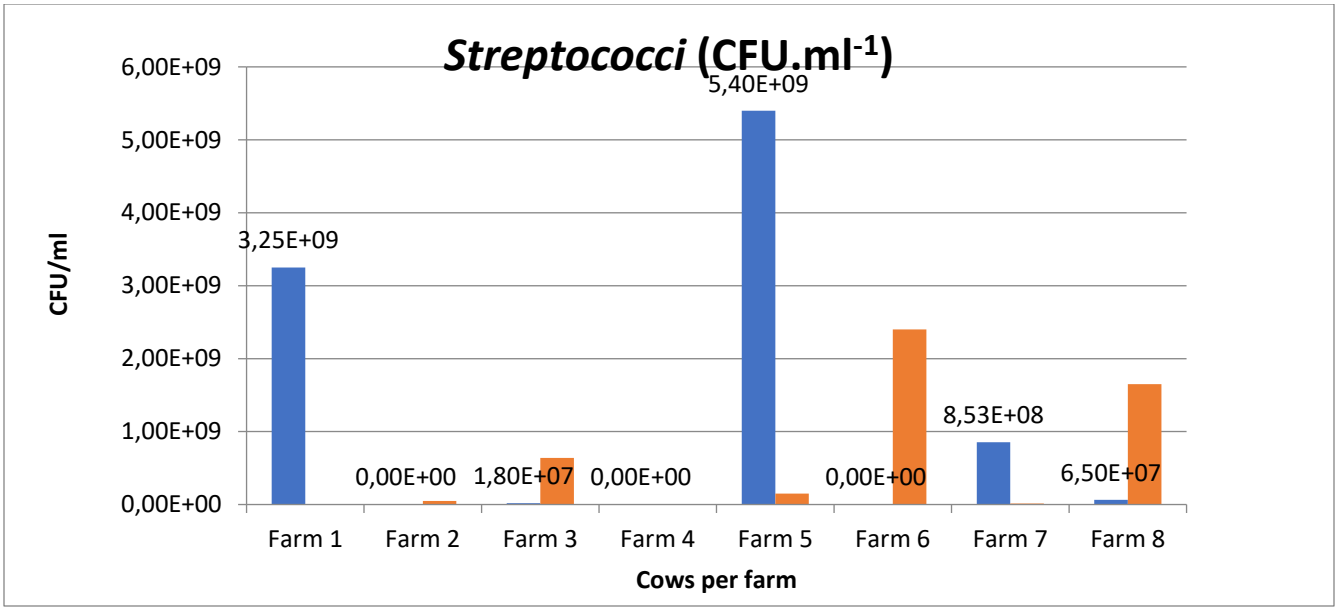

Figure 4.5: *Streptococcus* results for individual cows per farm (CFU.ml^{-1})

The presence of the *Streptococcus* species in raw milk may have been due to environmental factors such as the bedding of the livestock and workers' unhygienic practices. The *Streptococci* species is also responsible for aggressive neonate infections in both adults and children (Schuchat, 2001). This organism has lately been considered the predominant pathogen in dairy herds and that it on the increase in this environment (Kromker *et al*., 2014). Moreover, the *Streptococcal* species that is associated with mastitis infection in bovine herds is considered to be an environmental organism that causes environmental mastitis in cows (Taponen *et al.,* 2006). *S. uberis, S. agalactiae and S. dysgalactiae* are understood to be ubiquitous in nature and are mostly found in straw bedding and pastures, but they can also be found on bovines' skin and in the digestive system of cows. Because these organisms can persist in the udder of cows, some of the infections they cause are systemic rather than localised. In some cases,

these organisms tend to be resistant to antimicrobial agents and they thus cause significant rates of reinfection in bovine herds (Kromker *et al*., 2014).

4.4.3 Detection of species' specific genes by multiplex polymerase chain reaction (mPCR)

For the purpose of this study, 16 milk bacterial DNA were analysed using mPCR to simultaneously detect the five most predominantly observed mastitis-causing pathogens, namely *E.coli, S. aureus* and *Streptococci* spp. The results showed that, of the eight (8) bacterial DNA that were analysed, mPCR could detect only *E.coli* (i.e., the *alr* gene). For the utilisation of mPCR, DNA was extracted directly from all the samples that had been collected, irrespective of whether the samples had tested positive or negative for the CMT and SCC techniques. The DNA concentrations are depicted in Table 4.2 below, while the mPCR images showing the amplification of genes detected by this method are depicted in Images 4.6; 4.7; and 4.8 below.

Table 4.2: Concentration of DNA extracted from each analysed sample (ng/µl)

Sample Name	ng/µl	A260/280	A260/230
3	10.06	1.50	0.17
4	54.38	1.41	0.26
8	13.50	1.58	0.18
10	62.09	1.45	0.25
16	23.80	1.57	0.26
11	12.24	1.54	0.20
13	8.97	1.62	0.18
15	10.98	1.69	0.19

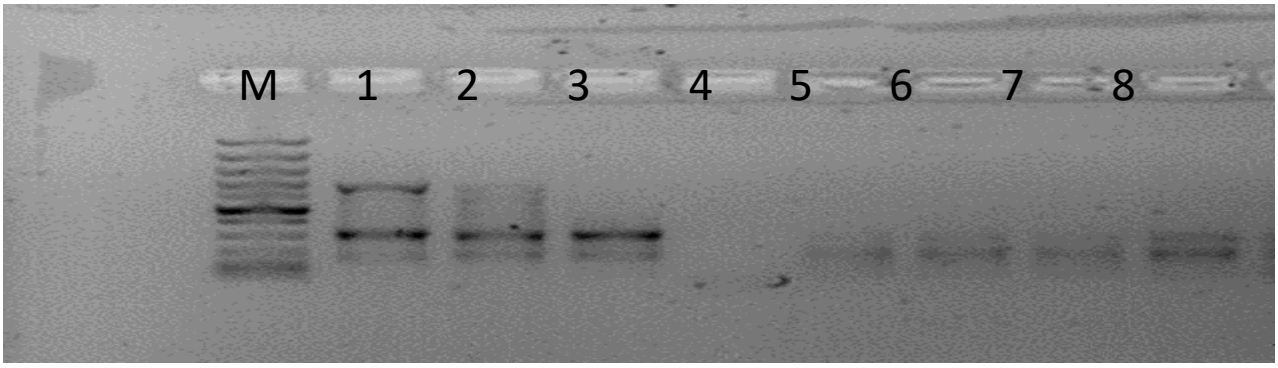

Figure 4.6: Agarose gel electrophoresis of amplified mPCR products.

Lane M: DNA Ladder (100 bp). **Lanes 1-3** and **5-8:**Multiplex amplicons. **Lane 4:** Negative control.

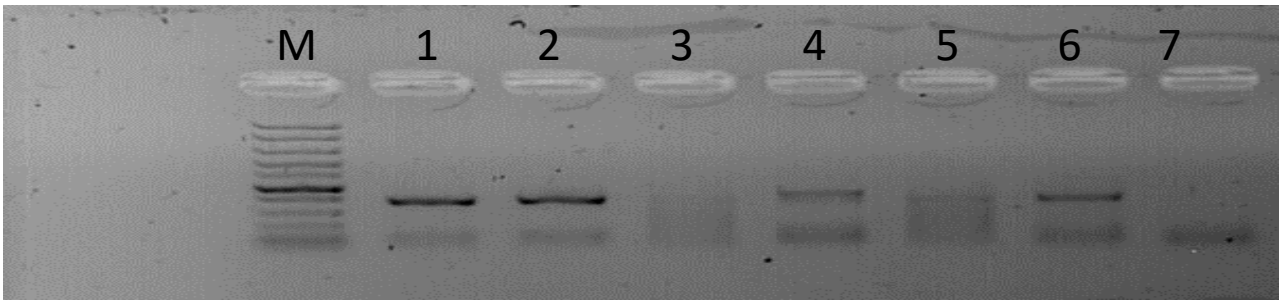

Figure 4.7: Agarose gel electrophoresis of amplified mPCR products.

Lane M: DNA Ladder (100bp). **Lane 1-6**: Multiplex amplicons. **Lane 7**: Negative control.

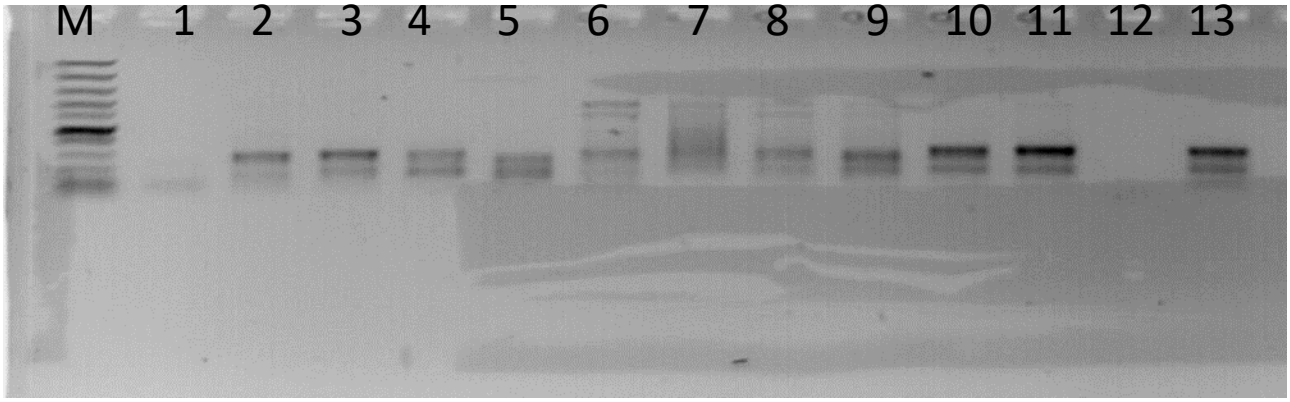

Figure 4.8: Agarose gel electrophoresis of amplified mPCR products.

Lane M: DNA Ladder (100 bp). **Lane 1**: Negative control. **Lane 2-13**: Multiplex amplicons

The results can be compared to those of Kalin *et al.* (2017), who detected lower numbers of these bacteria in the milk samples they tested: i.e., 26%, 12% and 6% for *S. aureus*, *S. agalactiae* and *E.coli* respectively.

The current study further investigated the sequences of all amplified genes to understand if they were true positives. Figure 4.9 below shows the phylogenetic tree for evolutionary relationships of all the amplified genes. The evolutionary history was inferred using the Neighbour-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of

branch length = 437.34375000 is shown. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the number of differences method as proposed by Nei and Kumar (2000) and are in the units of the number of base differences per sequence. These analyses involved seven nucleotide sequences. All positions containing gaps and missing data were eliminated (complete deletion option). There was a total of 255 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al.,* 2018).

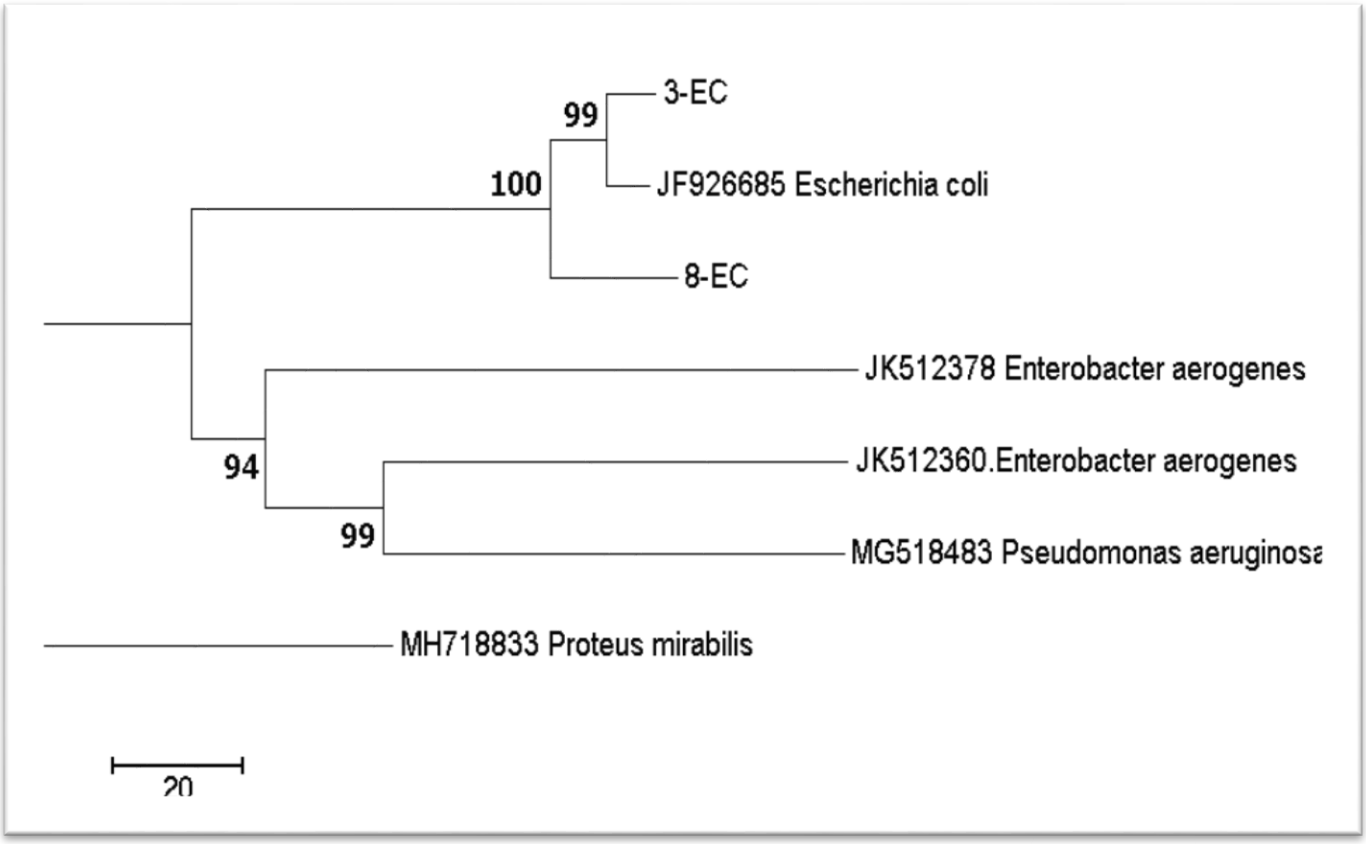

Figure 4.9: Evolutionary relationship of *E.coli* (*alr* gene)

The evolutionary tree shows that most of the detected genes were not true positives for *E.coli*. However, they were all Gram-negative bacteria that had been isolated in raw bovine milk and they could be linked to coliform mastitis. A study by El-Roos *et al.* (2013) investigated *P. aeruginosa* in raw milk and they revealed that about 40% of their raw milk samples contained this bacterium, which is of great concern due to its pathogenic nature and because of its ability to affect humans and animals. It has been identified as a major contributor to secondary community and nosocomial infections (Corona *et al.,* 2001).

