Identification of tick species and their bacterial pathogens from cattle in two provinces of South Africa

BY

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DECLARATION

I hereby declare that this dissertation, which I submit in fulfilment of the degree of Master of Science at the University of Pretoria, South Africa, is my own work. This work has not been submitted previously for any degree or examination at any other university.

……………………………………………………… ……………………
Bongekile Lungile Khoza

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LIST OF ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
°C	Degrees Celsius
%	Percentage
DNA	Deoxyribonucleic Acid
DVTD	Department of Veterinary Tropical Diseases
EDTA	Ethylenediaminetetraacetic acid
KZN	KwaZulu-Natal
FS	Free State
NGS	Next Generation Sequencing
PCR	Polymerase Chain Reaction
rRNA	Ribosomal Ribonucleic Acid
TBDs	Tick Borne Diseases
TBPs	Tick Borne Pathogens
BTBPs	Bacterial Tick-Borne Pathogens
BTBDs	Bacterial Tick-Borne Diseases
PBS	Phosphate Buffered Solution
TTBDs	Ticks and tick-borne diseases
OTU	Operational Taxonomic Unit
NCBI	National Centre for Biotechnology Information
DALRRD	Department of Agriculture Land Reform and Rural Development
AEC	Animal Ethics Committee
REC	Research Ethics Committee
ASVs	Amplicon Sequence Variants
SMRT	Single Molecule Real Time
Ng	nanogram
Bp	base pair
DADA2	Divisive Amplicon Denoising Algorith 2
PacBio	PacificBiosciences
ECF	East Coast Fever

TBF	Tick Borne Fever
GA	Granulocytic Anaplasmosis
HGE	Human Granulocytic Ehrlichiosis
gDNA	genomic Deoxyribonucleic Acid
V3	Variable 3
V4	Variable 4
SCM	SubClinical Mastitis
CCS	Circular Consensus Sequencing
NICD	National Institute for Communicable Diseases
PC0A	Principal Coordinate Analysis
NMDS	Non-metric Multidimensional Scale
ID	Identity

DISSERTATION SUMMARY

Identification of tick species and their bacterial pathogens from cattle in two provinces of South Africa

Candidate: Ms Bongekile Lungile Khoza

Supervisor: Dr Zamantungwa Mnisi

Co-supervisor: Prof Marinda Oosthuizen

Department: Veterinary Tropical Diseases

Degree: Master of Science Veterinary Science Tropical Diseases

In South Africa, resource-poor farmers are negatively affected by death and ill health of livestock due to high tick infestations. Tick infestations are associated with tick-borne pathogens causing various diseases that are a major constraint to cattle farming, a threat to human health and consequently the economy. This has been an ongoing concern for resource-poor farmers, mostly influenced by the inability to access veterinary care or proper education on the usage of veterinary products. This study sought to investigate the presence of ticks and their associated pathogens at three study sites, namely Harrismith and Phuthaditjhaba in the Free State province as well as Bergville in KwaZulu Natal. These are three neighbouring towns, where the point of intersection for livestock is the Drakensberg Mountains, which serve as a source of vegetation for grazing livestock. Between these three study sites there is uncontrolled translocation of livestock due to traditional practices and trade and thus the introduction of several tick species.

Ticks are recognised worldwide as major vectors of several disease-causing pathogens and are good indicators of pathogen distribution and epidemiology. However, global warming has result in climate change and consequently expanded tick distribution. Consequently, growing incidences of emerging and re-emerging tick-borne pathogens capable of causing tick-borne diseases (TBDs) of veterinary and

economic importance. These TBDs are major hindrances that constrain cattle farming, thus culminating in significant losses: threatening food security, global trade, eco-tourism, and affecting human and livestock health.

