FARM LEVEL SURVEY OF ENVIRONMENTAL CONTAMINATION BY PATHOGENIC BACTERIA: CASE STUDY OF UNIVERSITY OF LIMPOPO EXPERIENTIAL FARM

by

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DECLARATION

I declare that the dissertation hereby submitted to the university of Limpopo for the degree Master of Science in Agriculture (Animal Production) has not previously been submitted by me for a degree at this or any other university, that it is my own work in design and execution, and that all materials contained therein has been acknowledged.

Signature _____ Date_____

Shai K

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Dedication

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ABSTRACT

Identification of pathogens is a crucial step in the diagnosis of livestock diseases. An insight of bacterial diversity may enable decisions on appropriate treatment and shield farmers from tremendous losses due to disease outbreaks. The aim of this study was to investigate the occurence and diversity of pathogenic bacteria in the environment at University of Limpopo experiential farm using Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spctrometry (MALDI-TOF MS). The diversity of pathogenic bacteria were investigated in environmental samples collected from three different sampling locations within the farm (labelled according to the site of collections as cattle camp (CC), sheep camp (SHC), and goat camp (GC)). Total bacterial counts were calculated using the colony forming unit formula per gram of environmental sample (CFU/g) with the standard spread plate method. High bacterial counts were observed in CC: $7.25x10^7$ cfu/g and $4.5x10^6$ cfu/g for soil and faecal samples respectively while low counts were observed in SHC: $4.8x10^7$cfu/g for soil samples and $3.7x10^6$ cfu/g for faecal samples) for both soil and faecal samples. In soil samples, similar bacterial species were encountered at the different sampling locations. *Bacillus species* (*B. cereus, B. megaterium, B. simplex, B. mycoids, B. weihenstephansis, B. licheniformis and B. endophyticus*) were the most dominant across the different sampling locations in the soil samples. *Bacillus cereus* tested positive in 36 samples (33 %), however, the frequency of isolation dominated in the GC (50 %), SHC (41.6 %) CC with (28 %), and *Staphylococcus aureus* were the least isolated with a frequency of 1.4 % followed by *Listeria monocytogen*s with 2.8 % frequency in CC. In faecal samples, the occurrence and level of contamination with bacterial species varied across sampling locations with minor similarities observed in SHC and GC. *Escherichia coli* dominanted CC with 64.2 % frequency, and absent from SHC and GC. This study demonstrated the contamination of opportunistic, food borne bacteria like *B.cereus* and *L. monocytogenes* in the experiential farm environment and the need for good hygiene practices to prevent its entry into the food chain.

Key words: Livestock, Diseases, Soil, *Escherichia coli, Bacillus cereus.*

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1. CC -Cattle camp
2. SHC -Sheep camp
3. GC -Goat camp
4. SHCS -Sheep camp soil samples
5. GCS -Goat camp soil samples
6. CCS -Cattle camp soil samples
7. SHCF - Sheep camp faecal samples
8. GCF -Goat camp faecal samples
9. CCF -Cattle camp faecal samples
10. $^{+}$NSC - Number of positive samples
11. % FC - Percentage frequency in the camp
12. CFU -Colony forming unit
13. PCR - Polymarase chain reaction
14. ELISA - Enzyme-linked immunosorbent assay
15. MALDI-TOF MS - Matrix assisted laser desorption ionization-time of flight mass spectrometry

CHAPTER ONE

INTRODUCTION

1.1 Background

Small-scale livestock production in rural communities of South Africa contributes to food security as well as the provision of many social and economic attributes such as the promotion of livelihoods and socio-economic relief (Meissner *et al.,* 2013; Becker, 2015). Particularly in Limpopo Province, livestock production plays a significant role through the provision of meat, milk, manure and raw materials. Most households keep livestock for food security and religious purposes (Madziga *et al.,* 2013). However, livestock diseases that have devastating outcomes on animal health and that impact on national and international trade remain endemic in many parts of the world. Threats from old and new pathogens continue to emerge fuelled by changes in the climatic conditions. As a result, livestock from small scale farmers in rural areas suffer endemic diseases as they are kept under scavenging conditions which exposes them to virulent factors. In most cases, there is little attention to disease control and prevention in these livestock production systems (Gibbs, 2005).

According to the 'one health' principle which attempts to bring animal, human and environmental health under one umbrella, 60 % of all human infections are directly or indirectly linked to livestock disease outbreaks (FAO, OIE and WHO, 2008). Pathogenic bacteria are the aetiology of most diseases in small scale and commercial livestock farms. The extent of the diversity of microorganisms in soils determines the quality of pastures since a wide range of pathogenic bacteria are involved in pasture contamination from livestock manure (Bagge *et al.,* 2010). Young growing animals on pasture are especially prone to spore forming bacteria of the *Bacillus* and *Clostridium spp*. Livestock acquire the spores during grazing on contaminated pastures (Lange *et al.,* 2010; Cooper and Valentine, 2016). Once the bacteria are in abundance on pasture, cases of the disease usually occur frequently in susceptible herds and where disease outbreak is endemic, it is both an economic problem for farmers and an animal health problem.

Bacterial species have always been of considerable medical and economic importance and the world is faced with changing landscape of infectious diseases due to pathogenic bacteria that affect man and animals possessing significant threats to health and welfare of livestock (King *et al.,* 2010). Identification and differentiation of

the causative agent is crucial for implementation of prompt therapeutic and control measures since high morbidity of endemic diseases among small scale farmers reduces productivity. In this regard, there is a need to identify local pathogenic bacterial strains so that proper management and vaccination programmes can be instituted so as to minimise misuse of resources through vaccinating for non-prevailing bacteria (Neumann *et al*., 2002). Prevention and control of livestock diseases if often through commercial vaccines consisting of whole formularized cultures generally present as polyvalent formulation, Affected farms are usually those with irregular vaccination programs as farmers seek to minimise production costs (Maas, 2012).

1.2 Problem statement

In livestock disease endemic areas, pathogenic bacteria may be present in farm environments such as soil and faeces (Hang'ombe *et al.,* 2000). Christianssonn *et al*. (1999) reported bacterial contaminants of soil particularly *Bacillus cereus* to contaminate teats and udders of pasture fed cows leading to milk contamination and foodborne diseases. An analysis of the University of Limpopo experiential farm production records shows that diseases and conditions such as blackleg, pulpy kidney, abscesses, footrot and mastitis are among the endemic diseases at the farm. Consequently, production loss through livestock mortalities and reduced productivity are a serious threat to the viability of the farm. Considering the extensive production system practised at the farm, environmental contamination by pathogens such as bacteria could be a major source of disease outbreaks. Over the last few decades, several pathogens have either emerged or re-emerged (Jason, 2014). This study will address the problem of lack of documentation of sources of disease outbreak in the farm by identifying the pathogenic bacteria that contaminate the environment thereby posing a serious threat to successful livestock farming. Furthermore, identification of the environment contaminating pathogens will enable appropriate disease control strategies to be put in place at farm level.

1.3 Rationale of the study

This study applied a MALDI-TOF MS based method to detect and identify pathogenic bacteria in faecal and soil samples from the University of Limpopo experiential farm. The farm comprises different livestock species including beef cattle, small stock (sheep and goats), pigs and poultry. The focus of this study was on cattle, sheep and goats which are reared under an extensive production system According to McGuirk (2015) this production system increases the chances of disease outbreak as livestock will have access to pastures and water supplies that may be contaminated with disease causing agents such as bacteria. The farm relies on chemoprophylaxis as a disease management tool, however, the practice is irregular. The production system used may still allow livestock to shed bacteria into the environment through their faeces resulting in pasture contaminations. Bagge *et al.* (2010) reported manure to be the common pasture contaminant resulting in livestock infections. Therefore, this study screened the environment of the University of Limpopo experiential farm for pathogenic bacteria using MALDI-TOF MS test. This test was preffered as it is less laborious, very sensitive and is likely to give more accurate results since it can detect contaminating flora (Grenda and Kwiatek, 2010). Therefore, the study generated information on the prevalence and diversity of pathogenic bacteria prevailing in the livetock grazing environment at the University of Limpopo experiential farm. The information gathered from this study is a useful indicator of the risk of disease outbreak in the farm, which will enable the relevant stakeholders to design herd specific disease control programmes to improve herd health, safeguard animals grazing high-risk pasture and to develop good hygiene practices that will prevent the potential economic losses that come with pathogenic bacteria disease outbreaks (Fisher *et al.,* 1989).

1.4 Aim and objectives

The aim of the study was to carry out farm level investigation of the occurrence and diversity of pathogenic bacteria in the environment at University of Limpopo experiential farm.

The objectives of the study were to:

i. Determine the total bacterial counts in environmental samples collected from the cattle and small stock camps at the University of Limpopo experiential farm.
ii. Identify the pathogenic bacteria isolated from soil and faecal samples collected from cattle and small stock camps at the University of Limpopo experiential farm.

1.5 Research questions

i. Which livestock (cattle, sheep, goats) camp is more contaminated with pathogenic bacteria at the farm?
ii. Which pathogenic bacterial species are detectable with MALDI-TOF MS and responsible for the contamination of the livestock environment at the University of Limpopo experiential farm?

CHAPTER TWO

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2.1 Introduction

A gram of livestock manure or soil sample can habour millions of bacteria (Whitman,1998). Arable land and pastures contaminated with the faeces of sick animals, especially, contribute considerably to pathogen transfer resulting in farm environment pollution which is a major problem worldwide (Trawińska *et al.,* 2006; Nageswaran *et al*., 2012; Czekalski *et al*., 2012). Furthermore, the soil in high-production farms is commonly contaminated with microbial organisms, predominantly bacterial pathogens posing a significant threat to humans and animals (Godwin *et al*.,1997; Whitman, 1998; Douglas *et al.,* 2003). Stecher and Hardt (2008), reported bacteria in animal gastro-intestinal tract to aid digestion and deter pathogen colonization, but they can be excreted at high in faeces contaminating soils and pastures. These bacteria may persist due to differences in local soil conditions (Dapilly and Neyrat, 1999; Gale, 2004; Frey, 2007). The levels and types of pathogens occurring in livestock faeces vary with animal species, dietary sources, health status and age of the animals (Spiehs and Goyal, 2007; Hutchison *et al.,* 2005; Manyi-Loh *et al.,* 2016). Extensive livestock farming, which is the case at University of Limpopo experiential farm pose a threat to the environment, as livestock shed millions of bacteria through their faeces contaminating the soil and pastures and elevate the risk of disease breakouts. It is therefore apparent that these faeces or manure should be properly contained and managed so as to avoid posing a major epidemiological threat, though complete eradication of these bacteria cannot be achieved. Nonetheless, disease outbreaks can be prevented through vaccination when local strains are recognised and safeguard of human and animal health can be ensured (Radostitis *et al.*, 2000; Amin *et al.,* 2013; Manyi-Loh *et al.,* 2016).

2.2 Diversity of pathogenic bacterial species in farm environmental samples

Pathogenic bacteria are divided into Gram positive and negative. Furthermore, the Gram positive bacteria are divided into spore forming and non-spore forming bacteria of which some may be zoonotic, while some are non -zoonotic affecting only livestock. Several spore-forming bacteria are harmless ubiquitous bacteria commonly found in the soil as well as being natural colonizers of the gastro intestinal tract of warm-blooded animals (Postollecetal, 2012). Gram-positive organisms encompass more than 200 species that can form endospores, which make them resistant to extreme conditions

such as pressure, extreme heat or cold, drought, starvation, biocides, and UV radiation (Moeller *et al.,* 2008). Spore-forming bacteria belong to the phylum Firmicutes, which can further be divided into seven distinct classes: *Bacilli, Clostridia, Erysipelotrichia, Limnochordia, Negativicutes, Thermolithobacteria,* and *Tissierellia*, 13 orders, 45 families, and 421 genera (Seong *et al.,* 2018). Even with constant evolution and re-classifications, the *Bacillaceae* and *Paenibacillaceae* remained the most abundant families within the Firmicutes phylum, consisting of 67 and 56 species, respectively (Seong *et al.,* 2018). *Clostridium spp* grow only under anaerobic conditions (Gyles and Thoen, 1993; Songer and Post, 2005), while *Bacillus spp*, are facultatively aerobic (Quinn *et al.,* 1994). Their spores grow as vegetative cells under favourable conditions, but when growth conditions are poor or when deprived of nutrients, the bacteria can sporulate (Chauret *et al.,* 1999; Aitken *et al.*, 2005). These spores are highly resistant and can be transmitted by wind and water to the surrounding environment allowing colonization of new areas.