On the other hand, *E. aerogenes* is one of the causes of coliform/environmental mastitis in many areas of the world (Junaidu *et al.,* 2011). This bacterium infects the mammary gland by entering through its canal where it will multiply and cause infection. The latter bacterium is usually destroyed by the cow's immune system; however, it sometimes releases endotoxins that mostly cause a clinical form of mastitis. In subclinical infections, coliform bacteria remain in an infected teat/udder for longer periods (Maroney, 2005).

Because this study sought to detect subclinical and not clinical mastitis that is caused by pathogens, it is possible that the circumstance of not isolating all the species under investigation could have affected the limited detection using mPCR because there may have been no viable cells of the species under investigation (Ashraf *et al.,* 2017). A similar study by Goli *et al.* (2012) detected one pathogen in a mPCR assay at 43.5%, while only 3.8% was due to three pathogens. Rysanek et al. (2007) also recorded similar results. Although the current study did not detect multiple species in the extracted milk DNA, the

identification of these pathogens by multiplex PCR can still be helpful to get enough information regarding the causes of mastitis so that control measures can be appropriately implemented. However, it is acknowledged that factors such as PCR inhibitors can still play a role in the detection limit of mPCR, hence these factors need to be identified and removed in order to obtain more decisive results (Kalin *et al.,* 2017). Also, to increase the sensitivity of the mPCR assay, it is advisable that the samples be enriched to obtain enough bacterial DNA so that the pathogens can be detected (Phuektes *et al.,* 2001).

4.5 Conclusion

The study was undertaken to assess the prevalence and the extent of subclinical mastitis-causing pathogens on smallholding farms in the Maluti-a-Phofung Local Municipality in the vicinity of Harrismith. Both contagious and environmental mastitis were found to be common in all cases of mastitis. Contagious mastitis is mainly caused by *S. aureus* while environmental mastitis is caused by environmental *Streptococci,* including *E. coli* (Hussein *et al.,* 2017).

Upon visiting the selected farms, it was observed that the employees used their hands to clean the udders and milk the dairy cows. It may be argued that this practice may have resulted in the isolation and identification of *S. aureus* in almost all the collected samples of raw milk. However, based on the findings of their study, Mein *et al.* (2004) concluded that the epidemiological indicators of subclinical mastitis were better in hand milking practices than when a machine was used for milking. They argue that the mastitis

condition resulting from milking with a machine may be associated with the non-monitoring of the machine, failure to adequately clean the teat cups, or inadequate pressure from the vacuums.

The current study could not isolate all five subclinical mastitis-causing agents by utilising conventional microbiological techniques; however, the researcher was able to isolate, albeit to a minimal extent, *S. aureus* and *E. coli* as well as organisms of the *Streptococcal* species such as *S. mutans, S. pneumonia, S. Salivarius/vestibularis, S. avium, S. sanguis/gordinii* and *Enterococcus spp*. It should be noted that *Streptococci spp*. has been well studied in clinical cases and it may thus be concluded that the mode of contamination might have occurred through coughing or sneezing air droplets onto the milk or the hands of employees. Due to the fact that the predominant isolate was *S. aureus* was predominantly isolated, it can be concluded that contagious mastitis was prevalent in the cows under investigation. This conclusion is of enormous public health concern because, if the infection caused by the organism is not contained, consumers' health is seriously at risk. Therefore, to prevent and control mastitis both clinically and subclinically, various precautionary measures need to be implemented such as timely, specific tests for the identification of major bacteria, and strict monitoring tools at every farm in the Harrismith area. Demme and Abegaz (2015) state that poor hygienic practices on dairy farms, coupled with poor personal hygiene, may be factors that cause the spreading of bacteria from the environment to dairy herds. Humans (workers) also spread bacteria to raw milk and hence they should be appropriately trained to practise personal and husbandry hygiene at all times. The study could not isolate any environmental

streptococci, hence these bacteria required the mammary glands to survive, and it was here where they were detected. However, these bacteria can easily be eliminated by the use of antimicrobials/antibiotics such as penicillin (Zadoks and Watts, 2009). It was thus concluded that there is a need for dry cow therapy to control mastitis in the area under investigation.

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CHAPTER FIVE

CHARACTERISATION OF RAW MILK MICROBIOME USING 16S RIBOSOMAL RNA GENE SEQUENCING

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5.1 Abstract

Milk microbiota composition plays a vital role in determining the safety and quality of milk and products derived from milk. Merely a few studies have been conducted to investigate and understand the microbial community of raw milk in South Africa. The current study thus investigated raw milk microbiota in milk samples that were obtained from small-scale farms in the vicinity of Harrismith in the Free State Province, South Africa. This phase of the study utilised 16S rRNA gene sequencing. The results of the analyses showed that *Firmicutes* and *Actinobacteria* were the predominant phyla in raw milk. On the genus level, *Clostridium sensu stricto 1*, *Turicibacter* sp. and *Romboutsia* sp. were found in all the samples while, on species level, *Aerococcus* sp. (4.77%), *Clostriduem dispericum* (4.73%), *Turicibacter* sp. (4.01%), *Facklamia tabacinales* (0.38%) and *Enterococcus facials* (0.24%) were found. It was therefore evident that novel techniques such as next generation sequencing have the potential to fully elucidate prevailing microflora in milk and its products. Based on the findings, it may be argued that this study will assist farmers and their suppliers to understand the full spectrum of the microbial community associated with milk, and the information will subsequently improve the quality and safety of milk. However, it is urged that the presence of the bacteria detected by this study undergoes further characterisation and documentation to determine their ultimate effects on dairy herds as well as on humans who consume their milk.

Key words: Milk, Microbiota, Gene, Sequencing, Metagenomics

5.2 Introduction

Raw bovine milk is highly nutritious for humans as it contains almost all the essential nutrients such as proteins, fats, carbohydrates, vitamins, minerals and amino acids and has an almost neutral pH and a high water content. However, because raw milk contains these essential nutrients, it provides an ideal environment for all types of microorganisms to grow (Kim *et al.,* 2017). It is in this context that dairy microbiota have been characterised in various studies to determine their impact on milk and their possible effects on consumers. Bacterial genera such as *Lactococcus*, *Lactobacillus*, *Streptococcus* and *Propionibacterium* have been adequately explained with regards to their effects on sensory awareness, flavour, and organoleptic properties while others, such as *Bifidobacterium,* have been associated with their beneficial use for promoting human health (Debarry *et al.,* 2007; Fernandez *et al.,* 2013; Jost *et al.,* 2014). Most psychotrophic bacteria that are predominantly found in raw milk are Gram-negative of the genera *Pseudomonas*, *Achromobacter* or *Chromobacterium* and, in some instances, Gram-positive genera of *Streptococcus*, *Lactobacillus* or *Microbacterium* have also been found in this foodstuff.

The presence of milk-associated bacteria is important in determining the shelf life, aesthetic qualities and safety of milk and it is necessary to ensure good quality milk and its derived products (Ottesen *et al.,* 2013). Microorganisms are introduced in milk mainly through unhygienic sources of contamination on farms or by means of the udder of an infected animal (Oliver *et al.,* 2005). Furthermore, it has been well documented that

microorganisms are also introduced in milk through transportation, storage and processing. The location of a dairy farm, the manner in which the herd is housed, their feed, and bedding types also play a role in the bacterial composition in the udders of cows (Quigley *et al.,* 2013; Vacheyrou *et al.,* 2011; Angulo *et al.*, 2009).

Mokoena (2013) explains that the washing of milking cows' teats with contaminated water can also play a role in the microbial quality of raw milk. Milk pathogens have been regarded as a public health concern that particularly affects communities that consume raw milk that has not been exposed to any form of treatment. It is therefore vital that dairy producers consider environmental factors and the lactation period of their herds when they produce milk (O'Connell *et al.,* 2016; McInnis *et al.,* 2015). It is also vital that the impact of inappropriate storage conditions (such as duration and temperature) not be undermined when the microbial composition of raw milk is assessed. Moreover, the role of psychotrophic bacteria should also be taken into consideration as they have the ability to grow even in refrigeration temperatures and to produce lipases and proteases. It is for this reason that they are associated with milk spoilage (Hantsis-Zacharov and Halpern, 2007). These microorganisms have the ability to grow in temperatures of <6°C if stored over a considerable period of time. Moreover, inadequate cleaning of milk tanks before filling and failure to clean udders appropriately have also been found to be factors that cause milk contamination and spoilage (Doyle *et al.,* 2017).

Earlier, the quantification of undesirable microorganisms in raw milk was conducted by utilising traditional cultivation-based techniques. These methods revealed only the

presence or absence of bacteria based on their phenotypic qualities (Quigley *et al.,* 2013), and they thus only showed what was needed to be grown/cultured in a laboratory (Ward *et al.,* 1992). However, to overcome the shortcomings encountered by plate cultivation techniques, various culture-independent molecular techniques have been introduced and are well described by Liu *et al.* (2015). These techniques include denaturing gradient gel electrophoresis (DGGE), temporal temperature gel electrophoresis, quantitative real-time PCR, and Sanger sequencing of the 16S rRNA gene clone library (Liu *et al.,* 2015). This study utilised another form of independent molecular technique, namely the high-throughput next generation sequencing (NGS) technique. Compared to other molecular techniques, the utilisation of NGS in the characterisation of microbial communities has shown a great variety of microbial populations in contrast to traditional culture-based methods (Taioe, 2017). The application of metagenomics includes cloning of different bacterial genomes and their analysis without the need to culture them (Yun *et al.,* 2014). Even though this method was initially introduced for environmental microbiology, it has also been found to be efficient in the application or quantification of microbial communities in raw milk (Walsh *et al.,* 2016; Gschwendtner *et al.,* 2016). This study thus utilised a DNA-based technique, namely single-molecule real-time (SMRT) sequencing targeting 16S rRNA of the bacteria from raw milk. This technique provided a comprehensive insight into the microbiome of the raw milk that was analysed (Doyle *et al.,* 2017). The aim of this phase of the study was therefore to investigate and compare different microbial communities found in raw milk obtained from small-scale farms in the area of study by specifically targeting the 16S rRNA gene.

5.3 Materials and Methods

5.3.1 Sampling site and collection process

To obtain a true representation of core microbiota of raw milk from small-scale farmers in the vicinity of the study area, a representative of 3 samples were collected from three bulk milk takes of three poorly managed farms in Harrismith region in the Free State Province. The samples were collected using sterile 50 ml bottles and they were transported to the laboratory and analysed within 6-8 hours after sampling. During transportation, the samples were stored in a cooler box maintained at 4-6ºC.

5.3.2 DNA extraction

A QIAamp DNA Mini Kit was used for deoxyribonucleic acid extractions according to the manufacturer's instructions (QIAGEN, Hilden, Germany). Before extraction, 1 ml of the raw milk sample was inoculated in 9 ml of liquid media (nutrient broth) and incubated at 37ºC for 24 hours to obtain enough bacterial cells for analysis. After the 24-hour period, a maximum of $5x10^6$ cells was centrifuged at 190 rpm for 5 minutes. Thereafter the cells were resuspended in 200 µl of phosphate buffered saline (PBS) and 20 µl of proteinase K was then added to the mixture. Subsequently, 200 µl of Buffer AL was added and the mixture was vortexed thoroughly and incubated at 56ºC for 10 minutes. After incubating the mixture, 200 µl of 96%-100% ethanol was added and the mixture was vortexed thoroughly and pipetted into the QIAamp mini spin column, placed in a 2 ml collection tube, and then centrifuged at 8000 rpm for 1 minute. Thereafter, the flow-through and collection tubes were discarded. The spin column was then placed in a new 2 ml collection tube and 500 µl of Buffer AW1 was added and it was centrifuged at 8000 rpm for 1 minute.

After centrifugation, the flow-through and collection tubes were again discarded and the spin column was again placed into a new 2 ml collection tube and 500 µl of Buffer AW2 was added to the spin column and centrifuged at 14000 rpm for 3 minutes. The flow-through and collection tubes were discarded once again. Thereafter, the spin columns were transferred to a new 2 ml micro centrifuge tube and 200 µl of Buffer AE was added to elute the DNA in the spin column. It was incubated for 1 minute at room temperature. Subsequently, it was again centrifuged for 1 minute at 8000 rpm. The yielded DNA was determined using 1.5% agarose gel stained with ethidium bromide/GR green stainer and visualised under UV light. The DNA samples were frozen until needed.