Therefore, this study sought to identify ticks and detect bacterial tick-borne pathogens in the three neighbouring towns: Harrismith, Phuthaditjhaba and Bergville using a 16S rRNA next-generation sequencing (NGS) approach based on the PacBio sequencing platform. A total of n=50 blood samples were collected from cattle in each study site and n=418 ticks were collected from these cattle, comprising n=126 ticks from Harrismith, n=160 from Phuthaditjhaba and n=132 from Bergville. Ticks infesting cattle were identified morphologically to belong to the genera *Rhipicephalus* with six species and *Hyalomma* with only two species. Harrismith had *Rhipicephalus decoloratus, R. microplus*, *R. evertsi evertsi*, *Hyalomma truncatum*, *H. rufipes*, Phuthaditjhaba: *R. appendiculatus R. simus*, *R. evertsi evertsi*, *R. afranicus*, *H. rufipes* and Bergville: *R. evertsi evertsi*, *R. appendiculatus*, *H. truncatum.* Out of n=418 ticks collected, *R. evertsi evertsi* with n=332 was the most dominant tick species in the three study sites, whereas *R. decoloratus and R. microplus* tick species were only present in Harrismith. A full-length 16S rRNA gene was amplified and sequenced using PacBio technology for the identification of bacterial pathogens associated with these ticks. A total of 7,687,581 reads were obtained. Bacterial pathogens identified belonged to the genera *Anaplasma*, *Mycoplasma* and *Ehrlichia*. *Anaplasma* species detected were *A. marginale, A. centrale, A. phagocytophilum, A. platys* and *A. bovis. Mycoplasma* species were *M. wenyonii* and *M. bovis. Ehrlichia* species detected were *E. ruminantium* and *E. canis*. *Anaplasma marginale,* with a relative abundance of 43.5% in Harrismith, 54.2% in Phuthaditjhaba and 56.2% in Bergville, was the most abundant, followed by *A. platys* with 31.5% in Harrismith, 32.9% in Phuthaditjhaba and 22.6% in Bergville. *Mycoplamsa wenyonii* was 19.6% Harrismith, 7.8% in Phuthadijthaba and 14% in Bergville. The bacterial composition at the three sites aligned with the tick vectors identified at the three-study sites. The presence of *R. microplus* and *R. decoloratus* was reported for the first time in Harrismith, while *R. turanicus* was identified for the first time in Phuthaditjhaba. This shows that there has been an expansion in tick distribution because of climate change and possibly other ecological and anthropogenic factors.

This study underlines the critical impact of factors such as improper tick control measures, acaricide resistance, unregulated animal translocations, and climate change on resource-poor farmers. The findings emphasize the urgency of collaborative efforts in conducting comprehensive tick and tickborne pathogen surveillance studies. This collaborative approach is essential to inform and implement effective control strategies, ultimately enhancing our understanding of tick-borne pathogens and transmission dynamics for the benefit of both agricultural practices and public health.

CHAPTER 1

GENERAL INTRODUCTION

1.1. Introduction

Ticks belong to a group of arthropods and are recorded as a major worldwide problem with a huge impact on agriculture, the economy, human and animal health (Adenubi et al., 2018). Ixodid ticks are ranked second to mosquitoes as distributors of pathogens responsible for diseases of veterinary importance (Barker, 2010; Jongejan & Uilenberg, 2004). In South Africa, the economy is largely dependent on cattle farming involving the import and export of cattle and its by-products (Mtshali et al., 2022). Cattle and other ruminants are preferable tick hosts and are highly infested by ticks capable of transmitting a wide range of disease-causing agents. Transmission of bacterial pathogens from ticks to cattle is through direct contact during blood meal, thus subjecting cattle to various pathogens (Hotez et al., 2015). Ticks and the pathogens they transmit cause tick-borne illness and present a noteworthy peril to the well-being of humans and animals on a global scale (Rochlin & Toledo, 2020). Farmers residing in rural settings are the most affected and subjected to tick control challenges.

Tick burden and control are undoubtedly the most important setbacks in improving the economy and livestock health. This is due to numerous factors, which have led to a huge impact on agriculture and the economy at large. Factors contributing to this problem are inclusive of global warming effects, invasive tick species being introduced through animal translocation, the uncontrollable movement or interaction of animals, and incorrect usage of acaricides which culminates in acaricide resistance (Juache-Villagrana et al., 2023). Regarding the latter factor, ticks can acquire immunity towards specific acaricides. This phenomenon is of great concern in numerous geographical areas and underscores the significance of implementing comprehensive pest control strategies which entail the systemic rotation of diverse categories of acaricides (van Dalen & van Rensburg, 2023).