When penetrating and deep wounds are exposed to spores of these genus due to contact with soils, water or mud, the damaged tissues provide an anaerobic environment that allows the propagation of the bacteria and the production of its lethal toxin. Some of these bacteria are not only a problem in livestock but also affect human beings especially in the developing world where they are associated with high mortality. It is worth mentioning that vectors, such as flies and vermin, may also spread and cause subsequent infections to other animals with pathogens from mismanaged faeces which is common in extensive farming practices. The non-spore forming organisms constitute a large group of Gram-positive and Gram-negative bacteria that exist in the environment and as commensals on mucous membranes of animals and humans (Figueras and Borrego, 2010). Knowledge of these bacteria is incomplete, even though they are commonly implicated in necrotic and suppurative conditions.

2.3. Classification and description of the main bacterial pathogens in environmental samples associated with livestock infections

Manure and soil provide diverse biological and physiochemical environments to microorganisms. Livestock practices literally vary from one farm to another and from one geographical location to the other, but eventually, influence the microbial structure of faeces shed by the animals (Wang *et al.,* 2004; Manyi-Loh *et al.,* 2016). Both

animals and humans on and off farms are exposed to the potential health risks related to inadequate management of livestock faeces. The ability of these pathogens in manure to pollute, contaminate and infect the environment and livestock, depends on the pathogen's ability to survive in manure following excretion or the hygiene practices to prevent its entry into the food chain (Wang, 2004). The microbes presented below have been chosen based on their occurence and dissemination from environmental samples (soil and faecal) as well as their endorsed potential health threats to livestock and the environment as outlined by various researchers **(Table 2.1).**

2.3.1 Faecal samples

Studies on diversity of spore forming bacteria in cattle manure reported pathogenic bacteria of particular concern for animal health as, *Clostridium spp,* particularly *(C. chauvoei*, *C. botulinum*, *C. tetani*) and *Bacillus spp* (*B. anthracis* and *B. cereus*) (Bagge *et al.,* 2010; Adak *et al.,* 2002*;* Pachepsky *et al.,* 2006; Pell, 1997; Plaut, 2000 and Chauret *et al*.,1999). As well as bacteria like *Campylobacter spp (C. jejuni* and *C. coli), Salmonella enterica* (Adak *et al.,* 2002*;* Pachepsky *et al.,* 2006; Pell, 1997; Plaut, 2000). *Yersinia spp* (*Y. enterocolitica* and *Y. pestis*), *Leptospira spp* and *Coxiella burnetii* (Aitken *et al.,* 2005; Nightingale *et al*., 2004), *Mycobacterium avium* subsp, *paratuberculosis, Listeria monocytogenes* and *Escherichia coli* O157 (Robert *et al.*, 2017). All these bacterial species can cause serious clinical diseases in farm animals, when they are ingested in feed or water that has been contaminated with faeces, and/or saliva and nasal discharge such as in cases of Salmonellosis. Placental fluids from aborted foetus in sheep as a results of *Campylobacter* may results in pasture contaminations where animals graze, resulting in suffering or even death thus entailing production and economic losses for farmers (Zilbauer *et al.,* 2008). These bacteria are not only a threat to animals as they also cause foodborne diseases in humans. Bacteria like *L. mocytogenes* have been associated with human listeriosis (Nightingale *et al.,* 2004). It has been reported that manure from infected or shedding animals represent direct links between human infections and *L. monocytogenes* in farm animals and farm environments as a result of consuming animal-derived food products that are not processed before consumption like raw milk and raw foods of plant origin (Nightingale *et al.,* 2004). Several authors have commented on the fact that some of these organisms are zoonotic agents that can be shed into the faecal wastes generated by infected livestock (Jones, 1999). Though numerous pathogenic bacteria have been

discovered by various researchers in different regions, for the purpose of this study, only bacterial pathogens linked to livestock infections particularly cattle, sheep and goats will be discussed herein.

2.3.1.1 *Clostridium spp*

The class *Clostridia* consists of Gram-positive, anaerobic spore forming bacteria that are ubiquitous in the gastrointestinal tract (Marañón *et al.,* 2006; Prescott *et al.,* 2016; Smith *et al.,* 2014) and can be divided into two groups based on their ability to invade and multiply within living tissues. *Clostridium tetani* and *C. botulinum* belong to the the group that has little or no ability to invade and multiply in living tissues. However, their pathogenicity is manifested by the production of powerful toxins. The other group constitutes *C. chauvoei*, *C. septicum*, *C. haemolyticum*, *C. sordellii*, *C. perfringens*, *C. difficile* and *C. spiroforme* that can invade and multiply in living tissues or the intestines of the host animal. In addition, they produce less potent toxins in comparison to the former group (Bagge, 2009). Based on the individual species, some are responsible for diseases, including mastitis, tetanus, blackleg, haemoglobinuria, malignant oedema and infant botulism in cattle and humans (Williams, 2014).

Clostridium chauvoei occour in the soil, faeces, and the digestive tract of many animals, mainly in the form of spores that are highly resistant to environmental factors and many disinfectants (Cooper *et al.,* 2016; Habib *et al.,* 2015). Microscopically, *C. chauvoei* occur in pairs and rarely in short chains, but it is most frequently found as a single organism. Although traditionally considered a Gram-positive rod, the staining characteristics are variable and, particularly in old cultures, *C. chauvoei* may appear Gram negative. Most strains are motile, a feature that is associated with the presence of peritrichous flagella (Arndt *et al.,* 2016).The colonies of *C. chauvoei* are slightly raised, white-grey, and glossy, becoming increasingly opaque as incubation progresses. When spores are formed, usually after 24 hours of incubation, the colonies are completely opaque. They usually do not merge, but older cultures may show some degree of confluence (Arndt *et al.,* 2016).

The pathogenicity of *C. chauvoei* is linked to several toxins and virulence factors. Among these, several haemolysins result in cytolysis and haemolysis causing the characteristic lesions of blackleg (Popoff *et al.,* 2016; Uzal *et al.,* 2016). The main virulence factor of *C. chauvoei* is toxin A (CctA), β-barrel and pore-forming hemolysin

(Frey *et al., 2012;* Popoff*,* 2014*;* Popoff *et al.,* 2016; Uzal *et al.,* 2016). CctA is a soluble monomer that oligomerizes on the cell surface to form a polymer pore that perforates the cell membrane, resulting in disruption of membrane permeability and cell lysis. Novel experimental vaccines containing CctA demonstrate protective immunity in immunized guinea pigs (Frey *et al., 2012*). The role of commercial *Clostridium* and their associated factors in pathogenesis and pathology of blackleg infection was investigated after experimentally injecting gunea pigs and hill bulls (Vilei *et al.,* 2011). It was reported that toxins play a significant role in the pathogenic mechanism of blackleg infection. Infection of ruminants by *C. chauvoei* is caused by exposure of the animal to the pathogen present in form of spores in the soil of ‘‘poisonous’’ pastures (Wolf *et al.,* 2017). The bacterial spores are present in high numbers in the soil where cattle graze, thus, is a fatal disease of young cattle, sheep and other ruminant species. The infection starts after ingestion of endospores by susceptible animals. The spores pass the intestinal epithelium and enter the bloodstream to be lodged in the tissue of animal body especially in skeletal muscles, and lies latent till they are triggered and cause the disease (Habib *et al.,* 2015). Over exercise or injuries offer optimal environment for the germination of the endospores. The disease occurs globally among ruminants specified primarily as myonecrosis and characterized mainly by necrohemorrhagic myositis (Frey, 2015; Cooper and Valentine, 2016). The resulting blood poisoning leads to myonecrosis, oedemic lesions and fever, followed by lameness and death, causing significant economic losses in livestock production (Lange *et al*., 2010; Groseth *et al.,* 2011; Langroudi *et al.,* 2012; Frey and Falquet, 2015).

Most cases of blackleg occur in warm months of the year following excavation of soil, which can expose and activate latent spores. Also, the disease is enzootic in areas with history of flooding and is usually common that a large number of animals can be affected within a small-time frame (Lange *et al.,* 2010; Groseth *et al.,* 2011). Inam-ul-Haq *et al*. (2011) investigated epidemiological factors and economic losses related to blackleg infection in cattle. Their results showed that 16 % economic losses occurred due to morbidity and 84 % due to mortality. Although *C. chauvoei* is mainly considered to be specific to ruminants, rare fatal cases of fulminant human gas gangrene and neutropenic enterocolitis caused by *C. chauvoei* have been reported indicating possible zoonotic potential of the bacteria (Nagano *et al.,* 2008). It is assumed that

prevalence of *C. chauvoei* causing disease in human beings may be higher than currently diagnosed (Nagano *et al.,* 2008; Weatherhead *et al.,* 2012). Moreover, *C. chauvoei* has been detected in manure which represents a source of infection and can lead to contamination of pastures (Bagge *et al.,* 2009; Lange *et al.,* 2010).

Clostridium tetani natural inhabitat soil, but can also be isolated from faeces of domestic animals and humans (Smith, 2009). The incubation time varies from a few days to a month or more (Andrews *et al.,* 2004). Tetanus in ruminants is a fatal disease that often affects cattle and sheep, and is generally observed sporadically and with mortality rate of over 80 % (Radostits *et al.,* 2007). The disease in young ruminants and/or calves was reported to follow castration, shearing, tail docking, elastrator bands, dehorning, or infected umbilical cords as well as injections of pharmaceuticals and vaccines (Feqteau *et al.,* 2009). The spores of *C. tetani* are highly resistant and can be transmitted by wind and water to the surrounding environment allowing colonization of new areas. *Clostridium tetani* is not only a problem in livestock but does also affect human beings especially in the developing world where it is associated with high mortality (Dietz *et al.,* 1996). The organism remains at the site of entry, multiply, and produce a toxin that affects the nervous system, causing stimulation and contraction of skeletal muscles. The affected animal will appear stiff, the tail is held out, the head and neck stretched out, the third eyelid comes across the eye, and the nostrils are flared (Bruggemann, 2005). Eventually the animal will not be able to stand and goes into ‘tetanic’ convulsion. Respiratory failure ends in death. There is no natural immunity against tetanus. However, protection can be provided by active immunization with tetanus toxoid-containing vaccine (TT: formalin inactivated tetanus toxin) or administration of an anti-tetanus antibody (tetanus-specific immunoglobulin, TIG) (WHO, 2006).

Clostridium septicum, along with *C. chauvoei*, *C. perfringens* (*type A*), *C. sordelli* and *C. novyi* (type A) are the clostridia that produce malignant oedema (Sterne and Batty, 1975). Types of trauma that may predispose an animal to develop malignant oedema include intramuscular injections, parturition, shearing, castration, and tail docking (Mcguirk, 2015). *Clostridiu septicum* is found in the intestinal tract of most domestic livestock, and it is shed in their faeces, leading to pasture contamination. Toxins are produced at the entry site causing extensive swelling, with accumulation of bloody or clear fluid, and tissue death followed by gangrene. The affected area is extremely

painful. Toxins are absorbed into the bloodstream causing fever, weakness, trembling and then death. Vaccine is the most reliable form of control (Amimoto *et al.,* 2002).

Clostridium perfringens toxin types are widely distributed in nature and usually forms a part of the normal intestinal flora of man and animals though it can be found in the soil. *Clostridium perfringens* like all other bacterial species can be subdivided into strains according to the results of different typing methods. Although subdivision by serotyping was proposed in the past, division into strains according to the combination of toxin produced is still the most widespread and routinely used method. Genotyping is generally used only in PCR analysis of toxin genotype (Baums, 2004). *Clostridium perfringens* is the aetiology of enterotoxaemia an acute or peracute syndrome with a case fatality rate close to 100 percent, associated with an uncontrolled multiplication of *C. perfringens* in the small intestine (Songer and Uzal, 2005; Smith *et al.,* 2014; Prescott *et al.,* 2016). Sheep enterotoxaemia caused by *C. perfringens* (*types C and D*) is well documented. Different strains of syndromes include lamb dysentery, pulpy kidney and struck. The disease in adult sheep causes sudden death (Gkiourtzidis *et al.,* 2001; Savic *et al.,* 2012 and Goossens *et al.,* 2016). Unlike sheep enterotoxaemia, in cattle this condition has remained a poorly defined syndrome. Currently enterotoxaemia in cattle is characterised by sudden death, most often with haemorrhagic lesions at post-mortem and high colony counts of *C. perfringens* in small intestines (Prescott *et al.,* 2016). Field cases of enterotoxaemia remained difficult to confirm due to several toxins associated with the pathogenicity of *C. perfringens* until recent developments in molecular and biological techniques (Sawires and Songer, 2005; Sawires and Songer, 2006).