5.3.3 16S rRNA gene amplification and sample barcoding

The diversity of bacterial communities in milk samples from various farms was analysed using single molecule real-time PacBio sequencing technology (Pacific Biosciences, Menlo Park, CA, USA). Full-length 16S ribosomal RNA gene was amplified from gDNA using bacterial-specific primer 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (5TACGGYTACCTTGTTACGACTT). To allow multiplexing of amplicons, the 5`ends of the 16S rRNA forward and reverse primers were tagged with the universal M13F (TGTAAAACGACGGCCAGT) and M13R (GGAAACAGCTATGACCATG) sequences respectively. Furthermore, 5` block (5`NH4-C6) was added to 16S specific primers to ensure that carry-over amplicons from the first round PCR were not ligated to the SMRTbell adapters in subsequent steps. A set of five barcoded M13F and five barcoded M13R primers were designed to generate PacBio sequencing ready amplicons from 16S rRNA target sequence flanked by M13 universal overhangs. All primers were synthesised

and HPLC-purified (according to PacBio's SMRT sequencing recommendation) by Integrated DNA Technology (San Jose, CA, USA). First rounds of PCR were performed using M13-tagged 16S specific forward and reverse primers in a final volume of 25 (NEB Q5 hotstart mastermix) consisting of Q5 High-Fidelity 2X Master Mix (12.5 µl), 10 µM Forward Primer (1.25 µl), 10 µM Reverse Primer (1.25 µl), DNA template of 3 µl and Nuclease-Free Water of 7 µl. The conditions that were used for amplification were as follows: pre-incubation at 98°C for 2 min, followed by 10 cycles of denaturation at 98°C for 30 s, annealing at 66°C for 15 s, elongation at 72°C for 45 s, and 10 cycles of denaturation at 98°C for 30 s, annealing at 68°C for 15 s, elongation at 72°C for 45 s, and a final extension step at 72°C for 5 min. Prior to the seconds PCR amplification, PCR products from the first round were loaded on an agarose gel for visual inspection using Agilent 2100 Bioanalyzer System; thereafter, the second PCR amplification was ran using the same conditions. Barcoded 16S rRNA amplicons obtained from the secondary PCR were purified using Agentcourt AMPureXP magnetic beads (Beckman Coulter, Indianapolis, IN, USA) and quantified using a Qubit 2.0 Fluorometer and a Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Purified amplicons were then pooled in equimolar concentrations, and 500 ng of DNA was used for library preparation. Two PacBio libraries were constructed; each contained a pool of barcoded amplicons from nine samples. The SMRTbell adapters were ligated onto barcoded PCR products, and the libraries were sequenced on a PacBio RSII system using the P6-C4 polymerase and chemistry with a 360-min movie time.

5.3.4 Blast Protocol

High quality consensus CCS reads were generated on SMRT link. Every read was BLASTED and the resultant file was saved. NCBI blast version 2.3.0 was used with a cut-off e-value of 0.005. The top hit for every BLAST result (i.e., genus and species name) was counted and a record was kept of how many times each species appeared as a hit. The read count is the number of reads that matched the corresponding organism. In the event that no BLAST result was found for a particular read, that read count was recorded under 'No Hits'. Taxa information for every BLAST hit was recorded.

5.3.5 PacBio sequencing

PacBio raw reads were processed using RS ReadsOfInsert protocol in the SMRT Analysis software version 2.3 to obtain demultiplexed consensus sequences with a minimum of three full passes. Sequence data were processed using the software package QIIME version 1.9.174. Sequences shorter than 1000 nt were removed prior to downstream analyses. De novo chimeric detection was performed using the abundance-based algorithm implemented in UCHIME75 using a reference dataset from RDP71. The remaining sequences were clustered into OTUs based on an ‘open-reference’ OTU-picking method at 97% identity using UCLUST75. Taxonomy was assigned to the representative sequence of each OTU using the RDP Classifier22 retrained toward the Greengenes database (V13.8)76. Diversity analyses of the samples were performed using the QIIME pipeline.

5.4 Results and Discussion

The bacterial diversity in raw milk that had been collected from the bulk tanks on small-scale farms was determined for three farms immediately after the milking of the dairy cows. The phylogenetic and taxonomic assessment of the 16S rRNA showed that bacterial populations from the three selected farms were diverse and variable. In all the samples collected and analysed, taxa detected less than 1% relative abundance which accounted for more than 50% of the bacteria present. The variation in the bacterial population among all the samples showed that Farm 1 (Figure 5.1) had *Turicibacter* sp., *Clostriduem dispericum* and *Clostridium* sp. 87 (1.18%), 21 (0.28%) and 14 (0.19%) cluster sizes respectively. However, Farm 2 (Figure 5.2) showed that *Aerococcus* sp., *Turicibacter* sp. and *Facklamia tabacinales* were highly contained in the sample with 249 (4.77%), 102 (1.95%) and 20 (0.38%) cluster sizes respectively. Lastly, Farm 3 (Figure 5.3) contained *Turicibacter* sp., *Enterococcus facials* and *Clostridium disporicum* at 196 (2.13%), 24 (0.26%) and 22 (0.24%) cluster sizes respectively.

The results of this investigation were similar to those of Catozzi *et al.* (2017), who also found that milk samples from healthy cows contained *Turicibacter* sp., *Enterococcus* sp., *Aerococcus* sp., *Facklamia* sp. and *Clostridium* sp. together with other species of interest in dairy microbiology such as *Staphylococcus* sp. The current study is one of few that has reported such species in raw milk because most studies have reported the presence of *Lactobacilli*, *Pseudomoneae*, and the *Lactococcus* species (Von Neubeck *et al.,* 2016; Alnakip *et al.,* 2016). Even though this study did not take the seasonal variation of milk

microbiota into consideration, other studies have shown that the microbiota in milk manifest in considerable varieties in agricultural products as well as in raw milk and its derived products (Smits *et al.,* 2001). The cluster sizes of bacterial species per farm are shown below in Figure 5.1-5.3.

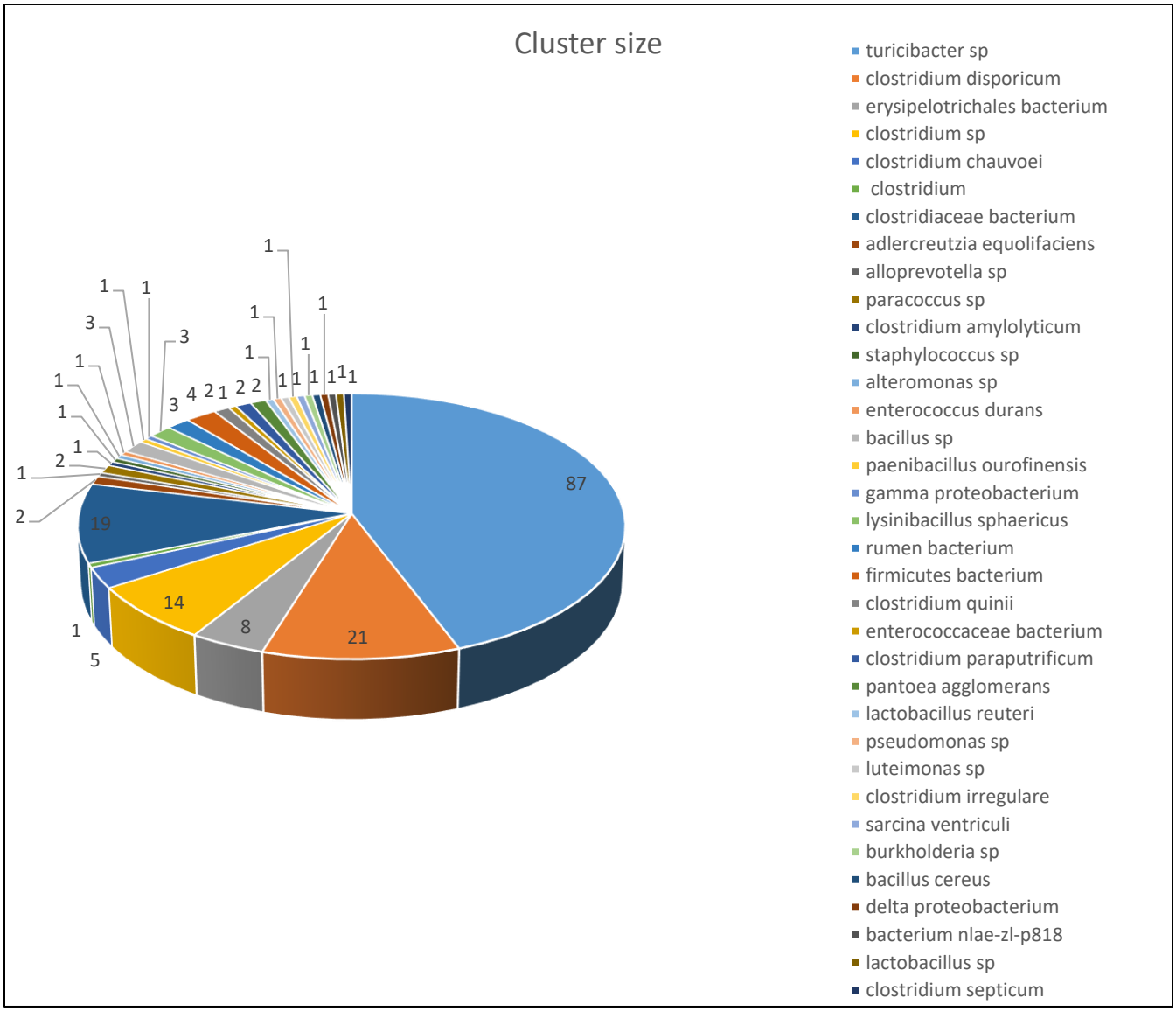

Figure 5.1: Abundance of bacteria at species level: Farm 1

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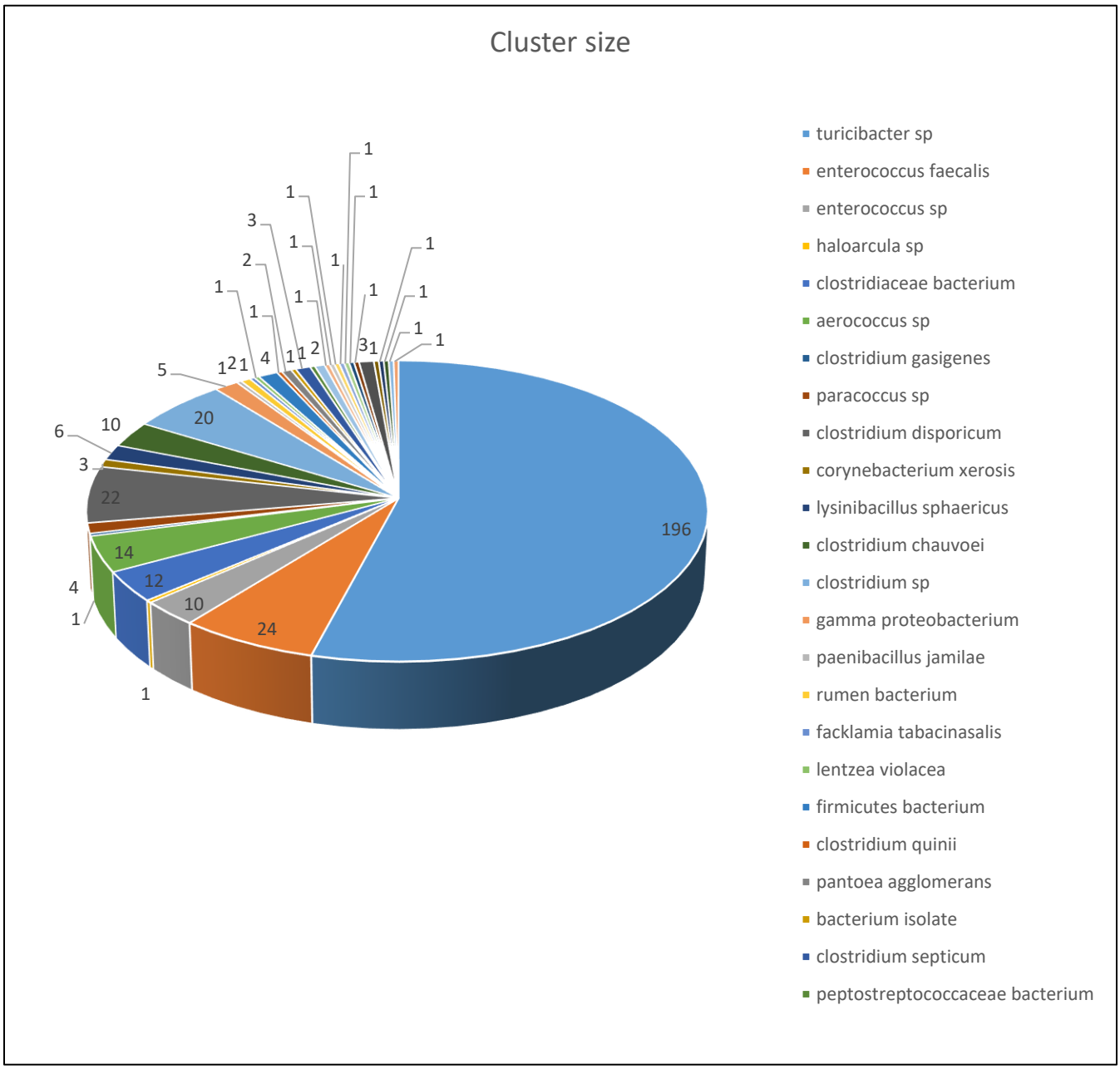