For a longest time, tick control consisted of utilization of acaricides in order to reduce tick infestations, however, vaccines are now deemed a more efficient and enduring strategy. Vaccines have transitioned from employing deactivated pathogens to recombinant proteins and vaccinomics methodologies. Tick targeted vaccines such as the recombinant vaccine based on Bm86 protein, TickGARD^{PLUS} (Intervet Australia, Australia), and Gavac® (Heber Biotec, Havana, Cuba) have viable anti-tick control properties. These vaccines possess holistic traits desirable in controlling ticks, such as prevention of tick infestations and transmission of tick-borne diseases, reduction of environmental contaminants and acaracide resistance (Rosario-Cruz et al., 2023). As such, the protein Bm86 has gained popularity as a target/reference candidate in the development of anti-tick vaccines (Rosario-Cruz et al., 2023).

To enhance the management of ticks, according to (Bishop et al., 2023) and (Gilbert, 2021), there are ongoing considerations of novel technologies and approaches, such as the manipulation of tick commensal bacteria and interventions that involve the alteration of parasite transmission by ticks. Furthermore, it is stated that it is imperative to establish standardized protocols for assessing the resistance of tick acaricides and to improve upon these protocols, along with the development of innovative molecular tests for detecting acaricide resistance. Surveillance plays a pivotal role in the monitoring of ticks, the assessment of the risk of tick-borne diseases, and the establishment of a solid foundation for risk control programs (de la Fuente, 2021). Generally, the development of vaccines and the implementation of more robust control strategies are indispensable in addressing the challenges posed by ticks and the associated tick-borne diseases (Gilbert, 2021).

This study targeted three neighbouring towns of the Free State and Kwa-Zulu Natal provinces in a rural setting of South Africa. The provinces are separated by the Drakensberg Mountain which is the source of vegetation for livestock. Mostly residents who survive through livestock farming occupy the towns. Between these towns, there is uncontrolled movement of livestock through trading, traditional practises and sharing of the grazeland. Additionally, there is notable lack and poor access to veterinary medicine and inadequate tick control measures.

Therefore, this study was designed to identify ticks serving as vectors of bacterial pathogens and to detect the bacterial tick-borne pathogens in cattle blood by using a 16S rRNA next-generation sequencing approach. Additionally, to conduct a comparative analysis of bacterial microbiome present at the three study sites.

1.2. Problem Statement

Ticks are known as vectors of several disease-causing pathogens in humans, domestic and wild animals, including viruses, rickettsiae, spirochaetes and parasitic protozoa (Andreotti et al., 2011). They act as reservoirs of these organisms and transmit several economically important diseases such as heartwater, bovine babesiosis, anaplasmosis, rickettsioses, tick-borne relapsing fever and theileriosis (De Vos, 1979; De Waal, 2000; Regassa et al., 2003; Marufu et al., 2010; Spickett et al., 2011).

There is a growing concern of emerging and re-emerging tick-borne pathogens that are of economic significance, and they infect livestock. The most affected livestock are those belonging to resource-poor farmers, largely due to poor veterinary services. The emergence and re-emergence of tick-borne pathogens are enabled by the inability of the farmers to buy veterinary medicines to control diseases, uncontrolled cattle movement, wildlife-livestock interface and regular burning of grazing land. The latter plays a big role in controlling free-living ticks on the vegetation (Trollope et al., 2002). Inappropriate use of veld as a management tool can lead to loss of basal cover and exposure to soil-to-soil erosion. These changes promote the emergence and re-emergence tick-borne pathogens in the environment where global warming is notable. Global warming has affected changes in the climate and disrupted the ecosystems in agriculture. Consequently, the tick-distribution and the pathogens they transmit.

The other factor is the competence of invasive tick species, such as the one-host tick (*R. microplus*) and the three-host tick (*A. variegatum*). These ticks have high adaptability and capacity to transmit several pathogens. In South Africa, *R. microplus*, is the vector of *Babesia bigemina, B. bovis* and *A. marginale.* The *R. microplus* has been reported to displace the indigenous tick, *R. decoloratus*, in South Africa

(Nyangiwe et al., 2013; 2017). *Amblyomma variegatum* is prevalent in various regions in the African continent and is the second most invasive tick species after *R. microplus* (Barré & Uilenberg, 2010). On the other hand, livestock translocation is among the contributing factors to the expansion of tick distribution and the pathogens they transmit (Nyangiwe et al., 2013; 2018).