2.3.1.2 *Escherichia coli*

Escherichia coli is a Gram-negative, facultative anaerobic, rod-shaped and motile bacterium that exists as a normal flora in the intestinal tract of both healthy animals and humans. It serves as a reliable indicator of faecal contamination. *Escherichia coli* O157:H7 found in cattle manure has been reported as the most notorious pathogen which produces a potent toxin that can cause serious infection in environmental mastitis resulting in sudden onset of fever, loss of appetite, diarrhoea and toxaemia (Shipigel *et al.,* 2008). Though the bacteria are isolated from environmental samples, high milk contamination has been reported resulting in significant economic loses. Ali

et al. (2010), reported the losses due to mastitis globally to amount to about 53 billion dollars annually. India and United States of America (USA) have been reported to contribute considerably to these losses due to cattle mastitis were by annualy approximately 2 billion dollars are lost in the USA and 526 million dollars in India. Subclinical mastitis accounts for 70 % whereas clinical mastitis is responsible for 30 % of dollar losses (Varshney and Naresh, 2004). Severe economic losses due to mastitis occur from reduced milk production, treatment cost, increased labour, milk with held following treatment and premature culling (Miller *et al.,* 1993). Early identification of pathogens can result in early recognition of disease risk, a greater portion of this loss can be avoided (Sharif *et al.,* 2009).

2.3.1.3 *Campylobacter species*

Campylobacter species are most commonly found in the intestinal tracts of animals such as cattle, pigs, chickens and wild animals where they are responsible for a variety of disorders (Baserisalehi *et al.,* 2007). Salihu *et al.* (2009), reported that *Ca. jejuni* and *Ca. fetus* cause abortion, stillbirths and birth of weak lambs in sheep during late pregnancy. The campylobacter species has higly been associated with feed contamination mostly in poultry, however, the contaminations are indirect where food become contaminated when coming into contact with animal faeces. This alarms a need to identify these species at farm level so as to put hygiene strategies like proper manure management in place to prevent the cross contaminations. Similarly, when transmitted to humans after consuming undercooked agricultural food products and contaminated water, it causes an infection called campylobacteriosis, which is a self-limiting and sporadic illness (Adekunle *et al*., 2009; Inglis *et al.,* 2010). In 2008, campylobacteriosis was the principal cause of zoonotic disease in humans with 190,566 reported confirmed cases (EFSA, 2010). In 2007, more than 200,000 confirmed cases of human campylobacteriosis were reported by the 24 member states, with an EU notification rate of 45.2 cases per 100,000 inhabitants.

2.3.2 Soil samples

Pelczar *et al.* (1993), defined soil as the region on the earth's crust where geology and biology meet, the land surface that provides a home to plant, animal and microbial life. The numbers and kinds of microorganisms present in the soil depend on many environmental factors such as amount and type of nutrients available, available

moisture, degree of aeration, pH and temperature (Prescott *et al.,* 1996). Bacterial species reported to be common in soil include: *Bacillus spp*, *Proteus, Pseudomonas, Corynebacterium Flavobacterium* (Nieminen, 2007), *Aeromonas, Staphylococcus, Chromobacterium, Nocardia, Micrococcus Streptococcus, Alcaligenes*, *Serratia marscens* (Ongunmwonyi *et al.,* 2008), *Clostridium* (Bagge, 2010) and *Listeria monocytogenes* (Nightingale *et al.,* 2004).

2.3.2.1 *Bacillus spp*

The *Bacillus* genus is part of the *Bacillaceae* family and is probably the oldest and most diverse genus of bacteria. Hong *et al.* (2009) reported *Bacillus spp* to have a remarkable range of physiological characteristics that renders appropriate categorization and generalizations impossible. Although the bacteria have been isolated in faecal samples, their main habitat has always considered to be the soil, and it is now being suggested that soil may simply act as a reservoir for *Bacillus spp* (Hong *et al.,* 2009). Their expansive physiology has allowed these species to colonize almost all-natural environments including soil, air, lake sediments, water, and fodders (Claus and Berkeley,1986). *Bacillus spp* have the tendency to form spores and persist in that resistant and dormant state when growth conditions are unfavourable but revert to vegetative cells through germination as growth conditions become favourable. *Bacillus species* identified in manure upto date include *B. anthracis, B. cereus, B. subtilis, and B. thuringiensis,* which are Gram-positive rods, aerobic spore formers and are mostly harmless and can persist for over years in the soil contaminated by manure (Manyi-Loh *et al*., 2016).The most peculiar of these species is *B. anthracis* that causes anthrax (a life threatening and dreaded disease) and *B. cereus* associated with foodborne diseases in human beings and mastitis in ruminants respectively (Bravata *et al.,* 2007).

The species of *Bacillus* and related genera have long been troublesome to food producers on account of their resistant endospores. Their spores may be present in various types of raw and cooked foods, and their ability to survive high temperatures makes it difficult to control (Anderson *et al.,* 1995; Ryu and Beuchat, 2005). The bacteria is asscociated with two types of illines the emetic and the diarrheal type resulting from consumption of contaminated starchy food such as rice and pasta. The accurate number of food poisonings caused by *B. cereus* in different countries is not known because it is not a reportable illness and is not always diagnosed (Kotiranta *et*

al., 2000). In the early 70's, outbreaks caused by *B. cereus* were only characterized by watery diarrhea, occurring 8–16 hours after ingestion of the contaminated food. However, later, a new form of *B. cereus* food-poisoning, characterized by nausea and vomiting was identified in United Kingdom (UK) due to the consumption of rice from Chinese restaurants and take-away outlets. As many as 192 such incidences involving more than 1,000 cases were reported in the UK between a period of 1971 and 1984 (Kramer and Gilbert 1989).

Other outbreaks of diseases have been reported in different parts of the world: in Hungary (117 outbreaks) between 1960 and 1968, followed by Finland (50 outbreaks), Netherlands (11 outbreaks) and Canada (9 outbreaks) (Gilbert 1979; Shinagawa 1990). Besides these, there are many reports of food-borne outbreaks of *B. cereus* from a large variety of foods in many countries including the USA (Bean and Griffin 1990), UK, Scandinavia, Japan (Johnson *et al.,* 1984) and Norway (Kotiranta *et al.,* 2000). In 1923, anthrax was reportedly responsible for the loss of 30 000- 60 000 animals in South Africa (Sterne, 1967; Turnbull, 1991; Gilfoyle, 2006). However, the introduction of anthrax vaccines reduced the outbreaks subsequently (Gilfoyle, 2006). This microbe is aerobic or facultative anaerobic, Gram-positive, non-motile rods measuring 1.0 µm wide and 3.0-5.0 µm long capable of forming central or terminal spores (endospores) (Dixon *et al.,* 2000). Clinical symptoms in animals include fever, shock, multiple organ failure, discharge/bleeding from various orifices, cardiac distress, respiratory distress and oedematous swelling of the tongue, face and throat and often, death occurs shortly after the appearance of the first symptoms (Leppla *et al.,* 2002; Vilas-Boas *et al.,* 2007; Hambleton *et al.,* 1984). *Bacillus cereus, B. pumilus and B. licheniforms* have also been reported to be species of life threatening to livestock (Quinn *et al.,* 1994 and Turnbull *et al.*, 1979). However, apart from *B. anthracis* and the previously mentioned species, most *Bacillus spp.* are harmless saprophytes.

2.3.2.2 *Listeria monocytogenes*

Listeria monocytogenes is a Gram-positive, facultative, intracellular, rod-shaped bacterium that grow over a wide range of environmental conditions (pH and temperature) thus a longer survival duration in the environment (Uzeh and Adepoju, 2013). Livestock farms serve as the main reservoir and elevated levels of the pathogen occur in animals fed with improperly fermented silage contaminated by growth on

manure-fertilized soils (Nightingale *et al.,* 2004). It is incriminated as the causative agent of severe illness in ruminants. *Listeria monocytogenes* can be shed in the faecal material of clinically affected animals, however, healthy animals also can be latent *L. monocytogenes* carriers (Pell, 1997). Although most animal listeriosis appears to be caused by ingestion of silage contaminated with elevated levels of *L. monocytogene*s, not all cases are feed borne (Wiedemann *et al.*,1997).

Table 2.1: Common bacterial pathogens in farm environmental samples (soil and faecal samples)

Pathogen type	Disease/ symptom	% Prevalence in environmental samples	References
Clostridium spp	Blackleg, Tetanus	0-16	Bagge *et al.* (2009), Frey and Falquet, (2015)
Escherichia coli	Mastitis	16	Chekabab *et al.* (2013)
Salmonella	Salmonellosis	0-3	You *et al*. (2006)
Campylobacter	Abortion and still birth	31	Hakkinen *et al.* (2007); Olson. (2001)
Bacillus spp	Anthrax, mastitis, arbotions	-	Nieminen, (2007); Manyi-Loh *et al.* (2016)
Listeria monocytogenes	Neurological signs, facial paralysis, an keratisis	24	Nicholson *et al.* (2005); Vivante *et al.* (2013)
Staphylococcus spp	Mastitis	10-12	Ongunmwonyi *et al.* (2008)

(-) The study was not focusing on percentage prevalence but rather on the surveillance of pathogenic bacteria in environmental samples.

2.4 Diagnostic techniques used in identification of pathogenic bacteria

An enormous array of diagnostic techniques exist in different laboratories, however, these techniques differ in terms of reliability, laborious, time consumptions, expensiveness and effectiveness and the researcher's decisions on which tests to use is often influenced by the availability of resources at local diagnostic laboratories. It is quite unrealistic to enumerate all the microbial pathogens present in cattle faeces because of the massive numbers of these pathogens that populate the gastrointestinal tract and the other systems in the animal (Manyi-Loh *et al.,* 2016).

Diverse groups of microbial pathogens are involved due to the vast array of physical, chemical and biological constituents contained in cattle faeces (Manyi-Lotch *et al.,* 2013). However, it is impossible for every individual pathogen that constitute the entire bacterial organization in faeces to be isolated, characterized and identified (Shroeder, 2003). These challenges may be linked to the limitations in some of the available methods failing to identify some of these pathogens owing to the state they may be exhibiting at that particular instance. In addition, differences in the DNA extraction and purification methods can affect DNA quality, which in due course can affect the interpretation of information regarding the microbial communities coexisting in environmental samples (Arbeli and Fuentes, 2007; Yamamoto *et al.,* 2009). Furthermore, some pathogens may require time-intensive tests and enrichment steps during analysis and detection, thereby making their quantification complex (Bagge, 2009).

It has been observed that different bacterial pathogens pose different symptoms in distinct species. Presumptive diagnosis of these diseases is achieved by clinical history, pathological findings, and histologic changes. Confirmation is usually performed by isolating, identification and characterization of pathogens from the clinical samples such as affected muscle piece, body fluids, faeces and contaminated soil samples using culture and PCR methods (Sasaki *et al.,* 2000). Conventional microbiological methods like gram staining, biochemical tests, animal pathogenicity tests and/or immunodetection methods, including fluorescence antibody test (FAT) and immunohistochemistry (IHC) can also be used (Naz *et al*., 2005; Abreu *et al.,* 2016). In realising the need to confirm the clinical and biological findings, microbiologists

developed molecular techniques in diagnosis of pathogenic bacteria with improvements occurring year on year (Sasaki *et al.,* 2000).

With these new developments, spending days in the laboratory in the process of identifying bacteria from clinical samples based on conventional phenotypic, time consuming method is slowly becoming an issue of the past (Chiu, 2014). For difficult to identify bacteria phenotypic methods might be inconclusive and the definitive identification is usually performed by sequencing of the 16 ribosomal RNA gene which has limitation to identify species in some genuses (Janda and Abbott, 2007). A variety of rapid typing methods have been developed for isolates from clinical samples, these include molecular techniques such as, enzyme-linked immunosorbent assay (ELISA) and matrix assisted laser desorption/ ionization time of flight mass spectrometry (MALDI TOF-MS) (Badri *et al.,* 2016).