Figure 5.2: Abundance of bacteria at species level in Farm 2

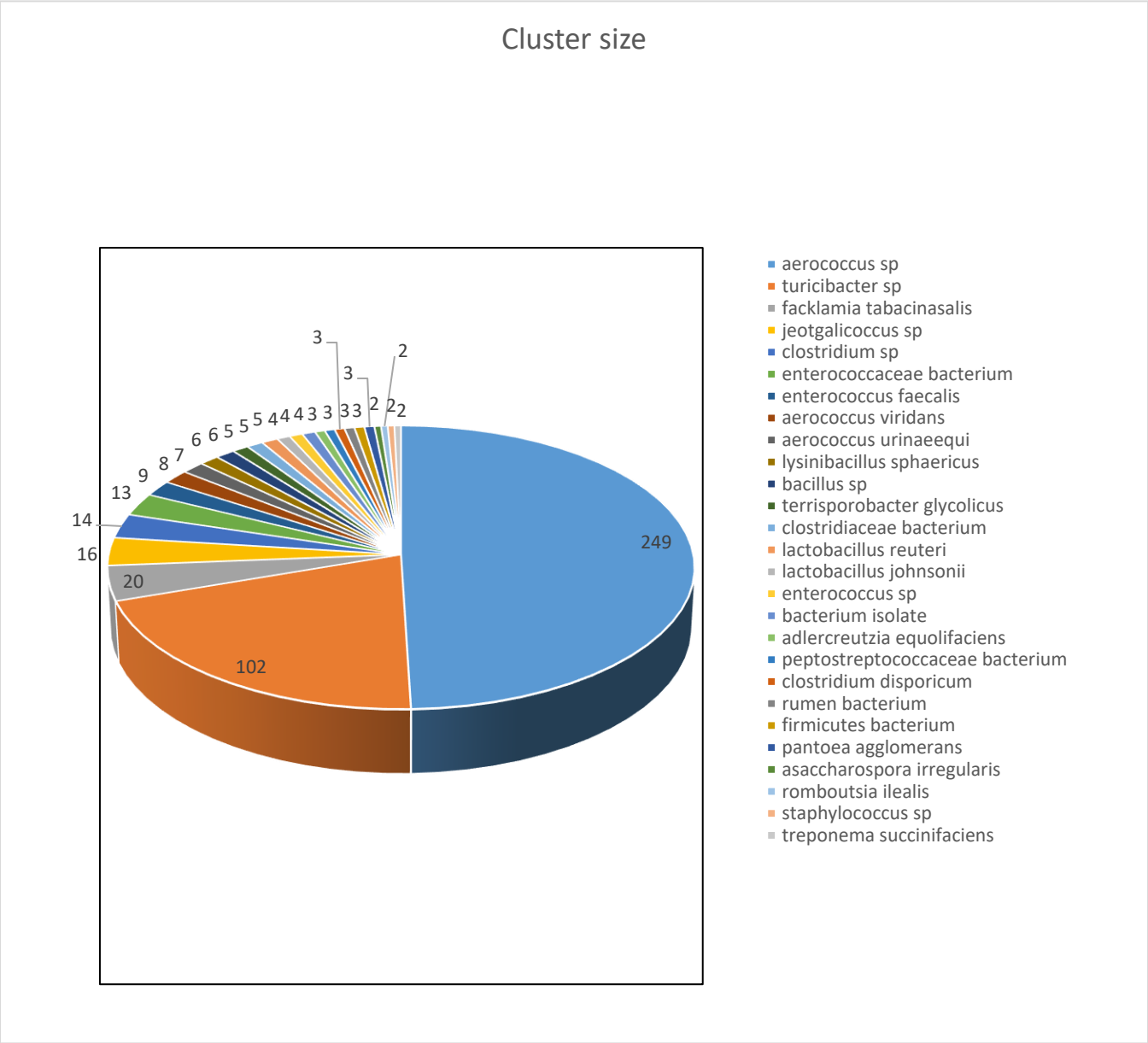

Figure 5.3: Abundance of bacteria at species level: Farm 3

Because microbiota in milk manifest in considerable varieties, Kable *et al.* (2016) argue that the difference in total bacteria in raw milk should be correlated with the differences in feeding and housing practices during sample collection. The latter authors argue that a reason for the differences in milk microbiome that have been reported by various studies could be that insufficient milk was collected and that the DNA sequence analyses could therefore not detect the distribution of taxa in each sample. However, irrespective of the sample-to-sample differences in microbiome, all the samples of the current study contained certain taxa that were represented in each of them, namely *Turicibacter* sp. and *Clostridia* sp. Endospore-forming bacteria such as *Clostridium* are also core microbiota of milk and encompass organisms that cause spoilage in both raw and pasteurized milk and milk-derived products.

5.4.2 Core microbiome of raw milk

Regardless of the variations in the bacterial species found in the raw milk samples, the normal flora associated with milk was also detected. A total of 13 (Figure 5.4) taxa were detected in the samples from all three the farms with *Clostridium sensu stricto 1*, *Turicibacter* sp. and *Romboutsia* sp. being the most prevalent. The most predominant phyla were *Firmicutes* and *Actinibacteria* at prevalence rates of 0.2%-100% (Table 5.1 and Figure 5.4).

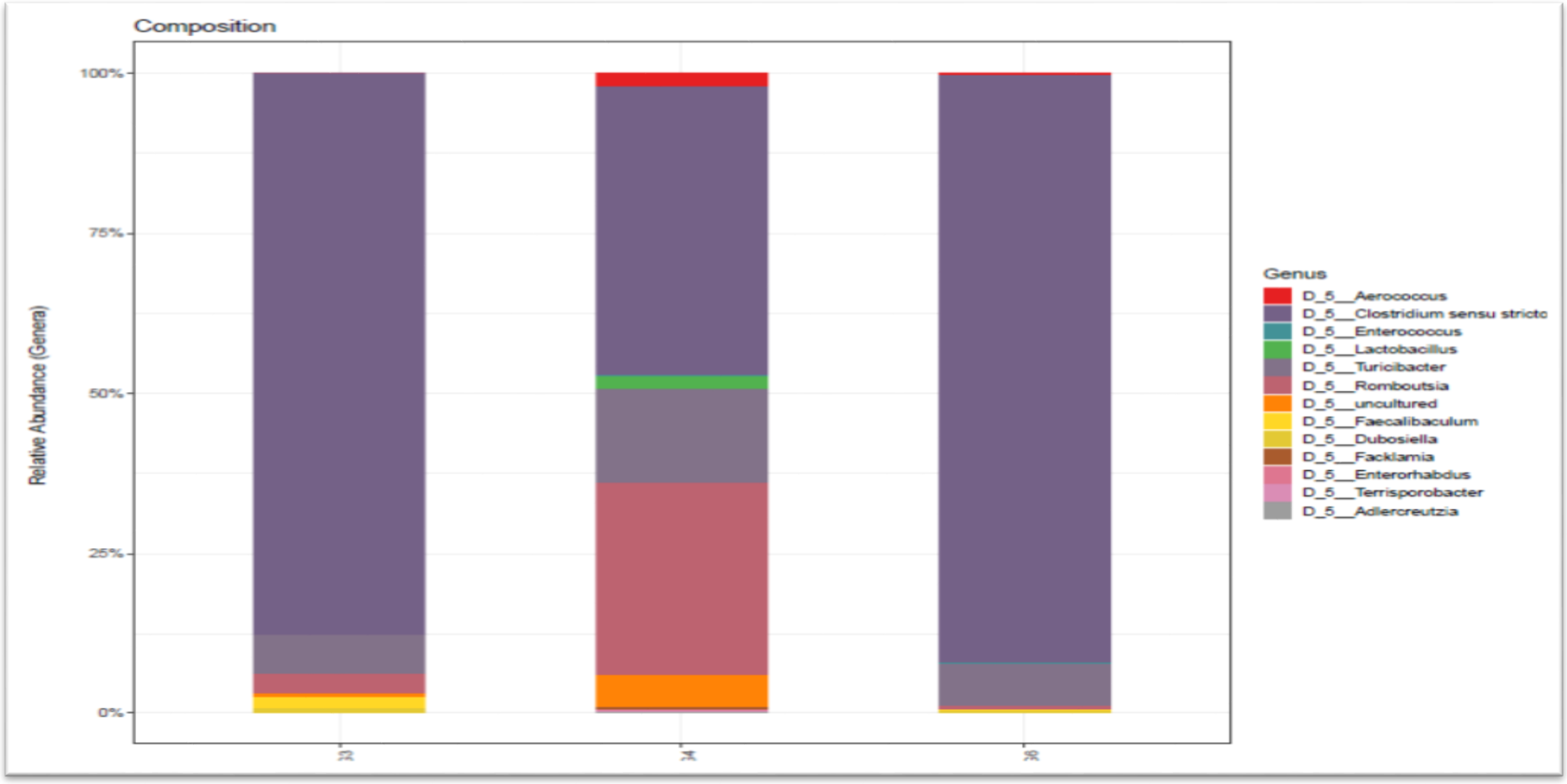

Figure 5.4: Composition of raw milk bacterial communities at genus level

Table 5.1: The relative abundance of each phylum per sample

OTU	Sample	Abundance	SampleName	Kingdom	Phylum
New.CleanUp.ReferenceOTU4980	G3	0	G3	Bacteria	Actinobacteria
New.CleanUp.ReferenceOTU1	G3	100	G3	Bacteria	Firmicutes
New.CleanUp.ReferenceOTU4980	G4	0,2260398	G4	Bacteria	Actinobacteria
New.CleanUp.ReferenceOTU1	G4	99,7739602	G4	Bacteria	Firmicutes
New.CleanUp.ReferenceOTU4980	G6	0	G6	Bacteria	Actinobacteria
New.CleanUp.ReferenceOTU1	G6	100	G6	Bacteria	Firmicutes

The results of this study were similar to those obtained by Quigley *et al.* (2013), who observed that the phylum *Firmicutes* was predominant in raw milk and its traditional fermented dairy products. Both these phyla (*Firmicutes* and *Actinibacteria*) and some of their associated microorganisms are found to be highly prevalent in animal environments and surroundings, including milking equipment. Several studies have also shown that *Firmicutes* still remains the most dominant phylum in raw milk and its fermented products (Raats *et al.,* 2011; Zhang *et al.,* 2015; Gao *et al.,* 2016).

Regarding the analyses of milk microbiota at genus level, this study found *Clostridium sensu stricto 1, Turicibacter sp.* and *Romboutsia* to be the predominant organisms in the milk sampled from the three farms. These results are corroborated by those of Delbe *et al.* (2007), who observed that, in raw milk, the most dominant taxa was *Firmicutes,* with orders of *Clostridiales* and the *Lactobacillales* being dominant in almost all the samples that were tested. The significance of *Clostridium sensu stricto*, *Clostridium botulinum* and *Clostridium tetani* in the food industry is mainly due to their neurotoxigenic properties. *Clostridium perfringens* is also associated with this threat (Wiegel *et al.,* 2006).

These species also contain a subgroup of bacteria known as Butyric acid bacteria (BAB) that is known for spoilage. O'Connell *et al.* (2016) state that these bacteria are noteworthy due to their high prevalence in bovine milk.

5.5 Conclusion

To this researcher's knowledge, the current study was the first to detect raw milk microbiome using culture-independent techniques to assess raw milk from a farming region in the Free State Province of South Africa. The study demonstrated that milk microbiota in the raw milk from small-scale farms were similar at both phylum and genus level; however, at species level the results differed significantly. Although this study did not take seasonal variability or any environmental factors into consideration when assessing the microbial communities of raw milk, other studies have adequately indicated that such factors could influence microbial communities.

Due to the fact that this study recorded microorganisms that are uncommon in raw milk such as *Clostridium sensu stricto* and *Romboutsia,* it implies that there is still an unexplored avenue that needs to be further investigated to determine milk microflora using novel techniques such as those utilised in the study. Moreover, this study also showed that few key genera, which are mostly associated with milk, warrant further scrutiny due to their ability to spoil food and produce heat stable enzymes. The findings of the study are congruent with other studies that also identified *Firmicutes* and *Actinobacteria* as core phyla within milk microbiota.

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CHAPTER SIX

GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

6.1 General Discussion

Recently the food industry has made significant progress concerning the healthy and safe production of food. However, the safety and quality of numerous fresh products remain a public health concern globally as unhygienic conditions and food spoilage persistently result in disease outbreaks that affect consumers and the economy (Nada *et al.,* 2012). One of the food products that is most susceptible to contamination is milk.

Milk can be contaminated through a variety of ways such as poor handling practices, unhygienic practices on dairy farms, and the infected udders of bovines. Research has also shown that the ineffective cleaning of milk containers and unsanitary working surfaces contribute to the contamination of milk. It is for this reason that research has predominantly focused on the contamination, transmission and prevention of foodborne infections based on the personal hygiene and handling practices within food establishments. These foci have highlighted the importance of quality control and food safety issues in the dairy industry because of its vulnerability to enhanced growth of various microorganisms. Against this background, the importance of information dissemination regarding reduced risks connected with the consumption of raw milk to milk production communities, and especially to farm owners and workers associated with milk production, cannot be over-emphasised.

It was in this context that the overarching aim of the study was to investigate the microbial quality of raw milk derived from small-scale farmers in the Harrismith area in the Free State Province, South Africa. The objectives of this study were to:

- enumerate and identify microbiota isolated from raw milk; and
- determine the prevalence of subclinical mastitis-causing pathogens in raw milk.

Chapter One outlined the general background of the study and elucidated the aim and specific objectives thereof. Chapter Two reviewed literature relating to microorganisms associated with raw milk and the health hazards it poses to the general population. Chapter Three reported on various microbial hazards associated with raw milk, with specific focus on TVCs (*Enterobacteriaceae* and *Streptococci* spp.). Chapter Four focused on the prevalence of subclinical mastitis-causing pathogens, namely *E. coli, S. aureus, S. agalactiae, S. dysgalactiae* and *S. uberis* by utilizing both culture methods and the novel multiplex polymerase chain reaction (mPCR). Chapter Five focused on the investigation into raw milk microbial communities through the utilisation of 16S rRNA gene sequencing using high throughput metagenomic sequencing techniques.

6.2 Concluding Remarks

Chapter One and 2 of this study were mainly dealing with the general background and introduction of the study. They focused on the literature review relating to dairy associated microorganisms and the hazards they pose in terms of raw milk. Chapter Three focused on the enumeration and identification of microbial hazards in raw milk with respect to

Enterobacteriaceae, Streptococci spp. and total viable count/total plate count. It was highlighted that the identification of *Enterobacteriaceae* such as *Shigella* spp. and *Pantoea* spp. entails the need for routine sampling of milk due to their association with human infections. It was also cautioned that they may contaminate milk through unhygienic personal practices. The study also showed high total viable counts for all the selected small-scale farms. These high counts are normally indicators of poor hygiene practices on farms.

Chapter Four investigated the prevalence of subclinical mastitis-causing pathogens using both culture and DNA-based techniques. The results that were discussed in this chapter showed that, based on screening techniques (CMT and SCC), the prevalence of this infection was 21% and 28% on CMT and SCC respectively. Various studies that have been conducted on the use of these screening tools for diagnosing subclinical mastitis have noted that researchers should not only depend on the screening of cows, but that they should supplement screening with the identification of pathogens using the culture method (McDougall *et al.,* 2010; Sharma *et al.,* 2010; Kaşikçi *et al.,* 2012).

This study further investigated five species that are predominantly identified in subclinical mastitis cases, namely *E. coli, S. aureus,* and environmental *Streptococcus* spp. (*S. agalactiae, S. dysgalactiae and S. uberis*). The results revealed that the raw milk that had been collected on the small-scale farms contained 95% *S. aureus*, followed by *Streptococcus* spp. and *E. coli* at 36.4% and 13.3% respectively. However, the study could not isolate any environmental *Streptococcus.* Therefore, it was concluded that the

threat of contagious mastitis was more prevalent in the milk due to the abundant prevalence of *S. aureus* in the raw milk samples.