Tick distribution has been a reliant indicator of the distribution of tick-borne diseases, thus demonstrating the presence of various pathogens. However, factors such as climate change, adaptation of invasive tick species and translocation of animals indicate that the tick distribution might not be a concise marker of tick transmitted diseases distribution. Thus, implying that the potential distribution of vectors might not always be indicative of the presence of diseases. For example, the vector may be present but the pathogen might either not have been reported or have been displaced by invasive tick species, while also other tick-borne pathogens might be mechanically transmitted by other fly bites or through contaminated objects (Estrada-Peña & Salman, 2013).

In view of the above, it is imperative to conduct ongoing and systematic survey studies on tick distribution and the associated pathogens across South Africa. Continuous research in this area is essential for refining and enhancing strategies aimed at the prevention and control of tick-borne diseases. By expanding our understanding of the dynamics of tick-borne pathogens and their prevalence, we can develop more targeted and effective measures to safeguard livestock health, protect communities reliant on agriculture, and contribute to the overall improvement of public health in the region.

1.3. Objectives

The main aim of this study was to investigate the presence of bacterial tick-borne pathogens in two provinces of South Africa, specifically focusing on a comparative analysis of the bovine blood microbiome in three neighbouring towns within those provinces.

In respect to the above, the following objectives were set for the study:

- Identification of ticks present at the three study sites using morphological keys.
- Detection of bacterial tick-borne pathogens using a 16S rRNA next-generation sequencing (NGS) approach based on the PacBio.
- To conduct a comparative analysis of bacterial microbiomes from the three study sites.

1.4. Dissertation Overview

Chapter 1: This chapter provides the introduction and defines the motivation and objectives of the study.

Chapter 2: This chapter provides a detailed review of the literature of ticks, tick vectors and tick-borne pathogens.

Chapter 3: This chapter entails the study design and discusses in detail the materials and methods used in the study.

Chapter 4: This chapter presents results of tick species and bacterial communities obtained from the three-study sites.

Chapter 5: This chapter discusses the results presented in Chapter 4. Additionally, concluding and recommendation remarks are stated in this chapter.

Chapter 6: This chapter is a list of all references for literature used in the study.

CHAPTER 2

LITERATURE REVIEW

2.1. Introduction

2.2. Ticks

The phylum Arthropoda, class Arachnida, subclass Acari, order Parasitoformes, and suborder Ixodida include ticks as obligatory ectoparasites. According to fossil evidence, ticks have existed for at least 90 years with more than 800 different species of ticks in the world (Klompen & Grimaldi, 2001). However, there are three tick families being the Ixodidae comprised of hard ticks, Argasidae comprised of soft ticks and Nuttalliellidae with one species *Nuttalliella namaqua* which occurs in Tanzania, Namibia, and South Africa (Diseko, 2018). They consume blood meals to stay alive and complete their intricate life cycles (Sonenshine & Roe, 2013). In the Ixodidae family, the capitulum protrudes forward from the body in the nymphs and adults (Balashov, 1972). The big spiracles lie right behind the coxae of the fourth pair of legs, and the eyes are near to the sides of the scutum (Cox, 2009).

In contrast to females and nymphs, the males possess a diminutive, shield-shape structure positioned below the capitulum. This structure is known as the scutellum because it serves as a rigid protective covering, enveloping the entirety of the dorsal surface in males. The act of attachment by the ixodid tick is typically not painful and the host cannot feel any form of discomfort as it often goes unnoticed by the host. These ticks remain attached to the host for extended periods, ranging from days to even weeks until they are fully fed (engorged) and ready to undergo the molting process. Some tick species prefer to detach from the host in order to molt in a secure environment, while some prefer to remain in the same host, this is mainly depended on the type of species, whether one, two or three-host (Sonenshine, 2005). Ticks are regarded as vectors for a variety of pathogens including viruses, bacteria, and protozoa linked to infectious diseases that are a risk to both human and animal health. The host and the environment have a critical role in the development and survival of ticks, which are extensively scattered throughout the planet. The second-largest

requirement for boosting survival after the host is warm temperatures in tropical and sub-tropical areas (Norval & Lightfoot, 1982).