These approaches enable subtyping of strains, but do so with different accuracy, discriminatory power, and reproducibility. However, some of these methods remain accessible to reference laboratories only and are difficult to implement for routine bacterial identification in a clinical laboratory. Irrespective of these challenges, bacterial detection and identification is a highly relevant issue in microbiological diagnostics. Conventional microbiology is an expensive but protracted diagnostic method and there are certain limitations associated with it (Malhotra *et al.,* 2014). Molecular methods have been increasingly incorporated in laboratories, particularly for the detection and characterization of isolates and for the diagnosis of diseases due to fastidious, slow growing, non-viable or non-cultivable organisms which cannot be detected by conventional culture techniques (Nolte, 2003). The diagnosis of diseases depends on observation of clinical symptoms, followed by immunological methods, such as immunofluorescence (Noble, 2007). However, these diagnostic methods are time consuming and laborious (Malhotra *et al.,* 2014).

2.4.1 Enzyme-linked immunosorbent assay (ELISA)

ELISA is a biochemical technique that combines an immunoassay with an enzymatic assay. An antibody bound to a solid matrix is used to capture the antigen from enrichment cultures and a second antibody conjugated to an enzyme is used for detection. The enzyme is capable of generating a product detectable by a change in colour (Cohen and Kerdahi, 1996; Jasson *et al.,* 2010). Detection using automated and

robotic ELISAs is widely used since they can reduce detection times after enrichment to as low as 1–3 hours (Thacker *et al.,* 1996). Thus, the results for fast growing bacteria can be obtained in two to three days instead of the 3–5 days needed by conventional methods (Leon-Velarde *et al.,* 2009). There are many commercial enzyme immunoassays for detecting the main pathogens and toxins in environmental samples. Bacteriophage recombinant protein technology can also be integrated in detection methods as part of improved immunological qualitative tests (Jasson *et al.,* 2010; Savoye *et al.,* 2011).

2.4.2 Polymerase chain reaction (PCR)

PCR is considered as an effective method for tracking and tracing of neurotoxigenic pathogens responsible for human and animal infections (Fach *et al.,* 2009). Nucleic acid amplification of a specific target region of the bacteria genome by PCR has increased in molecular diagnosis to the point where it is now accepted as the gold standard for detecting nucleic acid from several origins and it has become an essential tool in the research laboratory (Halm *et al.,* 2010). The potential of this technique to provide sensitive, specific and swift detection and quantification nucleic acids has made it an indispensable tool for state-of-the-art diagnosis of important human and animal bacterial pathogens for detection and diagnostic purposes (Frey *et al.,* 2012).

PCR can amplify DNA specifically from low numbers of bacteria, its simplicity, rapidity and reproducibility, offers advantage over conventional methods for identification (Millar *et al.,* 2002). Halm *et al.* (2010) reported excellent specificity (100 %) of real-time PCR when testing *Clostridium* species. Hundred percent of *C. chauvoei* or *C. septicum* strains were successfully identified by the real-time PCR assay. In one preliminary study, fresh samples of the injured muscle and liver were collected from one cattle, which was suspected of blackleg. These tissue samples were subjected to the immunofluorescence assay (IFA) and PCR. The PCR assay revealed the presence of *C. chauvoei* DNA in both tissues, while the IFA detected organisms only from muscle (unpublished data). This suggested that the one-step PCR could be useful as a diagnostic tool in the outbreaks of blackleg in ruminants, compared to the immunological method. Therefore, the effectiveness of vaccines with local strains

influenced, the aim of this study so to help vaccine developers in recognition of local strains, so as to prevent future production losses due to disease outbreaks.

2.4.3 Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS)

MALDI-TOF MS technology has proved to be a rapid and inexpensive method for the accurate identification of different microorganisms over the past years (Patel *et al.,* 2015). Several studies were carried out to analyse MALDI-TOF MS's ability to identify anaerobic isolates (Justesen *et al.,* 2011; Lee *et al.,* 2015). However, both the use of formic acid for the on-plate sample preparation and the updated Biotyper database (Bruker Daltonics, Bremen, Germany) have recently improved the identification of anaerobes and has allowed a rapid, specific, and low-cost identification of most common bacterial species of clinical interest within few hours (Bizzini *et al.,* 2010; Eigner *et al.,* 2009; Lagacé-Wiens *et al.,* 2012). Many studies have reported the successful identification with MALDI-TOF MS of *Streptococcus* and *Staphylococcus* with 99.3 % identification at the species level and only one strain identified at genus level 0.7 % (Szabados *et al.,* 2010; Dubois *et al.,* 2010; Cherkaoui *et al.,* 2011).

Gram-positive rod identified with the Brucker system revealed identification of 92.49 % at the species level (Alatoom *et al.,* 2012). Pavlovic *et al.* (2012); Risch *et al.* (2010); Steensels *et al.* (2011), identified anaerobic bacteria of members of the *Enterobacteriaceae* family with 82 % at the genus level and 79 % at species level using the Brucker Microflex Maldi-Tof instrument with Biotyper software. *Haemophilus, Aggregatibacter actinomycetemocomitans, Aggregatibacter aphrophilus, Cardiobacterium hominis, Eikenella corrodens,* and *Kingella* 93 % of these (HACEK) group were identified at the genus level using the brucker data base, and 100 % were identified at the genus level using a custom database that included clinical isolates (Couturier *et al.,* 2011; Culebras *et al.,* 2012; Fedorko *et al.,* 2012). However, information regarding the yield of MALDI-TOF MS in other difficult-to-identify bacterial isolates is not abundant (Alby *et al.,* 2013; Melo-Oliveira *et al.,* 2013; Seng *et al.,* 2013). Nevertheless, the successful identification of different bacterial species at genus and species level, divulge that MALDI-TOF MS can be used as a reliable tool for identification of bacterial species.

2.5 Methods for prevention and control of livestock diseases

2.5.1 Traditional methods

Traditionally, the Limpopo Province has been known to be among the provinces in the country with the highest livestock populations. However, its agricultural contribution to economic growth of the country is at the lowest. The majority of the inhabitants are pastoralists and, as a result, highly dependent on livestock and livestock products. Livestock productivity is however, frequently affected by different diseases including those of bacterial origin such as blackleg, mastitis and abortions. Since ancient times, plants have been used all over the world as unique sources of medicines for livestock (Ribeiro *et al.,* 2010). The same practice has also been witnessed in the Limpopo Province since modern livestock health care is less accessible due to inadequate veterinary services and reduced drug supply (Ribeiro *et al.,* 2010; Runyoro *et al.,* 2006). Consequently, small scale farmers often rely on their traditional knowledge and locally available materials (mainly plants) in the management of livestock diseases.

Frequent use of medicinal plants has been documented by several surveys in South Africa (Bisi-Johnson *et al.,* 2010; Mahwasane *et al.,* 2013; Mulaudzi *et al.,* 2013; Samie *et al.,* 2010). Ethnoveterinary practice in the province has, however, been affected by the acculturation and depletion of plants because of environmental degradation, deforestation and over exploitation of the medicinal plants. Alternative remedies such as plant materials (*Ricinus communis* and *Gadernia spatulufolia*) and non plant materials (potassium per manganate and hot iron) have also been reported to be popular among small scale livestock farmers in the management of blackleg (Moyo *et al.,* 2014). The depletion of medicinal plants recently due to increased population alarms the need to recognise local pathogen strains so that reliable drugs and vaccines can be produced for proper management of livestock diseases (Nishteswar, 2014).

2.5.2 Modern methods

Modern methods of preventing and controlling livestock diseases include chemotherapy and chemoprophylaxis (vaccination). Vaccination is a procedure that is a vital component of the health management of many livestock-producing operations. The advantages of vaccination are numerous (Pastoret *et al.,* 2004; Falquet *et al.,* 2013). Antibiotic and/or anthelmintic resistance, and the problem of pharmaceutical

residues, promote the use of vaccines rather than chemotherapy. Furthermore, vaccines are environmental friendly. Uzal (2012) reported clostridial vaccines as bacterins, and chemically toxoided culture supernatants that are generally available in polyvalent formulations. However, ineffective vaccination policy and lack of cold chain for vaccination storage limits disease control (Ushe *et al.,* 2010). Conventional vaccines and services are expensive and require veterinary technology in the storage and administration of the vaccine (Sori *et al.,* 2004; Harun-or Rashid *et al.,* 2010). Several studies conducted on effectiveness of vaccines for animal and public health, reported reduced cases of diseases and in some instances hundred percent disease eradication, with the best vaccine reported to be the one made from local pathogen strains isolated from the area (Pastoret *et al.,* 2004; Radostitis *et al.,* 2000). The evidence for the efficacy of vaccines is mostly subjective or based on measurement of antibody titers in vaccinated animals. Nevertheless, the limited information available shows that vaccines are highly effective in preventing bacterial diseases after natural exposure, and less effective against experimental challenge with *C. chauvoei* (Uzal, 2012). Thus, it is necessary that local pathogen strains should be recognised so that vaccines can be developed for better disease management at farm level.

2.6 Conclusion

Livestock production plays a key role in the livelihoods of rural farmers in ensuring food security and income. However, the benefits of livestock farming are not always realised as a result of disease outbreaks. Some livestock diseases are zoonotics thereby threatening the health of rural farmers. Previous research reported the prevalence of pathogenic bacteria to be high where there is no proper manure management and where vaccination is not practised, with few cases of the disease reported with vaccine inclusion. Information on the prevalence and diversity of pathogenic bacteria at farm level is limited. Thus, it is necessary to carry out farm level investigations of the prevalence and diversity of pathogenic bacteria in the environment so that vaccines can be developed from local pathogen isolates. This initiative can be used in combination with effective farm management programmes so as to reduce production and economic losses due to livestock diseases.

CHAPTER 3

METHODOLOGY AND ANALYTICAL PROCEDURES

3.1 Study area

The study was carried out at the University of Limpopo experiential farm. The farm is situated 9 km north-west of the university campus (23º49' S; 29 º41' E), in Limpopo Province, South Africa. The climate of the study site is classified as semi-arid with the annual precipitation of roughly ±495 mm per annum and the mean annual temperature of 25±1ºC (max) and 10±1ºC (min) (Moshia *et al.,* 2008). The farm is a multidisciplinary experiential farm, were research in agronomy, soil science, plant pathology, and animal production is being carried out. This farm comprises different livestock species including beef cattle, small stock (sheep and goats), pigs and poultry. However, this study focused only on beef cattle and small stock. During the study period, the farm comprised of sixty-three cattle of the Nguni breed, forty-six sheep of Dorper and Pedi breeds and forty-three goats of different breeds including the Boer goat, Cross bred red Kalahari, and Pedi goats. This farm comprise 1705 ha and of this 1600 ha are devoted for natural grazing as well as planted pastures for intensive sheep grazing have been established on 2 ha.

3.2 Collection of environmental samples

3.2.1 Soil samples

Soil samples used for this study were collected from three different camps at the farm. Hundred soil samples were collected for the isolation and identification of pathogenic bacteria over the period January to March 2018. The samples were labelled according to the site of collection as cattle camp samples (CCS) (60 samples), sheep camp samples (SHCS) (20 samples) and goat camp samples (GCS) (20 samples). The sampling locations were divided into resting points and from each resting point, samples were collected at random **(Figure 3.1 and Figure 3.3).** A zigzag format of sampling was used and approximately 100-200 gram soil samples were collected from 6-8 centimetre depth using a spade and transferred to sterile sealable plastic bags. The soil samples were brought to the Biotechnology laboratory at the University of Limpopo for analysis. In cases where samples were not attended to immediately, they were stored in a refrigerator at 4^0C for not more than 24 hours (Brooks, 2016).

3.2.2 Faecal samples

Cattle faecal samples were collected as cowpats on grassland while sheep and goat faecal samples were collected as pellets. Seventy faecal samples, cattle camp (30 samples); sheep camp (20 samples) and goat camp (20 samples) were collected. Approximately 100-200 gram faecal samples were collected in sterile sealable plastic bags around the livestock resting points and common pastures **(Figure 3.2, Figure 3.3 and Figure 3.4)**. The samples were then transported on ice, in a Styrofoam cooler box and transported to University of Limpopo, Biotechnology laboratory for the isolation and identification of pathogenic bacteria. The differences in the number of environmental samples collected was due to different camp sizes, cattle camp is much bigger than the sheep and goat camps resulting in easy access of additional samples.