The study further employed the use of multiplex PCR to simultaneously detect the genes encoding for *E. coli, S. aureus* and *Streptococci* spp. However, the genes could not be detected simultaneously, but the study did manage to detect the *alr* gene encoding for *E. coli* at 366 bp. This suggests that mPCR could be used to study bacterial detection in raw milk, but it must be cautioned that factors such as a high concentration of gDNA, primer sequences and length do play a role in the efficiency and reliability of the mPCR technique (Kalin *et al.,* 2017; Phuektes *et al.,* 2001).

Chapter Five of this study focused on the use of the 16S rRNA gene to investigate core microbiota of raw milk and the results were reported from phyla to specie level. At the phyla level, the study showed that raw milk from the study area comprised core milk phyla such as *Firmicutes* and *Actinobacteria*. Although the presence of core milk microbiota have been globally reported, there are organisms that still need to be explored further (De Jonghe *et al.,* 2011; Schmidt *et al.,* 2012), such as those that were detected by this study: *Turicibacter* sp., *Clostriduem dispericum*, *Clostridium* sp., *Aerococcus* sp., and *Facklamia tabacinales*. However, at species level most of these organisms have rarely been detected by other studies that investigated core microbiota in raw milk, therefore this study could serve as an avenue for investigating microbiota associated with milk.

Moreover, the study explored the use of next generation sequencing tools to understand milk microbiota, especially in milk derived from small-scale farmers, and it thus

encourages future studies to explore these techniques in more depth with a view to enhancing the safe consumption of milk by communities.

6.3 General Recommendations

The findings of this study have exposed alarming rates of microorganisms in raw milk that was collected from small-scale farms, hence the following recommendations are proposed to improve the quality and safety of milk:

- Farmers and farm workers should be trained with regards to the hygiene of raw milk in the interest of ensuring the health of the communities they serve.
- There should be regular inspections of dairy farms, more especially in terms of the building requirements of milking sheds as per the Regulations relating to the Hygiene Requirements for Milking Sheds, the Transport of Milk and Related Matters (R961 of 2012) (South Africa DoH, 2012).
- Farmers are encouraged to design a monitoring tool for the screening and testing of their cows for possible intramammary infections.
- Dry-cow therapy should be practised by all dairy farmers. This means that dairy cows should be subjected to antimicrobial testing on a regular basis as part of preventing mastitis infection.
- Regular check-ups of milk handlers and their hygiene practices should be conducted as a matter of course to minimise the possible contamination of the raw milk that they handle and to protect it from human infectious pathogens.

6.4 Future Research

Based on the findings of this study, the following were identified as possible future research opportunities:

- Large epidemiological studies should be conducted to understand the relationship between mastitis-causing pathogens and environmental/seasonal variability on small-scale farms.

- Future studies should focus on the use of molecular techniques to investigate core milk microbiota together with understanding the impact of microorganisms on the environment, with specific focus on air microbiota.

- Studies should focus on the knowledge, attitudes and practices of milk handlers with regards to food safety.

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APPENDICES

Appendix I: Alr gene (*E.coli*) Blasted sequences

Alignments

>gb|JF926685.1| Escherichia coli strain VMC 11 alanine racemase (alr) gene, partial cds

gi|354463156|gb|JF926686.1| Escherichia coli strain VMC 3 alanine racemase (alr) gene, partial cds

Length=366, Score = 526.1 bits (582), Expect = 2E-145, Identities = 309/321 (96%), Gaps = 3/321 (1%), Strand = Plus/Plus

TCGAAACCGCGTATTTCCGYCTGGGCSTAAGGCCGGAAAGGCTGAGGCKTTTTAT

CATTCGATACCCGTATGCACCGTCTGGGCGTAAGGCCGGAACAGGCTGAGGCGTT

TTATCATCKCCTGACCCMGTGCAAAAACKTTCGTCAGCCGGTGAATATCGTCAGCC

ATTTTGCGCGCCGCCTGACCCAGTGCAAAAACGTTCGTCAGCCGGTGAATATCGT

CAGCCATTTTGCGCGCGCGGATGAACCAAAATGTGGCGCAACCGAGAAACAACTC

GCTATCTTTAATACCTTTTGCGCGGATGAACCAAAATGTGGCGCAACCGAGAAACA

ACTCGCTATCTTTAATACCTTTTGCGAAGGCAAACCTGGTCAACGTTCCATTGCCG

CATCGGGTGGCATTCTGCTGTGGCCACAGGAAGGCAAACCTGGTCAACGTTCCAT

TGCCGCATCGGGTGGCATTCTGCTGTGGCCACAGTCGCATTTTGACTGGGTGCGC

CCGGGCATCATTCTTTATGGCGTCTCGCCGCTGGAAGATTCGCATTTTGACTGGGT

GCGCCCGGGCATCATTCTTTATGGCGTCTCGCCGCTGGAAGATCGCTCCACCGGT

GGCGATTTTCGCTCCACCGGTGGCGATTTT

>gb|CP032989.1| Escherichia coli strain W2-5 chromosome, complete genome

Length=4914512, Score = 521.6 bits (577), Expect = 1E-143, Identities = 308/321 (95%), Gaps = 3/321 (1%), Strand = Plus/Minus

TCGAAACCGCGTATTTCCGYCTGGGCSTAAGGCCGGAAAGGCTGAGGCKTTTTAT

CATTCGATACCGGTATGCACCGTCTGGGCGTAAGGCCGGAACAGGCTGAGGCGTT

TTATCATCKCCTGACCCMGTGCAAAAACKTTCGTCAGCCGGTGAATATCGTCAGCC

ATTTTGCGCGCCGCCTGACCCAGTGCAAAAACGTTCGTCAGCCGGTGAATATCGT

CAGCCATTTTGCGCGCGCGGATGAACCAAAATGTGGCGCAACCGAGAAACAACTC

GCTATCTTTAATACCTTTTGCGCGGATGAACCAAAATGTGGCGCAACCGAGAAACA

ACTCGCTATCTTTAATACCTTTTGC

GAAGGCAAACCTGGTCAACGTTCCATTGCCGCATCGGGTGGCATTCTGCTGTGGC

CACAGGAAGGCAAACCTGGTCAACGTTCCATTGCCGCATCGGGTGGCATTCTGCT

GTGGCCACAGTCGCATTTTGACTGGGTGCGCCCGGGCATCATTCTTTATGGCGTC

TCGCCGCTGGAAGATTCGCATTTTGACTGGGTGCGCCCGGGCATCATTCTTTATG

GCGTCTCGCCGCTGGAAGATCGCTCCACCGGTGGCGATTTTCGCTCCACCGGTGC

CGATTTT

Score = 55.4 bits (60), Expect = 1E-03 Identities = 42/50 (84%), Gaps = 0/50 (0%) Strand = Plus/Minus

CGCATTTTGACTGGGTGCGCCCGGGCATCATTCTTTATGGCGTCTCGCCG

CGCATTTTGACTGGGTTCGGCCTGGCATTATTTTGTATGGCGCTTCGCCG

>gb|CP032986.1| Escherichia coli strain BE2-5 chromosome, complete genome

Length=4677021 Score = 521.6 bits (577), Expect = 1E-143 Identities = 308/321 (95%), Gaps = 3/321 (1%) Strand = Plus/Minus

TCGAAACCGCGTATTT-CCGYCTGGGCSTAAGGCCGGAA-

AGGCTGAGGCKTTTTATCATTCGATACCGGTATGCACCGTCTGGGCGTAAGGCCG

GAACAGGCTGAGGCGTTTTATCATCKCCTGACCCMGTGCAAAAACKTTCGTCAGC

CGGTGAATATCGTCAGCCATTTTGCGCGCCGCCTGACCCAGTGCAAAAACGTTCG

TCAGCCGGTGAATATCGTCAGCCATTTTGCGCGCGCGGATGAACCAAAATGTGGC

GCAACCGAGAAACAACTCGCTATCTTTAATACCTTT

GCGGATGAACCAAAATGTGGCGCAACCGAGAAACAACTCGCTATCTTTAATACCTT

TTGCGAAGGCAAACCTGGTCAACGTTCCATTGCCGCATCGGGTGGCATTCTGCTG

TGGCCACAGGAAGGCAAACCTGGTCAACGTTCCATTGCCGCATCGGGTGGCATTC

TGCTGTGGCCACAGTCGCATTTTGACTGGGTGCGCCCGGGCATCATTCTTTATGG

CGTCTCGCCGCTGGAAGATTCGCATTTTGACTGGGTGCGCCCGGGCATCATTCTT

TATGGCGTCTCGCCGCTGGAAGAT

CGCTCCACCGGTGGCGATTTT

>gb|CP032892.1| Escherichia coli strain SCEC020022 chromosome, complete genome.

Length=4894694, Score = 521.6 bits (577), Expect = 1E-143, Identities = 308/321 (95%),

Gaps = 3/321 (1%), Strand = Plus/Minus

TCGAAACCGCGTATTTCCGYCTGGGCSTAAGGCCGGAAAGGCTGAGGCKTTTTAT

CATTCGATACCGGTATGCACCGTCTGGGCGTAAGGCCGGAACAGGCTGAGGCGTT

TTATCATCKCCTGACCCMGTGCAAAAACKTTCGTCAGCCGGTGAATATCGTCAGCC

ATTTTGCGCGCCGCCTGACCCAGTGCAAAAACGTTCGTCAGCCGGTGAATATCGT

CAGCCATTTTGCGCGCGCGGATGAACCAAAATGTGGCGCAACCGAGAAACAACTC

GCTATCTTTAATACCTTTTGCGCGGATGAACCAAAATGTGGCGCAACCGAGAAACA

ACTCGCTATCTTTAATACCTTTTGCGAAGGCAAACCTGGTCAACGTTCCATTGCCG

CATCGGGTGGCATTCTGCTGTGGCCACAGGAAGGCAAACCTGGTCAACGTTCCAT

TGCCGCATCGGGTGGCATTCTGCTGTGGCCACAGTCGCATTTTGACTGGGTGCGC

CCGGGCATCATTCTTTATGGCGTCTCGCCGCTGGAAGATTCGCATTTTGACTGGGT

GCGCCCGGGCATCATTCTTTATGGCGTCTCGCCGCTGGAAGATCGCTCCACCGGT

GGCGATTTTCGCTCCACCGGTGCCGATTTT

>gb|CP012781.1| Escherichia coli strain A18 genome, Length=4616722, Score = 521.6 bits (577), Expect = 1E-143Identities = 308/321 (95%), Gaps = 3/321 (1%)

Strand = Plus/Plus,

TCGAAACCGCGTATTTCCGYCTGGGCSTAAGGCCGGAAAGGCTGAGGCKTTTTAT

CATTCGATACCGGTATGCACCGTCTGGGCGTAAGGCCGGAACAGGCTGAGGCGTT

TTATCATCKCCTGACCCMGTGCAAAAACKTTCGTCAGCCGGTGAATATCGTCAGCC

ATTTTGCGCGCCGCCTGACCCAGTGCAAAAACGTTCGTCAGCCGGTGAATATCGT

CAGCCATTTTGCGCGCGCGGATGAACCAAAATGTGGCGCAACCGAGAAACAACTC

GCTATCTTTAATACCTTTTGCGCGGATGAACCAAAATGTGGCGCAACCGAGAAACA

ACTCGCTATCTTTAATACCTTTTGC

GAAGGCAAACCTGGTCAACGTTCCATTGCCGCATCGGGTGGCATTCTGCTGTGGC

CACAGGAAGGCAAACCTGGTCAACGTTCCATTGCCGCATCGGGTGGCATTCTGCT

GTGGCCACAGTCGCATTTTGACTGGGTGCGCCCGGGCATCATTCTTTATGGCGTC

TCGCCGCTGGAAGATTCGCATTTTGACTGGGTGCGCCCGGGCATCATTCTTTATG

GCGTCTCGCCGCTGGAAGAT

>gb|CP027205.2| Escherichia coli strain WCHEC025943 chromosome, complete genome, Length=4817293, Score = 521.6 bits (577), Expect = 1E-143, Identities = 308/321 (95%), Gaps = 3/321 (1%), Strand = Plus/Minus

TCGAAACCGCGTATTTCCGYCTGGGCSTAAGGCCGGAAAGGCTGAGGCKTTTTAT

CATTCGATACCGGTATGCACCGTCTGGGCGTAAGGCCGGAACAGGCTGAGGCGTT

TTATCATCKCCTGACCCMGTGCAAAAACKTTCGTCAGCCGGTGAATATCGTCAGCC

ATTTTGCGCGCCGCCTGACCCAGTGCAAAAACGTTCGTCAGCCGGTGAATATCGT

CAGCCATTTTGCGCGCGCGGATGAACCAAAATGTGGCGCAACCGAGAAACAACTC

GCTATCTTTAATACCTTTTGCGCGGATGAACCAAAATGTGGCGCAACCGAGAAACA

ACTCGCTATCTTTAATACCTTTTGCGAAGGCAAACCTGGTCAACGTTCCATTGCCG

CATCGGGTGGCATTCTGCTGTGGCCACAGGAAGGCAAACCTGGTCAACGTTCCAT

TGCCGCATCGGGTGGCATTCTGCTGTGGCCACAGTCGCATTTTGACTGGGTGCGC

CCGGGCATCATTCTTTATGGCGTCTCGCCGCTGGAAGATTCGCATTTTGACTGGGT

GCGCCCGGGCATCATTCTTTATGGCGTCTCGCCGCTGGAAGATCGCTCCACCGGT

GGCGATTTTCGCTCCACCGGTGCCGATTTT

>gb|CP022959.1| Escherichia coli ATCC 8739 chromosome, complete genome

Length=4746918, Score = 521.6 bits (577), Expect = 1E-143, Identities = 308/321 (95%),