Ticks and tick-borne diseases have a negative influence on livestock health and human livelihood. Ticks inhibit the production of cattle leading to mortality in tropical and subtropical regions of the world (Desta, 2016). Ticks infest livestock, particularly cattle, and release toxins during bites, leading to skin irritation and damage to hides. This infestation results in reduced milk production and exposes animals to bacterial, fungal, and screw-worm infections in the bite wounds, ultimately causing tick-borne diseases (Sackett et al., 2006). The relevance and presence of ticks alone and the infections they transmit in cattle result in significant veterinary and economic losses as well as causing grave economic damage in regions where cattle farming is mostly practiced for economic purposes, It has been found through research that farmers all over the world are still facing the biggest challenge of tick control, which is mostly caused by invasive tick species, along with native species. Due to climate and land change, animal imports, and an increase in global travel, the range of ticks has suddenly increased/expanded (Pérez de León et al., 2012). This can lead to public and veterinary problems as these invasive tick species can cause new infections and introduce endemic pathogens to new regions. According to a study by Nyangiwe (2018), the introduction of invasive tick species such as the one-host tick *R. microplus* and the three-host tick *A. variegatum,* also increases the risk of disease transmission because of their adaptability and capability to introduce a wide range of pathogens or spread pathogens to new areas. For example, *R. microplus* is slowly displacing the native tick *R. decoloratus* and has been reported to displace the indigenous tick *R. decoloratus* in South Africa (Nyangiwe et al., 2013). *Amblyomma variegatum* is prevalent in various regions in the African continent and considered the second most invasive tick species after the Asiatic blue tick, *R. microplus* (Barré & Uilenberg, 2010).

2.3. Life cycle of ticks

Ticks, regardless of the family classification they belong to, require a host for the completion of their life cycle which is also essential for survival and growth. Other ticks survive through the availability of a single, two, or three hosts. These ticks are named according to their dependence on the host, one-host tick, two-host tick as well and three-host tick respectively. The duration of the life cycle together with the number of hosts required for full development may vary per species. There are species capable of completing their cycle within months, while others may take several years (Estrada-Peña, 2015 & Lus, 2008). Four stages of equal importance form a complete life cycle. The stages include the eggs, larva, nymph, and the adult. The life cycle begins immediately upon the deposition of eggs by a mature female tick. The female tick is capable of laying a multitude of eggs simultaneously which are then deposited in protected regions such as leaf debris or soil and undergo the process of incubation (Sonenshine, 2005). Before incubation, the eggs emerge and subsequently hatch into larvae, which resemble adult ticks as they possess six legs. The larvae then initiate the process of host hunting for the next stage. The nymph forms part of the next stage in the life cycle of ticks. They have eight legs and exhibit a much larger size in comparison to larvae. Similar to the larvae, nymphs also require blood meal for further development. Nymphs attach to the host and engage in feeding through blood meal and detach right after and subsequently undergo a moulting process and develop into a final stage of an adult tick (Radolf et al., 2012). Adult ticks are generally larger than nymphs and more conspicuous and like other stages they also feed on blood meal for development wherein the female and male ticks mate (Klitgaard et al., 2019). After mating females become engorged and successively lay eggs, whilst the males die right after mating which vary with species.

2.3.1. One-host tick

After development into a larva, the tick finds a host and remains attached for as long as it may take to enable its full developmental stages (into a nymphal and adult) and only detaches from the host to lay eggs in the surrounding environment (Banks et al., 1998). Right after laying eggs, they quickly hatch into larvae and begin to hunt for a

host for attachment and feeding through blood meal. After feeding, the larvae molt into a nymph (unfed) and stay attached until they become engorged whilst still on the host molting into an adult tick that is sexually matured. The matured nymph will continue with its blood meal until it detaches from the host when it is ready to lay and deposit the eggs (Sonenshine & Roe, 2013).

2.3.2. Two-host tick

The tick voluntarily falls to the ground to lay eggs that hatches into larvae during the winter season. The larvae target a host (host one) for their next developmental stage while the larvae find a host and remain attached to it for feeding through blood meal until the nymphal (eight-legged) stage. Feeding continues until they become engorged and fall to the ground for a proper digestion of blood meal where they molt and develop into an adult tick. A hunt for an available host (host two) begins and the adults (both female and male) attach and have their blood meal and also mate. The engorged ticks (females) will fall to the ground for laying of eggs (Banks et al., 1998; Soneshine & Roe, 2013). The life circle of two-host ticks may take up to two years.