During sample collection, the environmental samples were collected from different resting points within camps in the farm. Figure 3.1 -3.4 depict the environment from where samples were collected.

Figure 3.1 Collection of soil samples from cattle grazing points in the cattle camp at the University of Limpopo experiential farm.

Figure 3.2 Collection of faecal samples from cattle grazing points in the cattle camp at the University of Limpopo experiential farm.

Figure 3.3 Water source for livestock at the University of Limpopo experiential farm from where environmental samples were collected.

Figure 3.4 Collection of faecal samples from small stock camps at the University Limpopo experiential farm.

3.3 Microbial analysis of environmental samples

3.3.1 Procurement of consumables

All the required consumables for the research, including, reagents, media, pipettes, gloves, face masks and petri-dishes were purchased from Prestige laboratories (Pty) Ltd, Durban, South Africa. The entire process of microbial analyses was performed under a sterile condition in a laminar flow with a Bunsen burner to avoid contamination from the environment. The collected samples were cultured on selective and general media for isolation of pathogenic bacteria.

3.3.2 Preparation of the media

The following media were prepared and used for detailed investigation of pathogenic bacteria according to the manufacturer's instructions. Reinforced Clostridia agar (51 g), Reinforced Clostridia broth (38 g), sheep blood agar (24.5 g), anaerobic basal broth (35.4 g) and nutrient agar (28 g) media were suspended into 1000 ml of distilled water, dissolved and sterilized by autoclaving at 121˚C for 15 minutes under $1.0342x10^5$ Pa; then allowed to cool at 45˚C and dispensed in sterilized petri dishes to form a thick layer and was allowed to solidify. Mitchell and Wimpenny (2007) reported the amount of solid medium in the plate has an impact on colony morphogenesis. Therefore, the height of solid media per plate was standardized to approximately 15 ml per 90 cm plate. The agar was poured into sterile petri dishes and allowed to solidify at room temperature. Both the agar and broth medium were kept in a refrigerator for later use. However, for sheep blood agar, 51 g of dehydrated blood agar base was suspended in 1000 ml of distilled water and boiled until dissolved completely. It was then sterilized by autoclaving at 121˚C for 15 minutes under $1.0342X10^5$ Pa. After autoclaving the media was allowed to cool at room temperature then enriched with 5 % defibrinated sheep blood. The medium was then poured in the sterile petri dishes in a volume of 10 ml quantities in each to form a thick layer and then kept at room temperature for solidification. For saline solution, 9 g of sodium chloride (NaCl) was suspended into 1000 ml of distilled water, autoclaved and allowed to cool.

3.3.3 Bacterial culture of environmental samples

The serial dilution method was used to reduce the bacterial concentration of the original soil sample to levels low enough for single colonies to be grown on agar plates, allowing for calculation of the initial counts of bacteria in the soil sample. One gram of the soil sample was weighed on a sterile plastic weighing boat and transferred immediately to the centrifuge tube containing 9 millilitres of normal saline. The contents of the tube were mixed thoroughly by vortexing at approximately 150 rpm for 1 minute and 1 millilitre of the aliquots were aseptically pipetted to 9 millilitres of the next test tube of normal saline. This continued up to the sixth test tube, dilution ratios included were 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} **(Figure 3.5)** (Koch, 1883). Two millilitres of the supernatant from the different dilutions were transferred to empty sterile tubes and heat at 65°C for 10 minutes and two millilitres of this were enriched in 10 millilitres Fastidious Anaerobic Broth (FAB, LabM, Bury, Lancashire, England), and heated at 65°C for 10 minutes. For plate counts, 0.1 millilitre of aliquots from different dilutions were transferred to petri dishes with corresponding labelling containing Reinforced Clostridia Agar (RCA) and spread over the surface by slowly shaking the plate and incubated anaerobically at 37ºC for 48 hours. After 48 hours, plates were removed from the incubator and characterization of the colonies and physical colony count was done.

A schematic represention of serial dilution of environmental samples into saline solution, so to reduce the concetration of bacteria in the sample to levels low enough to allow for bacterial colony growth on agar plates.

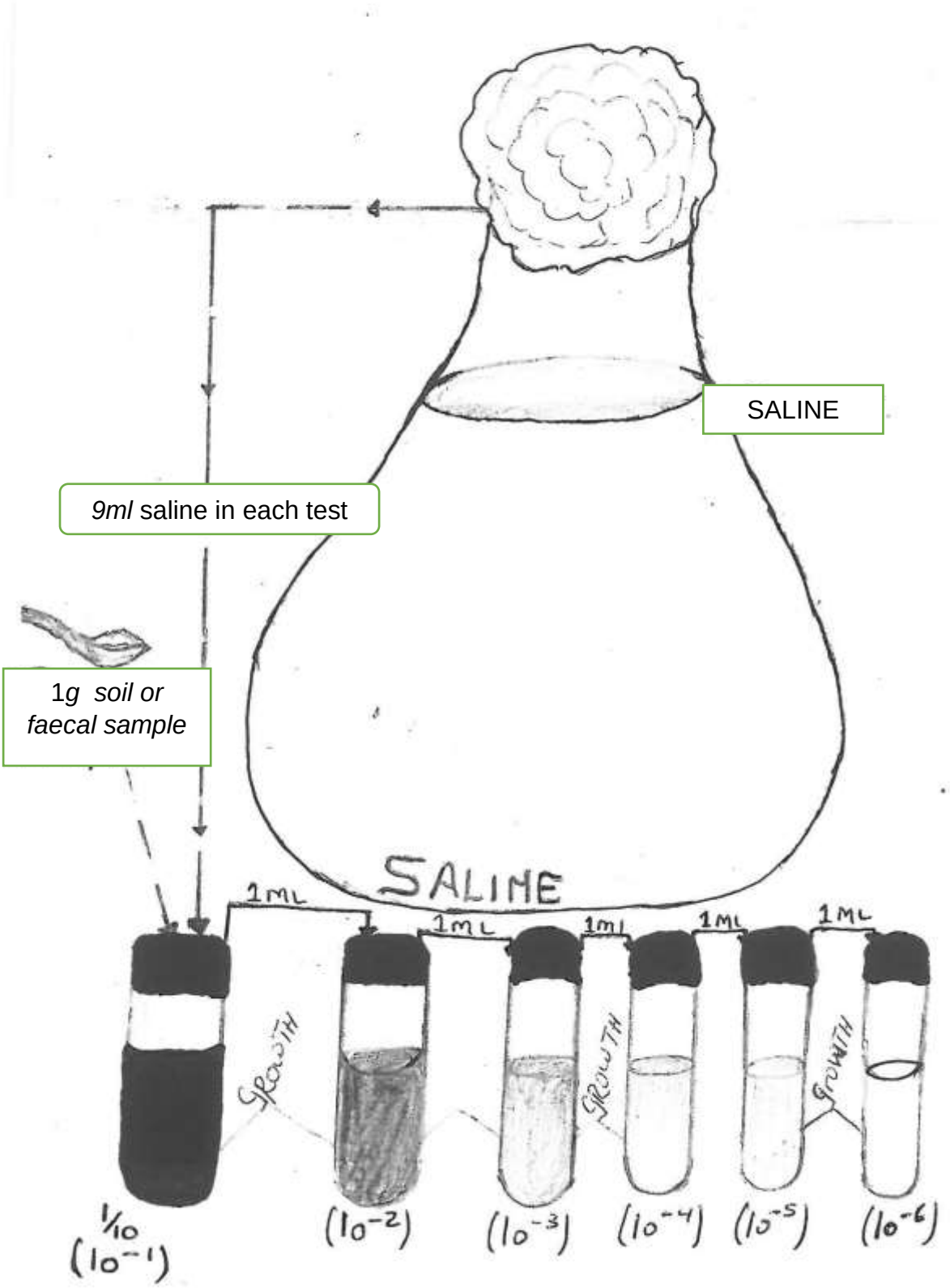

Figure 3.5: Serial dilution of environmental samples from the farm.

3.4 Purification of bacteria and determining total counts

After incubation of the plates at a temperature of 37°C for 24 to 48 hours, colonies were physically observed from petri dishes. Suspected colonies were picked using sterile inoculating loop and streaked on nutrient agar plates (using a four-way streaking method). The plates were incubated anaerobically at 37˚C for 48 hours to obtain a pure culture (Bagge *et al.,* 2009). Thereafter, manual colony count per plate was done to estimate the number of cells present. Routine manual counting was performed by two independent people with the hand counter. Counted colonies were marked with a marker on the plate cover to discriminate counted from uncounted colonies. Plates with over hundred colonies were counted by dividing the plates into two equal sections (Brugger, 2012). After counting one section, the count was multiplied with the total number of sections to estimate whole plate colonies and the data was recorded on an excel spreadsheet. Subsequently, one loopful of colony material was collected for proteomic identification using MALDI-TOF MS

3.5 Proteomic identification of colonies using MALDI-TOF MS

All bacterial isolates reported in this study were analysed by MALDI-TOF MS, using a Microflex LT bench top mass spectrometer (Bruker Daltonics, Maldi Biotyper). The software for the control of the instrument was FlexControl 3.3 and Maldi Biotyper 3.1 (Bruker Daltonics) for the analysis of the spectra and comparison with the database. A bacterial test standard provided by the manufacturer was included in every run for calibration purposes. Default settings (acquisition of mass spectra in the linear positive mode within the 2e20 kDa range, ion source 1 (IS1) 20 kV, IS2 18.05 kV, lens 6.0 kV, linear detector 2,560 V) were applied for all the detections. A rapid, on-plate method that requires less time and reagents for its performance was followed in this study (Matsuda *et al.,* 2012; Rodriguez-Sanchez *et al*., 2014). Fresh plates with isolated colonies of purified cultures grown on nutrient agar were selected. Different colony types matching colony description of different pathogenic bacteria with reference to literature (Holt *et al.,* 1994) were carefully chosen for bacterial identification using MALDI-TOF MS. Three hundred microliters of deionized water were pipetted into sterile Eppendorf tubes, and a small quantity of biological material (5-10 ml) between one single colony was placed into the tubes and mixed thoroughly through vertexing. Nine hundred microliters of absolute ethanol were added into the tubes and thoroughly

mixed. The tubes were centrifuged at maximum speed of (13,000 – 15,000 rmp) for two minutes. The supernatant was discarded, and thereafter the tubes were centrifuged again, and all the residual of the absolute ethanol were removed carefully by pipetting off waste without disturbing the pellet. The ethanol pellet was air dried at room temperature for three minutes, and 70 % formic acid (1-80 microliter) were added to the pellet and mixed very well by vertexing. Pure acetonitrile (1-8 microliter) was added and mixed carefully. The tubes were then centrifuged for two minutes at maximum speed such that all material is collected neatly in a pellet, and 1 microliter of the supernatant/ bacteria was drawn with a pipette and spotted on a polished steel MALDI- target plate and allowed to dry at room temperature. Once the spot was dried, it was overlaid with 1 microliter of HCCA solution (matrix) (a-cyano-4-hydroxycinnamic acid solution in 50 % acetonitrile and 2.5 % trifluoro acetic acid) prepared following the manufacturer's instructions. When the matrix was air dried, spectra were acquired by the mass spectrometer and compared with the database and the results were obtained within 10 to 15 minutes. Besides the cut-offs proposed by the manufacturer (≥3.0 and ≥ 1.9 for species and genus identification, respectively) score values ≥1.5 were established as the cut-off for low-confidence identification in this study, as these criteria have already been applied and accepted by other researchers (Hsu and Burnham, 2014; Fedorko *et al*., 2012; Rodríguez-Sanchez *et al.,* 2014). Isolates identified with score values below 1.5 were only considered when the first three identifications provided by MALDI-TOF MS were consistent. Otherwise, the identification was considered not reliable. The score values interpretation according to the instrument's instructions are: no reliable identification (0.000-1.669); probable genus identification (1.7-1.999); secure genus identification, probable species identification (2000-2.999); highly probable species identification (2.300-3.000).