Gaps = 3/321 (1%) Strand = Plus/Minus

TCGAAACCGCGTATTT-CCGYCTGGGCSTAAGGCCGGAA-

AGGCTGAGGCKTTTTATCATTCGATACCGGTATGCACCGTCTGGGCGTAAGGCCG

GAACAGGCTGAGGCGTTTTATCATCKCCTGACCCMGTGCAAAAACKTTCGTCAGC

CGGTGAATATCGTCAGCCATTTTGCGCGCCGCCTGACCCAGTGCAAAAACGTTCG

TCAGCCGGTGAATATCGTCAGCCATTTTGCGCGCGCGGATGAACCAAAATGTGGC

GCAACCGAGAAACAACTCGCTATCTTTAATACCTTTGCGCGGATGAACCAAAATGT

GGCGCAACCGAGAAACAACTCGCTATCTTTAATACCTTTTGCGAAGGCAAACCTGG

TCAACGTTCCATTGCCGCATCGGGTGGCATTCTGCTGTGGCCACAGGAAGGCAAA

CCTGGTCAACGTTCCATTGCCGCATCGGGTGGCATTCTGCTGTGGCCACAGTCGC

ATTTTGACTGGGTGCGCCCGGGCATCATTCTTTATGGCGTCTCGCCGCTGGAAGA

T

>gb|CP032237.1| Escherichia coli strain ECCWS199 chromosome, complete genome, Length=4737445, Score = 521.6 bits (577), Expect = 1E-143, Identities = 308/321 (95%), Gaps = 3/321 (1%), Strand = Plus/Minus

TCGAAACCGCGTATTTCCGYCTGGGCSTAAGGCCGGAAAGGCTGAGGCKTTTTAT
CATTCGATACCGGTATGCACCGTCTGGGCGTAAGGCCGGAACAGGCTGAGGCGTT
TTATCATCKCCTGACCCMGTGCAAAAACKTTCGTCAGCCGGTGAATATCGTCAGCC
ATTTTGCGCGCCGCCTGACCCAGTGCAAAAACGTTCGTCAGCCGGTGAATATCGT
CAGCCATTTTGCGCGCGCGGATGAACCAAAATGTGGCGCAACCGAGAAACAACTC
GCTATCTTTAATACCTTTTGCGCGGATGAACCAAAATGTGGCGCAACCGAGAAACA
ACTCGCTATCTTTAATACCTTTTGCGAAGGCAAACCTGGTCAACGTTCCATTGCCG
CATCGGGTGGCATTCTGCTGTGGCCACAGGAAGGCAAACCTGGTCAACGTTCCAT
TGCCGCATCGGGTGGCATTCTGCTGTGGCCACAGTCGCATTTTGACTGGGTGCGC
CCGGGCATCATTCTTTATGGCGTCTCGCCGCTGGAAGATTCGCATTTTGACTGGGT
GCGCCCGGGCATCATTCTTTATGGCGTCTCGCCGCTGGAAGATCGCTCCACCGGT
GGCGATTTTCGCTCCACCGGTGCCGATTTTCGCATTTTGACTGGGTGCGCCCGGG
CATCATTCTTTATGGCGTCTCGCCGCGCATTTTGACTGGGTTCGGCCTGGCATTAT
TTTGTATGGCGCTTCGCCG

>gb|CP023061.1| Escherichia coli strain FORC_069 chromosome, complete genome Length=5189917, Score = 521.6 bits (577), Expect = 1E-143, Identities = 308/321 (95%), Gaps = 3/321 (1%), Strand = Plus/Minus

TCGAAACCGCGTATTTCCGYCTGGGCSTAAGGCCGGAAAGGCTGAGGCKTTTTAT
CATTCGATACCGGTATGCACCGTCTGGGCGTAAGGCCGGAACAGGCTGAGGCGTT
TTATCATCKCCTGACCCMGTGCAAAAACKTTCGTCAGCCGGTGAATATCGTCAGCC

ATTTTGCGCGCCGCCTGACCCAGTGCAAAAACGTTCGTCAGCCGGTGAATATCGT

CAGCCATTTTGCGCGCGCGGATGAACCAAAATGTGGCGCAACCGAGAAACAACTC

GCTATCTTTAATACCTTTTGCGCGGATGAACCAAAATGTGGCGCAACCGAGAAACA

ACTCGCTATCTTTAATACCTTTTGCGAAGGCAAACCTGGTCAACGTTCCATTGCCG

CATCGGGTGGCATTCTGCTGTGGCCACAGGAAGGCAAACCTGGTCAACGTTCCAT

TGCCGCATCGGGTGGCATTCTGCTGTGGCCACAGTCGCATTTTGACTGGGTGCGC

CCGGGCATCATTCTTTATGGCGTCTCGCCGCTGGAAGATTCGCATTTTGACTGGGT

GCGCCCGGGCATCATTCTTTATGGCGTCTCGCCGCTGGAAGATCGCTCCACCGGT

GGCGATTTTCGCTCCACCGGTGCCGATTTT

>gb|CP030281.1| Escherichia coli strain E308 chromosome, complete genome

Length=4786360

Score = 521.6 bits (577), Expect = 1E-143, Identities = 308/321 (95%), Gaps = 3/321 (1%), Strand = Plus/Minus

TCGAAACCGCGTATTTCCGYCTGGGCSTAAGGCCGGAAAGGCTGAGGCKTTTTAT

CATTCGATACCGGTATGCACCGTCTGGGCGTAAGGCCGGAACAGGCTGAGGCGTT

TTATCATCKCCTGACCCMGTGCAAAAACKTTCGTCAGCCGGTGAATATCGTCAGCC

ATTTTGCGCGCCGCCTGACCCAGTGCAAAAACGTTCGTCAGCCGGTGAATATCGT

CAGCCATTTTGCGCGCGCGGATGAACCAAAATGTGGCGCAACCGAGAAACAACTC

GCTATCTTTAATACCTTTTGCGCGGATGAACCAAAATGTGGCGCAACCGAGAAACA

ACTCGCTATCTTTAATACCTTTTGC

GAAGGCAAACCTGGTCAACGTTCCATTGCCGCATCGGGTGGCATTCTGCTGTGGC

CACAGGAAGGCAAACCTGGTCAACGTTCCATTGCCGCATCGGGTGGCATTCTGCT

GTGGCCACAGTCGCATTTTGACTGGGTGCGCCCGGGCATCATTCTTTATGGCGTC

TCGCCGCTGGAAGATTCGCATTTTGACTGGGTGCGCCCGGGCATCATTCTTTATG

GCGTCTCGCCGCTGGAAGATCGCTCCACCGGTGGCGATTTTCGCTCCACCGGTGC

CGATTTT

>dbj|AP018808.1| Escherichia coli E2865 DNA, complete genome

Length=5678205, Score = 521.6 bits (577), Expect = 1E-143, Identities = 308/321 (95%), Gaps = 3/321 (1%), Strand = Plus/Plus

TCGAAACCGCGTATTTCCGYCTGGGCSTAAGGCCGGAAAGGCTGAGGCKTTTTAT

CATTCGATACCGGTATGCACCGTCTGGGCGTAAGGCCGGAACAGGCTGAGGCGTT

TTATCATCKCCTGACCCMGTGCAAAAACKTTCGTCAGCCGGTGAATATCGTCAGCC

ATTTTGCGCGCCGCCTGACCCAGTGCAAAAACGTTCGTCAGCCGGTGAATATCGT

CAGCCATTTTGCGCGCGCGGATGAACCAAAATGTGGCGCAACCGAGAAACAACTC

GCTATCTTTAATACCTTTTGCGCGGATGAACCAAAATGTGGCGCAACCGAGAAACA

ACTCGCTATCTTTAATACCTTTTGCGAAGGCAAACCTGGTCAACGTTCCATTGCCG

CATCGGGTGGCATTCTGCTGTGGCCACAGGAAGGCAAACCTGGTCAACGTTCCAT

TGCCGCATCGGGTGGCATTCTGCTGTGGCCACAGTCGCATTTTGACTGGGTGCGC

CCGGGCATCATTCTTTATGGCGTCTCGCCGCTGGAAGATTCGCATTTTGACTGGGT

GCGCCCGGGCATCATTCTTTATGGCGTCTCGCCGCTGGAAGATCGCTCCACCGGT

GGCGATTTTCGCTCCACCGGTGCCGATTTT

>dbj|AP018802.1| Escherichia coli E2863 DNA, complete genome Length=5357442, Score = 521.6 bits (577), Expect = 1E-143, Identities = 308/321 (95%), Gaps = 3/321 (1%), Strand = Plus/Plus

TCGAAACCGCGTATTTCCGYCTGGGCSTAAGGCCGGAAAGGCTGAGGCKTTTTAT

CATTCGATACCGGTATGCACCGTCTGGGCGTAAGGCCGGAACAGGCTGAGGCGTT

TTATCATCKCCTGACCCMGTGCAAAAACKTTCGTCAGCCGGTGAATATCGTCAGCC

ATTTTGCGCGCCGCCTGACCCAGTGCAAAAACGTTCGTCAGCCGGTGAATATCGT

CAGCCATTTTGCGCGCGCGGATGAACCAAAATGTGGCGCAACCGAGAAACAACTC

GCTATCTTTAATACCTTTTGCGCGGATGAACCAAAATGTGGCGCAACCGAGAAACA

ACTCGCTATCTTTAATACCTTTTGCGAAGGCAAACCTGGTCAACGTTCCATTGCCG

CATCGGGTGGCATTCTGCTGTGGCCACAGGAAGGCAAACCTGGTCAACGTTCCAT

TGCCGCATCGGGTGGCATTCTGCTGTGGCCACAGTCGCATTTTGACTGGGTGCGC

CCGGGCATCATTCTTTATGGCGTCTCGCCGCTGGAAGATTCGCATTTTGACTGGGT

GCGCCCGGGCATCATTCTTTATGGCGTCTCGCCGCTGGAAGA

Appendix II: Cluster sizes of microbial communities in raw milk: Farm 1

Organism/HIT	Cluster size	%	Accession	e-value	Query
No hits	28	0.38	No hits	0	N/A
bacterium clone	3866	52.46	gi|126673872|gb|EF406615.1| Uncultured bacterium clone infected_7days-A1 16S ribosomal RNA gene, partial sequence	0.0	m54271_181220_115634/4194482/ccs
bacterium gene	2658	36.07	gi|474443220|dbj|AB627595.1| Uncultured bacterium gene for 16S rRNA, partial sequence, clone: L77	0.0	m54271_181220_115634/4325720/ccs
turicibacter sp	87	1.18	gi|474441000|dbj|AB727348.1| Turicibacter sp. LA61 gene for 16S ribosomal RNA, partial sequence	0.0	m54271_181220_115634/4391114/ccs
bacterium partial	586	7.95	gi|218411191|emb|AM930363.1| Uncultured bacterium partial 16S rRNA gene, clone SMR57	0.0	m54271_181220_115634/4784713/ccs
clostridium disporicum	21	0.28	gi|219846899|ref|NR_026491.1| Clostridium disporicum strain DS1 16S ribosomal RNA gene, partial sequence	0.0	m54271_181220_115634/6619394/ccs

bacterium isolate	5	0.07	gi|148616202|gb|EF608156.1| Uncultured bacterium isolate DGGE gel band lcy20 16S ribosomal RNA gene, partial sequence	0.0	m54271_181220_115634/7013064/ccs
erysipelotrichales bacterium	8	0.11	gi|388556147|dbj|AB702912.1| Uncultured Erysipelotrichales bacterium gene for 16S rRNA, partial sequence, clone: M_Fe_Ery05	0.0	m54271_181220_115634/7733341/ccs
bacteria partial	1	0.01	gi|99643452|emb|AM265443.1| Uncultured bacteria partial 16S rRNA gene, clone ratBD030102C	0.0	m54271_181220_115634/7995641/ccs
clostridium sp	14	0.19	gi|693302917|gb|KM244808.1| Uncultured Clostridium sp. clone FecD015 16S ribosomal RNA gene, partial sequence	0.0	m54271_181220_115634/8520513/ccs
clostridium chauvoei	5	0.07	gi|219846422|ref|NR_026013.1| Clostridium chauvoei strain 2585 16S ribosomal RNA gene, complete sequence	0.0	m54271_181220_115634/32113563/ccs
clostridium	1	0.01	gi|630257000|gb|KJ722507.1| [Clostridium] glycolicum strain	0.0	m54271_181220_115634/32506199/ccs

			Nesulana6 16S ribosomal RNA gene, partial sequence		
clostridiaceae bacterium	19	0.26	gi|404321150|gb|JX645590.1| Uncultured Clostridiaceae bacterium clone O-116 16S ribosomal RNA gene, partial sequence	0.0	m54271_181220_115634/32571580/ccs
adlercreutzia equolifaciens	2	0.03	gi|292698367|dbj|AB434709.1| Adlercreutzia equolifaciens gene for 16S ribosomal RNA, partial sequence, strain: FJC-M48	0.0	m54271_181220_115634/32768655/ccs
alloprevotella sp	1	0.01	gi|728055999|gb|KM462157.1| Alloprevotella sp. feline oral taxon 309 clone UI031 16S ribosomal RNA gene, partial sequence	0.0	m54271_181220_115634/34472095/ccs
paracoccus sp	2	0.03	gi|766545705|gb|KP120808.1| Paracoccus sp. 91_16 16S ribosomal RNA gene, partial sequence	1.36049e-06	m54271_181220_115634/36635252/ccs
clostridium amylolyticum	1	0.01	gi|343205899|ref|NR_044386.1| Clostridium amylolyticum strain SW408 16S ribosomal RNA gene, complete sequence	0.0	m54271_181220_115634/37225152/ccs

organism clone	27	0.37	gi|319454940|gb|HQ747801.1| Uncultured organism clone ELU0024-T375-S-NIPCRAMgANb_000090 small subunit ribosomal RNA gene, partial sequence	0.0	m54271_181220_115634/39322488/ccs
staphylococcus sp	1	0.01	gi|559104865|emb|HG313909.1| Staphylococcus sp. KB2.4R partial 16S rRNA gene, isolate KB2.4R	4.07819e-07	m54271_181220_115634/39977817/ccs
alteromonas sp	1	0.01	gi|619328192|dbj|AB924621.1| Alteromonas sp. BAKZL1107 gene for 16S ribosomal RNA, partial sequence	1.53055e-06	m54271_181220_115634/40108309/ccs
enterococcus durans	1	0.01	gi|917638659|gb|CP012384.1| Enterococcus durans strain KLDS 6.0930, complete genome	0.0	m54271_181220_115634/40239965/ccs
bacillus sp	3	0.04	gi|189913522|gb|EU685817.1| Bacillus sp. PK-8 16S ribosomal RNA gene, partial sequence	9.90881e-09	m54271_181220_115634/41747044/ccs
paenibacillus ourofinensis	1	0.01	gi|167508917|gb|EU257517.1| Paenibacillus ourofinensis strain AC13MSD 16S ribosomal RNA gene, partial sequence	3.2897e-08	m54271_181220_115634/43189058/ccs

gamma proteobacterium	1	0.01	gi|4406419|gb|AF114581.1| Uncultured gamma proteobacterium DCM-ATT-12 16S ribosomal RNA gene, partial sequence	1.06815e-07	m54271_181220_115634/50529163/ccs
lysinibacillus sphaericus	3	0.04	gi|398307810|gb|JX406328.1| Lysinibacillus sphaericus strain ARg 16S ribosomal RNA gene, partial sequence	5.84561e-11	m54271_181220_115634/51905028/ccs
rumen bacterium	3	0.04	gi|896685276|gb|KR068416.1| Uncultured rumen bacterium clone YAK-M46 16S ribosomal RNA gene, partial sequence	0.0	m54271_181220_115634/52887866/ccs
firmicutes bacterium	4	0.05	gi|694178833|gb|KM200426.1| Uncultured Firmicutes bacterium clone T2-196 16S ribosomal RNA gene, partial sequence	0.0	m54271_181220_115634/55378690/ccs
bacterium 16s	2	0.03	gi|14586434|emb|AJ308392.2| Uncultured bacterium 16S rRNA gene, clone S25-5	0.0	m54271_181220_115634/58065369/ccs
clostridium quinii	2	0.03	gi|219846557|ref|NR_026149.1| Clostridium quinii strain DSM 6736	0.0	m54271_181220_115634/58196501/ccs