2.3.3. Three-host tick

Ticks that require multiple hosts are usually classified to be ixodid and their life circle may take up to three years. The laying of eggs to the ground or environment followed by hatching into the larvae stage takes place during the winter seasons. The larvae attach themselves to a host (host one), usually these are small animals. The larvae will feed on the host and become engorged and detach from the host during summer for full digestion of the blood meal and molting for the next nymphal stage. During spring, the engorged nymph will target small rodents as possible hosts (host two) for development into an adult tick. The following year during spring, the adult tick emerges and attaches to a new host (host three). The third host is usually bigger than the first and second host, for example, cattle. Mating between the female and male tick followed by blood meal feeding will occur until the female tick becomes engorged

dropping to the ground to lay eggs. Meanwhile, the male ticks remain attached awaiting mating with other female ticks (Sonenshine & Roe, 2013).

Therefore, with climate change, the stages forming the complete life circle of ticks are often disturbed for example, adult ticks are mostly abundant throughout warm and wet summer periods. The larvae are abundant in colder, dry, late autumn and winter seasons while the nymph is more abundant in winter and spring (Horak et al., 2017).

2.4. Tick vectors of veterinary importance in South Africa

2.4.1. *Hylomma* species

2.4.1.1. *Hyalomma rufipes*

This tick species is known as brown-legged simply because of the dark brown colour, well known as the large, coarse bont-legged tick with long mouthparts. The legs are shiny and brightly bended with colourful rings, this is based on descriptions by (Estrada-Peña et al., 2017). These ticks are widely distributed but mostly occur in drier seasons. According to findings by (Walker, 1991), the species is commonly found in Savannas, grasslands and other regions that may have the hosts preferable. *Hyalomma rufipes* are two-host ticks feeding on either goat, cattle, sheep, to mention a few. The tick species are equally important vectors of disease-causing agents that threaten the health of both humans and livestock, particularly in southern Africa. They transmit *A. marginale* an aetiological agent of bovine anaplasmosis also termed gall-sickness in cattle as well as the viral agent of Crimean-Congo haemorrhagic fever in human.

2.4.1.2. *Hyalomma truncatum*

This tick species is usually small with shiny smooth bont-legged, unlike *H. rufipes.* Adult ticks have a dark brown colour, mouthparts are very long, and the legs have ivory-coloured rings. Male ticks have numerous large punctations on the posterior region of the scutum. *Hyalomma truncatum* is predominantly found in Africa and

causes damage to tissues of their host and bacterial infections that result in the formation of abscesses and, is a vector of *A. marginale* a pathogen causing bovine anaplasmosis, and can also transmit *Babesia caballi*, the causative agent of equine piroplasmosis (Walker, 2003).

2.4.2. *Rhipicephalus* species

2.4.2.1. *Rhipicephalus appendiculatus*

This tick species are brown ear ticks also found in the eastern, central and South African (Mtshali, 2012; Khumalo, 2017). Adult ticks are medium sized with short mouthparts and the males tend to increase in leg sizes from the first up to the fourth pair, while fed ticks have a slender caudal process. They are widely distributed across other regions of the world. Cattle is the most preferred host for the tick species and in most cases is heavily infested throughout the developmental stages. Previous studies have revealed that the tick species has been identified (or reported) from many provinces of South Africa including Limpopo, North-west, Mpumalanga, KwaZulu Natal and Gauteng. This is the primary vector of *Theileria parva* responsible for causing East Coast Fever (ECF) in cattle in East Africa, January disease in Zimbabwe and Corridor disease in South Africa (Perry et al., 1991; Maboko et al., 2001; Estrada-Peña, 2015).

2.4.2.2. *Rhipicephalus decoloratus*

Rhipicephalus decoloratus is a single host tick, also known as the blue tick, simply due to the hue of engorged females. The adults are very tiny, unnoticeable, with slender legs and short mouth parts. Male ticks are brownish yellow in colour and are typically smaller than the female ticks. Through the sclerotized scutum, their darker coloured intestines and discernible. Areas of the body they usually target when parasitizing their host, which is preferably cattle are the face, the shoulders, neck as well as the escutcheon and usually paired with females (Walker, 2003). The *R. decoloratus* tick species is widespread in the majority of South Africa's more humid region, except for

those regions where the invasive tick species *R. microplus* has displaced it. This tick species is also found in frigid mountainous locations such as the Drakensberg range and is regarded as the most significant external parasite affecting livestock, mostly cattle. Furthermore, it is reported as an endemic tick to the African continent with a vast presence in tropical and subtropical regions (Sevinç & Xuenan, 2015). They are capable of transmitting *B. bigemina* the causal agent of bovine babesiosis, *A. marginale* causing bovine anaplasmosis (Horak et al., 2015).