3.6 Data presentation

The numbers of viable bacteria per gram were obtained using a mathematical formula for colony forming units (CFU) per gram of environmental sample. Diversity, occurrence and frequency of isolation of pathogenic bacteria identified in environmental samples using MALDI-TOF MS are presented with descriptive statistics (tables and charts). Microsoft Excel, 2013 was used to compare the level of contamination in faecal and soil samples.

$$\text{CFU/g} = \frac{number\ of\ colonies\ per\ agar\ plate\ X\ Dilution\ factor}{volume\ of\ culture\ plated}$$

CHAPTER 4

RESULTS

4.1 Purification of bacteria and total colony counts

Environmental samples were cultured on different medium thus, Reinforced Clostridia agar and sheep blood agar in different dilution factors of 10^{-0} to 10^{-6}. Samples cultured on sheep blood agar grew and triggered haemolyses within 24 hours of incubation at 37˚C. The isolates appeared flat and raised with the shape being filamentous to irregular, round and punctiform. They were smooth or wrinkled and the colour ranged from grey, cream to white and with few colonies being yellow (**Figure 4.1-4.3**). Different colonies subcultured on nutrient agar for purification were able to grow under anaerobic conditions within 24 to 36 hours of incubation at 37˚C. Plates of pure cultures taken for bacteria count demonstrated that each gram of environmental samples produced a different number of colonies per plate. Some plates had colonies which were too numerous to count (TNTC) and were discarded. These are colonies which were above 300 per plate and others were below the limit of quantification (LOQ) with less than 30 colonies per plate. Plates with a dilution factor of 10^{-5} were selected since the colony counts per plate were falling within the number of colonies allowed on agar plates (Keil *et al.,* 2015). The average colony counts per plates were used to determine the colony forming units per gram of environmental sample (CFU/g). The average colony forming units per camp in soil samples ranged $5X10^7$ to $9.5x10^7$ of CC, $4.9X10^7$ to $6.1X10^7$ of GC, and $3x10^7$ of SHC (Figure 4.4).

For faecal samples the average colony forming unit per camp ranged from $3.9X10^6$ to $5.2X10^6$ of CC, $3x10^6$ to $5.1X10^6$ of GC, and $3.3X10^6$ to $4X10^6$ of SHC (Figure 4.5). Overally, there were differences in colony counts. These differences were observed with the different sampling locations, and with different samples. Cattle camp soils produced more colony counts per plate as compared to the sheep and goat camps. Again, there were differences in colony counts of soil samples as compared with the ones for faecal samples.

Figure 4.1 Plates of colonies cultured on sheep blood agar

Plate 4.1a

Plate 4.1b

Figure 4.2 Plates of colonies cultured on Reinforced Clostridia and nutrient agar

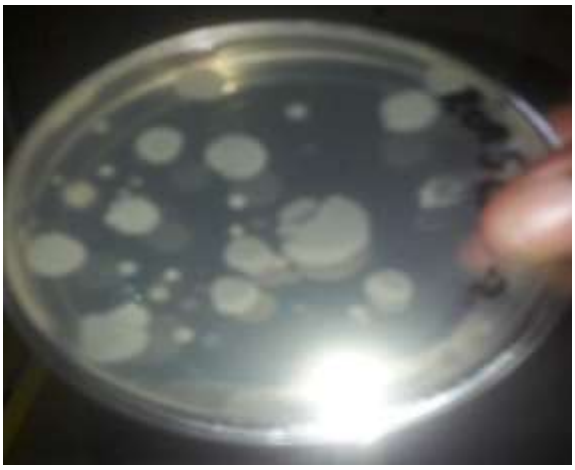

Plate 4.2a

Plate 4.2b

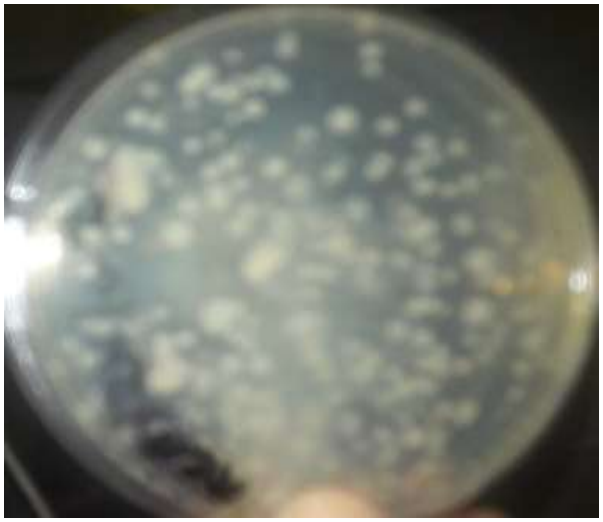

Plate 4.2c

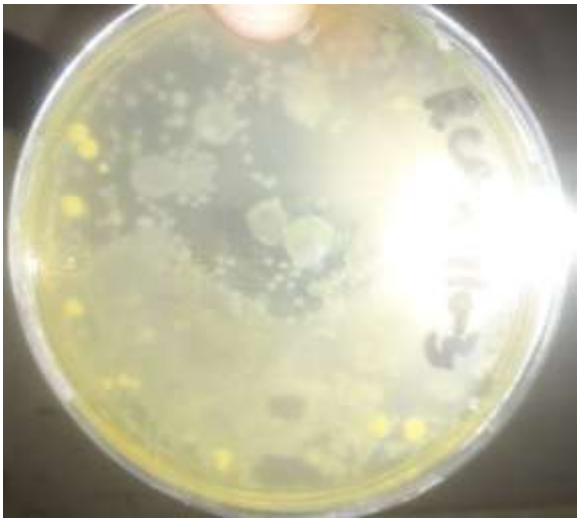

Plate 4.2d

Figure 4.3 Plates with a four-way streaking of pure cultures on nutrient agar

Plate 4.3a

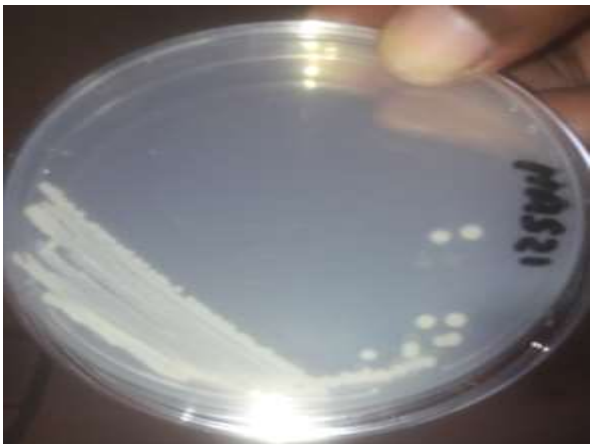

Plate4.3b

4.2. Colony forming units (CFU) per gram of environmental sample (soil and faecal)

Colony forming units were calculated using the formula of Harley (2005) based on average colony counts per plate. This was done to estimate the number of viable bacteria per gram of environmental sample. Based on these calculations, there were differences in the average colony forming units of different sampling locations. The results indicated that the cattle camp had a higher level of contamination as compared to the goat and sheep camps for both soil and faecal samples. The highest counts were observed in samples from the cattle camp and the lowest counts were observed in samples from the sheep camp for both soil and faecal samples. In soil samples the colony forming units per gram of environmental sample avereged 7.25 $X10^7$ of CCS, 5.5$X10^6$ of GCS and 4.8$X10^6$ of SHCS **(Figure 4.4)**. and for faecal samples, the average colony forming units per gram of environmental samples was at 4.6$X10^6$ of CCS, 4.05$X10^6$ of GCS and 3.7$X10^6$ of SHCS **(Figure 4.5)**.

The average numbers of viable bacteria per gram of soil sample were calculated based on mean colony counts per plate. All samples were of similar weight (1 gram) and exposed to same dilution factors of 10^{-5}, 0.1 millitre of bacterial culture were plated per agar plate and the results are presented in **Figure 4.4** as average colony forming units per gram of soil sample (CFU/g).

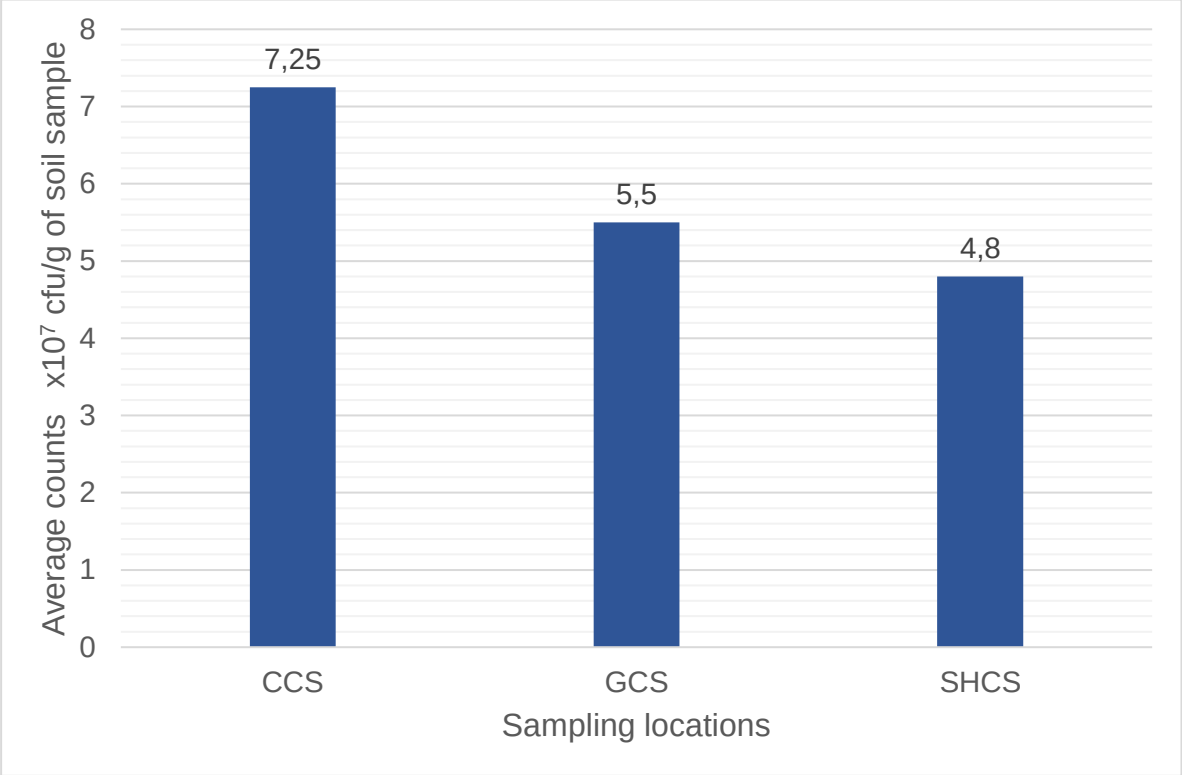

Figure 4.4. Average colony forming units (CFU) per gram of soil sample from different camps within the farm

The average numbers of viable bacteria per gram of faecal sample were calculated based on mean colony counts per plate. All samples were of similar weight (1 gram) and exposed to same dilution factors of 10^{-5}, 0.1 millitre of bacterial culture were plated per agar plate and the results are presented in **Figure 4.5** as average colony forming units per gram of faecal sample (CFU/g).