			16S ribosomal RNA gene, partial sequence		
enterococcaceae bacterium	1	0.01	gi|371500977|gb|JN680640.1| Uncultured Enterococcaceae bacterium clone SL121 16S ribosomal RNA gene, partial sequence	0.0	m54271_181220_115634/9110346/ccs
clostridium paraputrificum	2	0.03	gi|219846543|ref|NR_026135.1| Clostridium paraputrificum strain ATCC 25780 16S ribosomal RNA gene, partial sequence	0.0	m54271_181220_115634/10289687/ccs
pantoea agglomerans	2	0.03	gi|144679022|gb|EF523432.1| Pantoea agglomerans strain IGCAR-18/07 16S ribosomal RNA gene, partial sequence	2.47245e-09	m54271_181220_115634/61276945/ccs
lactobacillus reuteri	1	0.01	gi|336447599|gb|CP002844.1| Lactobacillus reuteri SD2112, complete genome	0.0	m54271_181220_115634/63635794/ccs
pseudomonas sp	1	0.01	gi|34525856|emb|AJ551142.1| Pseudomonas sp. An1 partial 16S rRNA gene, isolate An1	1.53055e-06	m54271_181220_115634/64684580/ccs
luteimonas sp	1	0.01	gi|539360362|gb|KF500869.1| Uncultured Luteimonas sp. clone	1.46678e-06	m54271_181220_115634/66388448/ccs

			SPU:DMSN172 16S ribosomal RNA gene, partial sequence		
clostridium irregulare	1	0.01	gi|437756|emb|X73447.1| Clostridium irregulare 16S rRNA gene, strain DSM 2635	0.0	m54271_181220_115634/68420284/ccs
sarcina ventriculi	1	0.01	gi|5852402|gb|AF110272.1|AF110272 Sarcina ventriculi 16S ribosomal RNA gene, complete sequence	0.0	m54271_181220_115634/69010349/ccs
burkholderia sp	1	0.01	gi|571054944|gb|KF248549.1| Uncultured Burkholderia sp. clone ANWF3X 16S ribosomal RNA gene, partial sequence	1.46678e-06	m54271_181220_115634/69862021/ccs
bacillus cereus	1	0.01	gi|753292923|gb|CP009300.1| Bacillus cereus D17, complete genome	0.0	m54271_181220_115634/12190659/ccs
delta proteobacterium	1	0.01	gi|166407099|gb|EU104843.1| Uncultured delta proteobacterium clone 2R2U24 16S ribosomal RNA gene, partial sequence	1.90494e-08	m54271_181220_115634/23397060/ccs
bacterium nlae-zl-p818	1	0.01	gi|379364073|gb|JQ607647.1| Bacterium NLAE-zl-P818 16S ribosomal RNA gene, partial sequence	0.0	m54271_181220_115634/24838716/ccs

lactobacillus sp	1	0.01	gi|58040990|gb|AY862434.1| Lactobacillus sp. ID9203 16S ribosomal RNA gene, partial sequence	0.0	m54271_181220_115634/25691033/ccs
clostridium septicum	1	0.01	gi|748584437|dbj|LC019777.1| Clostridium septicum gene for 16S ribosomal RNA, partial sequence, strain: JCM 8151	0.0	m54271_181220_115634/26018086/ccs

Appendix III: Cluster size of microbial communities: Farm 2

Organism/HIT	Cluster size	%	Accession	e-value	Query
bacterium clone	2870	54.87	gi|238068950|gb|FJ881155.1| Uncultured bacterium clone R-9170 16S ribosomal RNA gene, partial sequence	0	m54271_181220_115634/4391811/ccs
bacterium gene	1154	22.06	gi|803377068|dbj|AB969396.1| Uncultured bacterium gene for 16S rRNA, partial sequence, clone: LH9	0	m54271_181220_115634/4260289/ccs
bacterium partial	605	11.57	gi|157690526|emb|AM183072.1| Uncultured bacterium partial 16S rRNA gene, clone SMB5	0	m54271_181220_115634/5243761/ccs
aerococcus sp	249	4.76	gi|315002343|emb|FR691452.1| Aerococcus sp. R-38529 partial 16S rRNA gene, strain R-38529	0	m54271_181220_115634/4260177/ccs
turicibacter sp	102	1.95	gi|474441000|dbj|AB727348.1| Turicibacter sp. LA61 gene for 16S ribosomal RNA, partial sequence	0	m54271_181220_115634/5440125/ccs
organism clone	41	0.78	gi|319492806|gb|HQ785667.1| Uncultured organism clone ELU0104-T246-S-NI_000244 small subunit ribosomal RNA gene, partial sequence	0	m54271_181220_115634/35914413/ccs
No hits	28	0.54	No hits	0	N/A
facklamia tabacinasalis	20	0.38	gi|662570985|gb|KJ733869.1| Facklamia tabacinasalis strain Fse17 16S ribosomal RNA gene, partial sequence	9.55998E-41	m54271_181220_115634/6881781/ccs
jeotgalicoccus sp	16	0.31	gi|760236190|gb|KP183066.1| Uncultured Jeotgalicoccus sp. clone 12L_86 16S ribosomal RNA gene, partial sequence	0	m54271_181220_115634/7799670/ccs
clostridium sp	14	0.27	gi|332656110|gb|JF733419.1| Uncultured Clostridium sp. clone	0	m54271_181220_115634/4849925/ccs

enterococcaceae bacterium	13	0.25	LC06st4 16S ribosomal RNA gene, partial sequence gi|371500977|gb|JN680640.1| Uncultured Enterococcaceae bacterium clone SL121 16S ribosomal RNA gene, partial sequence gi|333353442|gb|JF772098.1|	0	m54271_181220_115634/40108118/ccs
enterococcus faecalis	9	0.17	Enterococcus faecalis strain FCC120 16S ribosomal RNA gene, partial sequence gi|318054042|gb|HQ425688.2|	0	m54271_181220_115634/6095634/ccs
aerococcus viridans	8	0.15	Aerococcus viridans strain DSD-PW4-OH13 16S ribosomal RNA gene, partial sequence gi|343202949|ref|NR_043443.1|	0	m54271_181220_115634/34996964/ccs
aerococcus urinaeequi	7	0.13	Aerococcus urinaeequi strain IFO12173 16S ribosomal RNA gene, partial sequence gi|398307810|gb|JX406328.1|	0	m54271_181220_115634/40895023/ccs
lysinibacillus sphaericus	6	0.11	Lysinibacillus sphaericus strain ARg 16S ribosomal RNA gene, partial sequence gi|238835938|gb|FJ957618.1|	1.13389E-07	m54271_181220_115634/5047022/ccs
bacillus sp	6	0.11	Uncultured Bacillus sp. clone JPL-S3_E15 16S ribosomal RNA gene, partial sequence gi|645322288|ref|NR_119074.1|	0	m54271_181220_115634/45548402/ccs
terrisporobacter glycolicus	5	0.1	Terrisporobacter glycolicus strain DSM 1288 16S ribosomal RNA gene, partial sequence gi|209360611|gb|FJ234923.1|	0	m54271_181220_115634/32637186/ccs
clostridiaceae bacterium	5	0.1	Uncultured Clostridiaceae bacterium clone TUM-dMbac-MR4-B1-KC-30 16S ribosomal RNA gene, partial sequence gi|526120653|gb|CP006603.1|	0	m54271_181220_115634/46268710/ccs
lactobacillus reuteri	5	0.1	Lactobacillus reuteri TD1, complete genome gi|41584196|gb|AE017198.1|	0	m54271_181220_115634/48300421/ccs
lactobacillus johnsonii	4	0.08	Lactobacillus johnsonii NCC 533, complete genome	0	m54271_181220_115634/35455129/ccs

enterococcus sp	4	0.08	gi|78128495|gb|DQ232854.1| Uncultured Enterococcus sp. clone F28 16S ribosomal RNA gene, partial sequence	0	m54271_181220_115634/50594540/ccs
bacterium isolate	4	0.08	gi|148616202|gb|EF608156.1| Uncultured bacterium isolate DGGE gel band lcy20 16S ribosomal RNA gene, partial sequence	0	m54271_181220_115634/59114276/ccs
adlercreutzia equolifaciens	3	0.06	gi|292698367|dbj|AB434709.1| Adlercreutzia equolifaciens gene for 16S ribosomal RNA, partial sequence, strain: FJC-M48	0	m54271_181220_115634/42206002/ccs
peptostreptococcaceae bacterium	3	0.06	gi|323433447|gb|HQ853235.1| Uncultured Peptostreptococcaceae bacterium clone JL12_2009_9 16S ribosomal RNA gene, partial sequence	0	m54271_181220_115634/50135701/ccs
clostridium disporicum	3	0.06	gi|219846899|ref|NR_026491.1| Clostridium disporicum strain DS1 16S ribosomal RNA gene, partial sequence	0	m54271_181220_115634/52953750/ccs
rumen bacterium	3	0.06	gi|50788892|dbj|AB185612.1| Uncultured rumen bacterium gene for 16S rRNA, partial sequence, clone: F24-D12	0	m54271_181220_115634/57803541/ccs
firmicutes bacterium	3	0.06	gi|291332218|gb|GU958750.1| Uncultured Firmicutes bacterium clone CF2-153 16S ribosomal RNA gene, partial sequence	0	m54271_181220_115634/64749762/ccs
pantoea agglomerans	3	0.06	gi|144679022|gb|EF523432.1| Pantoea agglomerans strain IGCAR-18/07 16S ribosomal RNA gene, partial sequence	5.46255E-09	m54271_181220_115634/68092234/ccs
asaccharospora irregularis	2	0.04	gi|662235825|dbj|AB971797.1| Asaccharospora irregularis gene for 16S ribosomal RNA, partial sequence, strain: JCM 1425	0	m54271_181220_115634/38469786/ccs
romboutsia ilealis	2	0.04	gi|672239008|ref|NR_125597.1| Romboutsia ilealis strain CRIB 16S ribosomal RNA gene, partial sequence	0	m54271_181220_115634/42271203/ccs

staphylococcus sp	2	0.04	gi|238835864|gb|FJ957544.1| Uncultured Staphylococcus sp. clone JPL-2_E02 16S ribosomal RNA gene, partial sequence	0	m54271_181220_115634/42795133/ccs
treponema succinifaciens	2	0.04	gi|328447254|gb|CP002631.1| Treponema succinifaciens DSM 2489, complete genome	0	m54271_181220_115634/56033979/ccs
eggerthella sp	1	0.02	gi|338903436|dbj|AP012211.1| Eggerthella sp. YY7918 DNA, complete genome	0	m54271_181220_115634/34079703/ccs
feedlot manure	1	0.02	gi|12751282|gb|AF317386.1| Uncultured feedlot manure bacterium B87 16S ribosomal RNA gene, partial sequence	0	m54271_181220_115634/36897073/ccs
methylobacteriaceae bacterium	1	0.02	gi|728797615|emb|LN614855.1| Uncultured Methylobacteriaceae bacterium partial 16S rRNA gene, isolate PbD, clone Pbk42	2.69125E-20	m54271_181220_115634/36897277/ccs
clostridium vincentii	1	0.02	gi|219846744|ref|NR_026336.1| Clostridium vincentii strain DSM 10228 16S ribosomal RNA gene, partial sequence	0	m54271_181220_115634/37748925/ccs
corynebacterium xerosis	1	0.02	gi|520729903|gb|KF177173.1| Corynebacterium xerosis strain NS4 16S ribosomal RNA gene, partial sequence	0	m54271_181220_115634/40895318/ccs
ruminococcaceae bacterium	1	0.02	gi|851161231|emb|LN866991.1| Ruminococcaceae bacterium mt9 partial 16S rRNA gene, strain mt9	0	m54271_181220_115634/50136037/ccs
aerococcaceae bacterium	1	0.02	gi|162296235|gb|EU289078.1| Uncultured Aerococcaceae bacterium clone 8817-D4-C-2C 16S ribosomal RNA gene, partial sequence	0	m54271_181220_115634/54919465/ccs
jeotgalicoccus huakuii	1	0.02	gi|636560490|ref|NR_116550.1| Jeotgalicoccus huakuii strain NY-2 16S ribosomal RNA gene, partial sequence	0	m54271_181220_115634/56164801/ccs
gamma proteobacterium	1	0.02	gi|4406419|gb|AF114581.1| Uncultured gamma proteobacterium DCM-ATT-12 16S	3.19832E-08	m54271_181220_115634/57017163/ccs

			ribosomal RNA gene, partial sequence		
lactobacillus sp	1	0.02	gi|58040990|gb|AY862434.1| Lactobacillus sp. ID9203 16S ribosomal RNA gene, partial sequence	0	m54271_181220_115634/57409740/ccs
bacilli bacterium	1	0.02	gi|583826832|emb|HG917260.1| Uncultured Bacilli bacterium partial 16S rRNA gene, clone H207	0	m54271_181220_115634/59900341/ccs
clostridium chauvoei	1	0.02	gi|219846422|ref|NR_026013.1| Clostridium chauvoei strain 2585 16S ribosomal RNA gene, complete sequence	0	m54271_181220_115634/62653415/ccs
lactobacillus acidophilus	1	0.02	gi|388270627|gb|JX047330.1| Lactobacillus acidophilus strain KR 16S ribosomal RNA gene, partial sequence	0	m54271_181220_115634/67371604/ccs
enterococcus rotai	1	0.02	gi|566084797|ref|NR_108137.1| Enterococcus rotai strain CCM 4630 16S ribosomal RNA gene, complete sequence	0	m54271_181220_115634/68289312/ccs
salinicoccus roseus	1	0.02	gi|219846719|ref|NR_026311.1| Salinicoccus roseus strain DSM 5351 16S ribosomal RNA gene, complete sequence	0	m54271_181220_115634/70910692/ccs
compost bacterium	1	0.02	gi|295018141|emb|FN667213.1| Uncultured compost bacterium partial 16S rRNA gene, clone FS2275	0	m54271_181220_115634/73794185/ccs
proteobacterium partial	1	0.02	gi|440583473|emb|HE774687.1| Uncultured proteobacterium partial 16S rRNA gene, clone TWC 14	6.92315E-05	m54271_181220_115634/15139366/ccs
enterococcus avium	1	0.02	gi|110811551|gb|DQ779961.1| Enterococcus avium 16S ribosomal RNA gene, partial sequence	0	m54271_181220_115634/18416026/ccs
acidobacteriaceae bacterium	1	0.02	gi|641399953|gb|KJ589986.1| Uncultured Acidobacteriaceae bacterium clone ASTS_SIM_1000m_383 16S ribosomal RNA gene, partial sequence	3.19832E-08	m54271_181220_115634/18678155/ccs

bacteria partial	1	0.02	gi|99643452|emb|AM265443.1| Uncultured bacteria partial 16S rRNA gene, clone ratBD030102C	0	m54271_181220_115634/19006019/ccs
enterococcus hirae	1	0.02	gi|157907322|dbj|AB362590.1| Enterococcus hirae gene for 16S rRNA, partial sequence, strain: NRIC 0101	0	m54271_181220_115634/19268021/ccs
soil bacterium	1	0.02	gi|409109686|gb|JX490001.1| Uncultured soil bacterium clone B093 16S ribosomal RNA gene, partial sequence	0	m54271_181220_115634/19661671/ccs
clostridium difficile	1	0.02	gi|291482251|emb|FN668941.1| Clostridium difficile BI1 chromosome, complete sequence	0	m54271_181220_115634/20906520/ccs
jeotgalicoccus halotolerans	1	0.02	gi|219846053|ref|NR_025643.1| Jeotgalicoccus halotolerans strain YKJ-101 16S ribosomal RNA gene, partial sequence	0	m54271_181220_115634/23200399/ccs
nostoc sp	1	0.02	gi|296244782|gb|GU563896.1| Nostoc sp. PCC 7120 16S ribosomal RNA gene, partial sequence	0.000252558	m54271_181220_115634/25690379/ccs
luteimonas sp	1	0.02	gi|539360362|gb|KF500869.1| Uncultured Luteimonas sp. clone SPU:DMSN172 16S ribosomal RNA gene, partial sequence	6.77614E-10	m54271_181220_115634/27197871/ccs
burkholderia sp	1	0.02	gi|571054944|gb|KF248549.1| Uncultured Burkholderia sp. clone ANWF3X 16S ribosomal RNA gene, partial sequence	8.76549E-09	m54271_181220_115634/27525211/ccs
paenibacillus sp	1	0.02	gi|82940475|emb|AM162312.1| Paenibacillus sp. JA-08 partial 16S rRNA gene	0	m54271_181220_115634/29032928/ccs
bacteroidales bacterium	1	0.02	gi|388556248|dbj|AB702730.1| Uncultured Bacteroidales bacterium gene for 16S rRNA, partial sequence, clone: M_Fe_Bac13	0	m54271_181220_115634/30671194/ccs
aerosphaera taetra	1	0.02	gi|17906971|emb|AJ279038.1| Aerosphaera taetra 16S rRNA gene, strain CCUG 43036T	0	m54271_181220_115634/31785698/ccs

Appendix IV: Cluster size of microbial communities: Farm 3

Organism/HIT	Cluster size	%	Accession	e-value	Query
No hits	29	0.32	No hits	0	N/A
turicibacter sp	196	2.13	gi|474441000|dbj|AB727348.1| Turicibacter sp. LA61 gene for 16S ribosomal RNA, partial sequence	0.0	m54271_181220_115634/4194595/ccs
bacterium gene	3392	36.91	gi|474443073|dbj|AB627546.1| Uncultured bacterium gene for 16S rRNA, partial sequence, clone: C129	0.0	m54271_181220_115634/4325613/ccs
bacterium clone	4875	53.04	gi|192980615|gb|EU774638.1| Uncultured bacterium clone EAC_1aaa03d09 16S ribosomal RNA gene, partial sequence	0.0	m54271_181220_115634/4325719/ccs
bacterium partial	515	5.60	gi|218411206|emb|AM930378.1| Uncultured bacterium partial 16S rRNA gene, clone SMR144	0.0	m54271_181220_115634/4587761/ccs
enterococcus faecalis	24	0.26	gi|157907332|dbj|AB362600.1| Enterococcus faecalis gene for 16S rRNA, partial sequence, strain: NRIC 0111	0.0	m54271_181220_115634/6226115/ccs
organism clone	17	0.18	gi|319515839|gb|HQ808700.1| Uncultured organism clone ELU0161-T363-S-NIPCRAMgANa_000331 small subunit ribosomal RNA gene, partial sequence	0.0	m54271_181220_115634/6292261/ccs
enterococcus sp	10	0.11	gi|78128495|gb|DQ232854.1| Uncultured Enterococcus sp. clone F28 16S ribosomal RNA gene, partial sequence	0.0	m54271_181220_115634/6750413/ccs
haloarcula sp	1	0.01	gi|157057885|gb|EU080979.1| Uncultured Haloarcula sp. clone	6.32974e-05	m54271_181220_115634/6750853/ccs

clostridiaceae bacterium	12	0.13	HKTR18-12 16S ribosomal RNA gene, partial sequence gi|404321150|gb|JX645590.1| Uncultured Clostridiaceae bacterium clone O-116 16S ribosomal RNA gene, partial sequence gi|760236124|gb|KP183000.1|	0.0	m54271_181220_115634/7078736/ccs
aerococcus sp	14	0.15	Uncultured Aerococcus sp. clone 12S_41 16S ribosomal RNA gene, partial sequence gi|5453309|gb|AF143692.1|AF143692	0.0	m54271_181220_115634/7144318/ccs
clostridium gasigenes	1	0.01	Clostridium gasigenes 16S ribosomal RNA gene, partial sequence gi|766545705|gb|KP120808.1|	0.0	m54271_181220_115634/8323274/ccs
paracoccus sp	4	0.04	Paracoccus sp. 91_16 16S ribosomal RNA gene, partial sequence gi|219846899|ref|NR_026491.1|	1.46678e-06	m54271_181220_115634/32965312/ccs
clostridium disporicum	22	0.24	Clostridium disporicum strain DS1 16S ribosomal RNA gene, partial sequence gi|701216539|gb|KF928790.1|	0.0	m54271_181220_115634/33423768/ccs
corynebacterium xerosis	3	0.03	Corynebacterium xerosis strain GD34 16S ribosomal RNA gene, partial sequence gi|398307810|gb|JX406328.1|	0.0	m54271_181220_115634/33751267/ccs
lysinibacillus sphaericus	6	0.07	Lysinibacillus sphaericus strain ARg 16S ribosomal RNA gene, partial sequence gi|219846422|ref|NR_026013.1|	2.40181e-09	m54271_181220_115634/35586392/ccs
clostridium chauvoei	10	0.11	Clostridium chauvoei strain 2585 16S ribosomal RNA gene, complete sequence gi|259221050|gb|GQ868399.1|	0.0	m54271_181220_115634/36503808/ccs
clostridium sp	20	0.22	Uncultured Clostridium sp. clone BBC617 16S ribosomal RNA gene, partial sequence	0.0	m54271_181220_115634/39125650/ccs

gamma proteobacterium	5	0.05	gi|4406419|gb|AF114581.1| Uncultured gamma proteobacterium DCM-ATT-12 16S ribosomal RNA gene, partial sequence	2.40181e-09	m54271_181220_115634/39387527/ccs
paenibacillus jamilae	1	0.01	gi|343201283|ref|NR_042009.1| Paenibacillus jamilae strain CECT 5266 16S ribosomal RNA gene, partial sequence	2.36463e-57	m54271_181220_115634/42074559/ccs
rumen bacterium	2	0.02	gi|283982346|gb|GU304514.1| Uncultured rumen bacterium clone L406RT-6-A12 16S ribosomal RNA gene, partial sequence	0.0	m54271_181220_115634/43779030/ccs
facklamia tabacinasalis	1	0.01	gi|219846890|ref|NR_026482.1| Facklamia tabacinasalis strain GF112B 16S ribosomal RNA gene, partial sequence	0.0	m54271_181220_115634/46006916/ccs
lentzea violacea	1	0.01	gi|183228388|gb|EU593726.1| Lentzea violacea strain 173540 16S ribosomal RNA gene, partial sequence	0.0	m54271_181220_115634/48300574/ccs
firmicutes bacterium	4	0.04	gi|291331851|gb|GU958383.1| Uncultured Firmicutes bacterium clone TF1-87 16S ribosomal RNA gene, partial sequence	0.0	m54271_181220_115634/50529273/ccs
clostridium quinii	1	0.01	gi|219846557|ref|NR_026149.1| Clostridium quinii strain DSM 6736 16S ribosomal RNA gene, partial sequence	0.0	m54271_181220_115634/55509472/ccs
pantoea agglomerans	2	0.02	gi|144679022|gb|EF523432.1| Pantoea agglomerans strain IGCAR-18/07 16S ribosomal RNA gene, partial sequence	8.38438e-09	m54271_181220_115634/56099147/ccs
bacterium isolate	1	0.01	gi|148616202|gb|EF608156.1| Uncultured bacterium isolate DGGE gel band lcy20 16S ribosomal RNA gene, partial sequence	0.0	m54271_181220_115634/56689385/ccs

clostridium septicum	3	0.03	gi|219846429|ref|NR_026020.1| Clostridium septicum strain Pasteur III 16S ribosomal RNA gene, complete sequence	0.0	m54271_181220_115634/11076039/ccs
peptostreptococcaceae bacterium	1	0.01	gi|323433475|gb|HQ730635.1| Uncultured Peptostreptococcaceae bacterium clone JL12_2009_6 16S ribosomal RNA gene, partial sequence	0.0	m54271_181220_115634/65733016/ccs
pseudomonas sp	2	0.02	gi|619328198|dbj|AB924627.1| Pseudomonas sp. BAKZL1113 gene for 16S ribosomal RNA, partial sequence	3.79229e-08	m54271_181220_115634/65929808/ccs
enterococcaceae bacterium	1	0.01	gi|371500977|gb|JN680640.1| Uncultured Enterococcaceae bacterium clone SL121 16S ribosomal RNA gene, partial sequence	0.0	m54271_181220_115634/66323288/ccs
bacillus cereus	1	0.01	gi|145578085|gb|EF535591.1| Bacillus cereus strain CECRI-22/07 16S ribosomal RNA gene, partial sequence	1.403e-06	m54271_181220_115634/68747872/ccs
enterococcus rivorum	1	0.01	gi|358247443|emb|FR746103.1| Enterococcus rivorum partial 16S rRNA gene, strain HAMBI 3119	0.0	m54271_181220_115634/70124155/ccs
bacteroidetes bacterium	1	0.01	gi|291332106|gb|GU958638.1| Uncultured Bacteroidetes bacterium clone CTF1-21 16S ribosomal RNA gene, partial sequence	0.0	m54271_181220_115634/12189791/ccs
terrisporobacter glycolicus	1	0.01	gi|775465134|dbj|LC036317.1| Terrisporobacter glycolicus gene for 16S ribosomal RNA, partial sequence, strain: JCM 1401	0.0	m54271_181220_115634/14156384/ccs
bacterium bakzl1152	1	0.01	gi|619328205|dbj|AB924634.1| Bacterium BAKZL1152 gene for 16S ribosomal RNA, partial sequence	4.28419e-12	m54271_181220_115634/15401319/ccs

clostridium tertium	1	0.01	gi|310975222|ref|NR_037086.1| Clostridium tertium strain 795 16S ribosomal RNA gene, partial sequence	0.0	m54271_181220_115634/17630166/ccs
bacillus sp	3	0.03	gi|268373831|gb|GU136567.1| Bacillus sp. S110(3)-1 16S ribosomal RNA gene, partial sequence	0.0	m54271_181220_115634/21496576/ccs
staphylococcus sp	1	0.01	gi|559104865|emb|HG313909.1| Staphylococcus sp. KB2.4R partial 16S rRNA gene, isolate KB2.4R	1.48585e-08	m54271_181220_115634/25624935/ccs
bacillus drentensis	1	0.01	gi|459377144|gb|KC679987.1| Bacillus drentensis strain QAU54 16S ribosomal RNA gene, partial sequence	1.19962e-07	m54271_181220_115634/26935756/ccs
psychrobacter sp	1	0.01	gi|307828803|gb|HM246327.1| Psychrobacter sp. 22F07-MB2-7 16S ribosomal RNA gene, partial sequence	3.9738e-08	m54271_181220_115634/28902146/ccs
aerococcus viridans	1	0.01	gi|373279808|gb|JN713500.1| Aerococcus viridans canine oral taxon 331 clone 1D024 16S ribosomal RNA gene, partial sequence	0.0	m54271_181220_115634/29164363/ccs
carnobacterium sp	1	0.01	gi|164653349|gb|EU344922.1| Uncultured Carnobacterium sp. clone Hg5-12 16S ribosomal RNA gene, partial sequence	0.0	m54271_181220_115634/30081701/ccs
bacterium 16s	1	0.01	gi|18693144|emb|AJ408995.1| Uncultured bacterium 16S rRNA gene, clone HuCB15	0.0	m54271_181220_115634/31195328/ccs