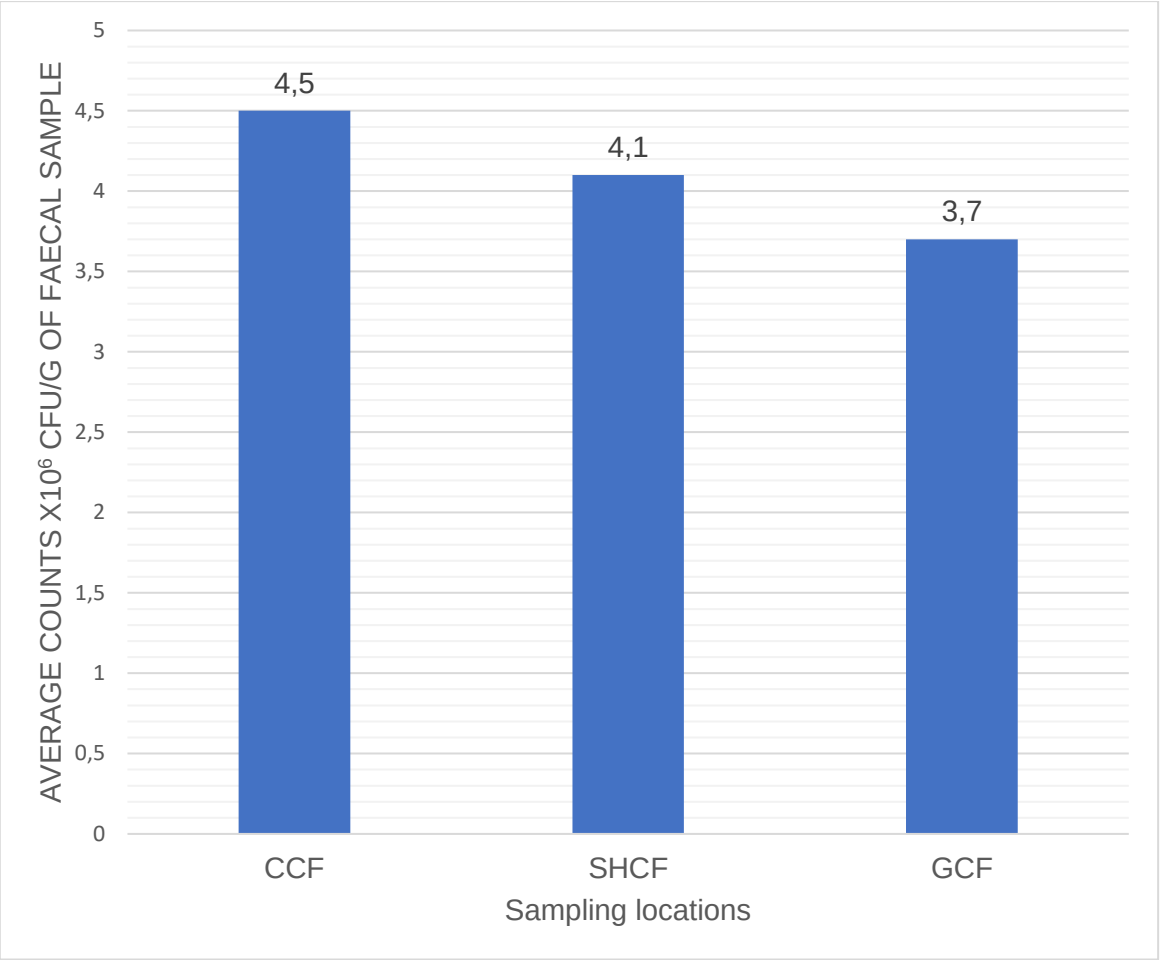

Figure 4.5. Average colony forming unit per gram of faecal sample from different camps in the farm.

4.3 Diversity of bacterial species identified in soil and faecal samples using MALDI-TOF MS

During the study period, a total of hundred and seventy environmental samples collected for microbial analysis of pathogenic bacteria were analysed by MALDI-TOF MS using the simplified on-plate method. A total of hundred and thirty-four (79 %) of the samples were successfully analysed by MALDI-TOF MS and tested positive with different bacterial species. The remaining thirty-six samples (21 %) could not be identified as MALDI-TOF MS could not detect enough peaks to reliably identify the isolates. Twenty (15 %) of the isolates were falling between the scores (0.00-1.599) indicating that there was no reliable identification for these isolates, eighty-eight (66 %) with score (1.6-1.999) were identified at probable genus identification, twenty-five isolates (19 %) with score (2.300-2.299) were identified at secure genus level and probable species level and there are no isolates that were identified at the highly probable species identification with score (≥3) **(Table 4.1 and Table 4.2)**. Based on

these analyses, it was observed that different farm environments harbour different bacterial species. However, minor similarities were observed with few bacterial species where *B. cereus* and *E. coli* were isolated in both manure and faecal samples. *Bacillus cereus* were identified as the most dominant species isolated from soil samples in 36 positive samples (33 %), followed by *B. mycoides* in 24 positive samples (22 %) **(Table 4.3)**. However, with regard to faecal samples, 26 positive samples were identified with *E. coli* being the most prevalent species, identified in 9 positive samples (34.6 %), followed by *Acinetobacter baumannii* identified in 8 positive samples (30.8 %), *Acinetobacter baumanii* in 5 positive samples (19.2 %), *B. cereus* and *B. psedomycoides* being the least dominant with (7.7 %) each **(Table 4.4)**. The levels of contamination with pathogenic bacteria in this study varied greatly with respect to sampling location. However, there were similarities with species prevailing across sampling locations. Molecular charecterization of this strains was not carried out, however, it is deduced from literature that these species have pathogenic strains, supplementary confirmation of vilurence factors with PCR in future studies is essential. In soil samples, a total of nineteen distinct strains of bacteria were identified. Eleven strains (58 %) were recorded from the cattle camp soils (CCS), with *Bacillus* genera being the most dominant. *Bacillus cereus* were frequently isolated with (28 %), *B. weihenstaphansis* (17 %) and *B.mycoides* (33 %), followed by *E. coli* with (5.6 %) and other bacteria species were the least with identification in one or two positive isolates with (0-1.4 %). In sheep camp soils (SHCS), four strains (21 %) were recorded with *Bacillus spp* being the most dominant (41.6 %) followed by *Psuedomonas resinovarans* (8.3 %) . In goat camp soils (GCS) eight samples tested positive (42 %) with *bacillus spp* being the most dominant with (50 %) followed by seratia marcescens with (20.8 %) **(Table 4.3)**. Not all bacterial contaminants of the environmental samples identified herein are pathogenic to livestock, pathogenic species are presented with an asterisk (*) **(Table 4.3 and Table 4.4)**. High contamination was recorded in soil samples as compared to faecal samples. Out of the 22-bacterial species identified with MALDI-TOF MS MS, seventeen (77 %) were from soil samples and three (14 %) from faecal samples. However, two (9 %) species were common in both soil and faecal samples.

Table 4.1 Bacterial species identified from soil samples by MALDI-TOF MS using the simplified on-plate method

		Cut off values proposed for this study			
Bacteria species	No of positive Sample/s	≥3	2.00-2.99	1.6-1.99	0.00-1.599
Bacillus cereus[*]	36	-	6	30	-
Listeria monocytogens[*]	2	-	-	-	2
Lysinibacillus fusiformis	4	-	-	2	2
Bacillus megaterium	1	-	-	1	-
*Staphylococcus aureus**	1	-	-	1	-
Enterobacter cloacae	4	-	-	3	1
Enterobacter cancerogenus	1	-	-	-	1
Bacillus simplex	1	-	-	1	-
Enterobacter absburiae	2	-	-	2	-
Bacillus mycoids	24	-	-	20	4
Bacillus weihenstephanesis	12	-	-	8	4
*Escherichia coli**	4	-	3	1	-
*Seratia marcescens**	5	-	2	2	1
*Bacillus licheniformis**	4	-	-	3	1
Bacillus endophyticus	1	-	-	1	-
Pseudomonas resinovarans	1	-	-	-	1
Pseudomonas aeruginosa[*]	2	-	-	2	-
Pseudomonas monteilii	2	-	-	1	1
Pseudomonas corrugate	1	-	-	1	-
Total	**108**	**0**	**11**	**79**	**18**

[*]Pathogenic bacteria

Table 4.2 Bacterial species identified from faecal samples by MALDI-TOF MS using the simplified on-plate method

Bacteria species	No of positive sample/s	Cut off values proposed for this study			
		≥3	2.00-2.999	1.699-1.999	0.00-1.599
*Escherichia coli**	9	-	8	1	-
*Bacillus cereus**	2	-	-	2	-
Bacillus pseudomycoides	2	-	-	-	2
Acinetobacter genomospecies	8	-	2	6	-
Acinetobacter baumanii	5	-	4	1	-
Total	**26**	**0**	**14**	**10**	**2**

*Pathogenic bacteria

Table 4.3 Occurrence and percentage frequency of bacteria from soil of different camps in the farm

Bacterial species	No of positive samples in the farm	% Frequency in the farm	Occurrence			No of positive samples in the camp and % Frequency					
			CCS	SHCS	GCS	CCS		SHCS		GCS	
						+NSC	%FC	+NSC	%FC	+NSC	%FC
*Bacillus cereus**	36	33.3	+	+	+	20	28	6	50	10	41.6
*Listeria monocytogenes**	2	1.9	+	-	-	2	2.8	0	0	0	0
Lysinibacillus fusiformis	4	3.7	-	+	+	0	0	1	8.3	3	12.5
Bacillus megaterium	1	0.9	+	-	-	1	1.4	0	0	0	0
*Staphylococcus aureus**	1	0.9	+	-	-	1	1.4	0	0	0	0
Enterobacter cloacae	4	3.7	+	-	-	4	5.6	0	0	0	0
Enterobacter cancerogenus	1	0.9	+	-	-	1	1.4	0	0	0	0
Bacillus simplex	1	0.9	+	-	-	1	1.4	0	0	0	0
Enterobacter absburiae	2	1.9	+	-	-	2	2.8	0	0	0	0
Bacillus mycoids	24	22.2	+	-	-	24	33	0	0	0	0
Bacillus weihenstephanesis	12	11.1	+	-	-	12	17	0	0	0	0
*Escherichia coli**	4	3.7	+	-	-	4	5.6	0	0	0	0
*Serratia marcescens**	5	4.6	-	-	+	0	0	0	0	5	20.8
*Bacillus licheniformis**	4	3.7	-	+	-	0	0	4	33.3	0	0
Bacillus endophyticus	1	0.9	-	-	+	0	0	0	0	1	4.2
Pseudomonas resinovarans	1	0.9	-	+	-	0	0	1	8.3	0	0
*Pseudomonas aeruginosa**	2	1.9	-	-	+	0	0	0	0	2	8.3
Pseudomonas monteilii	2	1.9	-	-	+	0	0	0	0	2	8.3
Pseudomonas corrugate	1	0.9	-	-	+	0	0	0	0	1	4.2
Total	**108**	**100**				**72**	**100**	**12**	**100**	**24**	**100**

CCS=Cattle camp soils; **SHCS**=Sheep camp soils; **GCS**=Goat camp soils; +**NSC=** Number of positive samples in camp; **% FC**= Percentage frequency in the camp; ***** Pathogenic bacteria; **+** =present ; **-** = absent.

Table 4.4 Occurrence and percentage frequency of bacteria from faecal samples of different camps in the farm

Bacteria	No of positive samples in the farm	% Frequency in the farm	Occurrence			Number of positive samples in the camp and % Frequency					
			CCF	SHCF	GCF	CCF		SHCF		GCF	
						$^{+}$NSC	%FC	$^{+}$NSC	%FC	$^{+}$NSC	%FC
*Escherichia coli**	9	34.6	+	-	-	9	64.2	0	0	0	0
*Bacillus cereus**	2	7.7	+	-	-	2	14.3	0	0	0	0
Bacillus psedomycoides	2	7.7	+	-	-	2	14.3	0	0	0	0
Acinetobacter baumannii	8	30.8	+	+	+	1	7.1	3	100	4	44.4
Acinetobacter genomospecies	5	19.2	-	-	+	0	0	0	0	5	55.6
Total	**26**	**100**				**14**	**100**	**3**	**100**	**9**	**100**

CCF= Cattle camp faeces; **SHCF**=Sheep camp faeces; **GCF**= Goat camp faeces; **+**= Present **-** = Absent , ***** Pathogenic bacteria; $^{+}$**NSC=** Number of positive samples in the camp; **% FC**= Percentage frequency in the camp

Percentage frequencies in Table 4.3 and Table 4.4 were calculated using the formula indicated below :

$$\textbf{\% Frequency} = \frac{\boldsymbol{Number\ of\ samples\ from\ a\ camp\ positive\ for\ a\ bacterial\ species}}{\boldsymbol{Total\ number\ of\ positive\ samples\ from\ the\ farm/camp}} X100$$

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

This study utilised MALDI-TOF MS to identify bacterial isolates from environmental samples. The results indicated that 134 (79 %) environmental samples of the 170 collected tested positive with MALDI-TOF MS identification. Urwlyer and Glaubitz. (2015) reported MALDI-TOF MS to have a revolutionized speed and precision of microbial for clinical isolates to outperform conventional methods. This is evidenced in their study were MALDI-TOF MS showed lowest number of false identification (4 %) and 60 % accuracy at genus level. In contrast, the biochemical-based system assigned 25 % of genera incorrectly. In this study, MALDI-TOF MS provided identification at the genus level of 67 % of the bacterial isolates. This agrees with Dupont *et al*. (2010), Justesen *et al*. (2011), Nagy *et al*. (2012), Wieser *et al*. (2012), Lee *et al.* (2015) and Florio *et al*.(2018) who reported the capacity of MALDI-TOF MS to identify bacterial microorganisms at the genus and species level according to the cut of values in this study (score value 2.000 - 2.299) ranging from 65.2 % to 83.9 %. Average colony forming units in both soil and faecal samples fell within the range reported by previous researchers for environmental samples (Okoh *et al*., 1999 and Ogunmwonyi *et al*., 2008). Total bacterial counts were relatively higher in soil samples suggesting high contaminations as compared to faecal samples.

These results agree with the reports of Trawińska *et al*. (2006) who indicated that soil in the vicinity of high-production farms is commonly microbial-contaminated as arable land and pastures contaminated with the faeces of sick animals, especially, contribute considerably to pathogen transfer into the soil. Boes *et al*. (2005) and Ngole *et al*. (2006) reported that micro-organisms survival in the soil environment is favoured by high temperature and moisture. Oliver *et al*. (2006) stated that rainfall events result in faecal microbes being washed from the cowpats into the surrounding soils where their survival could be enhanced. This finding is further supported by the results of Muirhead (2009) who reported an increase in soil *E. coli* concentrations on the grazed camps which they believed coincided with an increase in rainfall during their study period. Furthermore, Kress and Gifford. (1984) and Stoddard *et al*. (1998) reported that heavy rain or irrigation on a fresh cow pat is likely to result in far greater microbial mobilization and leaching. Mobilization and leaching of bacteria during rainfall could be the reason for high percentage prevalence of bacteria species in soil compared to faecal samples recorded in the current study. Bacteria leach with water into the soil, where they incubate and multiply resulting in contamination of pastures were animals graze (Kress and Gifford, 1984).

In the present study, Bacillus species was identified to be the most prevalent species in soil samples. This is in agreement with Gutiérrez- Mañero *et al*. (2003) and Amin (2015), who reported bacillus as naturally occurring soil bacteria and most abundant genus in the rhizosphere of soil. From this genus, the most dominant species appeared to be *B. cereus*, which when allowed an opportunity to invade mammalian tissues is an opportunistic pathogen that may cause severe or local systematic infections such as endophthalmitis and septicaemia (Kotiranta *et al* ., 2000). *B. cereus* has been reported to cause serious clinical diseases in farm animals, thereby causing economic loses to farmers. Nieminen *et al*. (2007) and Salih (2015) reported *Bacillus spp* to occasionally cause mastitis in cattle. *B. cereus* has been reported as an important food borne pathogen. Tewari and Abdullah (2015) reported highly toxic strains of *B.cereus* responsible for food-related fatalities. Ranieri *et al*. (2009), Gundogan and Avci. (2014) reported *Bacillus spp* to be ubiquitous in nature. The ability of its spores to tolerate different environmental conditions including elevated temperatures result in these species being the most common isolated bacteria from food, air, soil, and faecal samples. However, the reports of Bavykin *et al*. (2004) indicating that bacillus is the most common genus in faecal samples are in contrast with the findings of this study as these bacteria tested positive only in faecal samples of cattle with a frequency occurrence of 35 %.

In faecal samples, *E. coli* was the most frequently isolated pathogen. However, no molecular characterisation and test for virulence were carried out in this study. The dominance of *E.coli* in faecal samples reported in our study agrees with reports by previous researchers (Tahamtan *et al*., 2006; Raji *et al*., 2006; Hiko *et al*., 2008; Hashemi *et al*., 2010). Hogan *et al*. (1999) and Abakpa *et al*. (2015), reported *E. coli* to be naturally present in faeces of warm blooded animals. *E. coli* occurrence has been previously used as an indicator of faecal contamination, signaling the possible presence of faecal pathogens such as Salmonella and Shigella species (Odonkor and Addo, 2018). In a study by Rodrigues *et al*. (2015), MALDI TOF-MS identified 83 % *E. coli* prevalence in faecal samples, however, the pathogenicity of the strains were not reported. Schmidt *et al*. (2015) reported *E. coli* to be free-living commensals in animal intestines. The prevalence of this bacterial species was found to be high in the cattle camp in comparison with the small stock camps. Sima *et al*. (2009), Kiranmayi *et al*. (2010) and Rahimi *et al*. (2012) reported domestic animals as the sources of *E. coli.* However, the major animal carriers seem to be healthy domesticated ruminants, primarily cattle and, to a lesser extent sheep.

These findings agree with the results of our study as *E. coli* were only identified in cattle faeces with zero percentage prevalence in sheep and goat faecal samples. However, large variations have been described in the shedding patterns of individual animals, the proportion of shedding animals on farms that harbor them and, over time, in the amount of shedding on the same farm (Smith *et al*., 2010). Among cattle, shedding occurs intermittently (Hancock *et al*.,.1997; Kulow *et al*., 2012; Sharma *et al*., 2012), and it was reported that, at any time, up to 50 % of the healthy animals excrete *E. coli* in their stool (Lim *et al*., 2010). Freshly deposited faeces contain the nutrients required by bacteria, and replication presumably depends on the faeces retaining water and attaining suitable temperatures for growth. Cow pats are able to retain moisture when exposed to sunlight. The pats quickly form a skin, which thickens to a well-defined crust within 48 hours favouring bacterial growth (Van Kessel *et al*., 2007). However, exposure of sheep and goat pellets to the sun results in drying of the pellets due to size and less moisture contained making the environment unfavourable for bacterial growth. Topp *et al*. (2003) studied the relationship between soil moisture content and prevalence of *E. coli* bacteria and concluded that elevated soil moisture levels were associated with increased number of bacteria in the soil. This could explain the low counts of *E. coli* in soil samples of the current study as soil samples were collected in early summer where moisture content in soil is believed to be relatively low. Of the twenty-two bacterial isolates isolated from the environmental samples in this study, some species have been reported previously to carry virulent strains which are responsible for livestock infections particularly in cattle, sheep and goats. These pathogenic bacteria include: *E. coli* (Olson, 2001; Chekabab *et al*., 2013), *B.cereus* (Nieminen, 2007; Manyi-Loh, 2016; Robert *et al*., 2017), *L. monocytogens* (Nightingale *et al*., 2004; Nicholson *et al*., 2005), *Staphylococcus aereus* (Toroitich, 2013), *Pseudomonas aeruginosa* (Radostits *et al*., 2000), *Seratia marcescens* and *B. licheniformis* (Olson, 2001).

Pathogenic bacteria in farm environments pose a major epidemiological threat (Amin *et al*., 2013). Although *E. coli* have been reported as harmless commensals of the intestines of warm blooded animals, the strain *E. coli* O157:H7 has been reported to be harmful (Titilawo *et al*., 2015). Shearer *et al*. (2003), reported *E. coli* as one of the major bacterial pathogens associated with livestock infections in farms. Healthy colonized cattle and other ruminants are the most significant animal reservoir harboring *E. coli* (Ferens and Hovde ,2011). Previous studies linked approximately 75 % of the human *E. coli* outbreaks to food

products of bovine origin (Callaway *et al*.2009; Munns *et al*., 2016). Other reservoirs that may impact transmission include sheep (La Ragione *et al*. 2012; Soderlund *et al*.2012; Gencay 2014), goats La Ragione *et al*.(2009), Mersha *et al*.(2010), Alvarez-Suarez *et al*.(2016) and Swift *et al*.(2017).

According to Tomita *et al*. (2001) *E. coli* bacteria possess several pathogenic factors responsible for their pathogenicity, among which exotoxin A (toxA) and exoenzyme S (exoS) are the two major fatal toxins which are associated with subclinical mastitis infection in bovines. Toroitich. (2013) in a cross-sectional study to determine the prevalence of mastitis and identify the associated risk factors reported the most predominantly isolated bacterium to be S*taphylococcus aureus* with a prevalence of 36 % followed by *E. coli* with a prevalence of 27.2 % and *Pseudomonas* were least isolated with less than 1 % prevalence.

In the present study, *L. monocytogenes* tested positive in cattle camp soils (2.8 %). This falls in the same range as the findings of Mohammed *et al*. (2009) (5.4 %) and less compared to the findings from previous studies by Moshtaghi *et al*. (2003) (17.7 %), Nightingale *et al*. (2004) (22.2 %) and Locatelli *et al*. (2013) (38.1 %). Several animal derived *L. monocytogenes* contaminated food products, including raw milk, pasteurized milk, chocolate milk, butter, soft cheeses, and processed meat and poultry products, have been implicated as sources of human listeriosis cases and outbreaks (Buchanan *et al*., 2017). It has been reported that manure from infected or shedding animals represent direct links between human infections and *L. monocytogenes* in farm animals and farm environments as a result of consuming animal-derived food products that are not processed before consumption such as raw milk and raw foods of plant origin (Nightingale *et al*., 2004).

Previous studies reported Campylobacter species and Clostridium species to be pathogenic bacteria of livestock commonly isolated from environmental samples (Baserisalehi *et al*., 2007; Bagge, 2009). However, in the present study, all samples tested negative for Campylobacter species. Clostridium species were detected in 2 samples (5 %). Bandelj *et al*. (2016) reported a 10 % prevalence of *clostridium difficile* in cattle faecal samples. The use of selective media may have disadvantaged growth of other bacteria species in the samples. Olson. (2001), reported the Campylobacter genus to have fastidious growth requirements making conventional detection and identification difficult.

This could be the reason why all samples in the present study tested negative for Campylobacter species. The differences in bacterial contamination in the sampling locations in this study may be due to different management practices in the livestock camps were cattle are kept in their camp continuously resulting in piling of faecal samples increasing microbial habitat. In comparison with sheep and goats which are housed at night resulting in few fresh pellets in their camps. Spiehs and Goyal (2007), Hutchison *et al*. (2005) and Manyi-Loh *et al*.(2016) indicated that the levels and types of pathogens occurring in livestock faeces vary with animal species, dietary sources, health status and age of the animal.

5.2 Conclusions

This study showed that diverse bacterial species contaminate the livestock grazing environment of University of Limpopo experiential farm. Although samples were collected from different locations within the farm, the distinct types of bacteria isolated from soil samples were generally similar. However, with regard to faecal samples, bacterial isolates differed from one camp to the other entailing that different livestock harbour different bacteria in their faeces. Of the bacterial species isolated in the study, some genus have been reported to be pathogenic resulting in tremendous loses to farmers. Even though it is possible that several bacterial species in the university farm livestock environment may have been missed in this study, *Bacillus sp* in soil and *E. coli* in faecal samples could be readily assigned dominant. This study demostrated the contamination of opportunistic, food-borne bacteria like *B. cereus* and *L. monocytogenes* in the experiential farm environment and the need for good hygiene practices to prevent its entry into the food chain.

5.3 Recommendations

It is impossible for every individual pathogen that constitute the entire bacterial organization in the environment to be isolated. Furthermore, some pathogens may require time-intensive tests and enrichment steps during analysis and detection, thereby making their quantification complex. As the livestock farm has long been known to be an incubator for infectious diseases, a high contamination of farm environments with bacterial pathogens poses a health risk to both livestock and human beings. The use of more sensitive tests like PCR, ELISA, nuclei acid probes and molecular characterization of the isolated bacterial species to link their toxins with infectious diseases in livestock should be

a focus of extension of this investigation in future. MALDI-TOF MS can be used to identify potential disease risk in an environment and allow for appropriate control management. The sensitivity and specificity of MALDI-TOF MS compared with traditional microbiology identification methods needs to be investigated. Bacterial colonies not identified with MALDI-TOF MS can be identified and characterised using classical microbiology methods and included as positive controls to broaden the scope of bacteria the MALDI-TOF MS can detect. We can deduce from literature that the best method to control pathogenic bacteria populations on farm environments is through proper manure management as it presents with several benefits that stand to reduce economic losses due to disease outbreaks and safe guard public health from zoonotic diseases.

CHAPTER 6

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