2.4.2.3. *Rhipicephalus evertsi evertsi*

The medium-sized, dark-brown tick *R. evertsi evertsi*, one of the 74 species in the genus, has reddish-orange legs. They have a dark scutum and contrasting legs and adults are simple to distinguish (Walker, 2003). *Rhipicephalus evertsi evertsi* is a two-host tick species and the preferred hosts are both wild (zebras) and domestic animals such as horses, donkeys, cattle, goats and sheep. On livestock, this powerful and destructive tick is prevalent throughout African regions. The tick species is associated with *A. marginale* which is responsible for the disease bovine anaplasmosis. It is also a vector of *Babesia caballi* and *Theileria equi*, the causative agents of piroplasmosis in horses.

2.4.2.4. *Rhipicephalus microplus*

This is an Asian blue tick also recorded as an invasive tick species in South Africa, known to displace *R. decoloratus* and has high adaptability and capability to transmit an array of pathogens causing diseases of economic and veterinary importance (Nyangiwe et al., 2017). Despite its high adaptability, the study by (Nyangiwe et al., 2023) has shown that the species is currently undergoing a rapid expansion of its geographical distribution into regions where it previously did not prevail, which raises concerns in terms of disease transmission. Although the adult ticks of *R. microplus* are slightly larger and significantly redder in colour compared to *R. decolorus* adults, they generally look similar. This tick species is distributed throughout the coastal regions of the Eastern Cape province and can also be found at numerous locations in the

Northeastern portion of the Northern Cape province, and as well as in the coastal regions of the eastern Cape province (Nyangiwe, 2017). They prefer cattle and other animal species as host. Also, vectors of *B. bovis,* the causative agent of bovine babesiosis also termed the Asiatic redwater, *B. bigemina* causative agent of bovine babesiosis also termed African redwater, *A. marginale* causative agent of bovine anaplasmosis. Its capacity to transmit both *B. bovis* and *B. bigemina* in cattle and other livestock threatens production of cattle and other livestock (Nyangiwe et al., 2017). The tick species has been found to be expanding its host preference wherein goats were listed to be infested by the species, this implies and gives confirmation that the species is indeed undergoing through the process of adaptation in hosts either than cattle (Nyangiwe & Horak, 2007). Furthermore, (Nyangiwe & Horak, 2007) stated that ticks such as *R. microplus* detected from goats could potentially have an impact on the epidemiology of diseases threatening and causing a burden in cattle, consequently serving as carriers of zoonotic pathogens.

2.4.2.5. *Rhipicephalus simus*

Rhipicephalus simus are three-host ticks with adults having a huge dark brown colour. The males have a glossy scutum appearance and is covered in four longitudinal rows of huge punctations (Walker, 2003). The preferred domestic hosts of adult ticks are horses, cattle, and dogs. These ticks are widely distributed across southern Africa, from South Africa in the south to Angola, Zambia, Zimbabwe, Malawi, and Mozambique in the north, particularly in regions with moderate to heavy rainfall. The tick vector transmits *A. marginale and A. centrale* responsible for causing bovine anaplasmosis, *Babesia trautmanni* which is the causal agent of porcine babesiosis, also it excretes toxins which may result in paralysis in both lambs and calves (Norval & Mason, 1981).

2.4.2.6. *Rhipicephalus afranicus* n species formerly known as *R. turanicus*

Rhipicephalus afranicus is a three-host tick species, medium-sized with reddish-brown colour. The adult tick is usually found during late wet to early dry seasons (Walker,

2003), and primarily infests and feed on goats, dogs, cattle, sheep and lions and on other occasions horses are likely to be infested (Bakkes et al., 2020). According to an investigation carried out by Bakkes et al. (2020), the species previously identified as *R. turanicus* in different African nation was indeed a separate and unique species, namely *R. afranicus*. Bakkes et al. (2020) further went on and revealed that this differentiation was established through the examination of both morphological charecteristics and genetic assessment and is widely distributed across numerous countries within the Afrotropical region, encompassing Angola, Botswana, Cameroon, Malawi, Namibia, Nigeria, Sinegal, South Africa, Sudan, Tanzania, Uganda, Zambia, and Zimbabwe. However, the reclassification carries significant implications for enhancing the comprehension of the epidemiology of diseases spread by ticks, given that precise species identification is essential for efficient disease management and prevention measure. Furthermore, the findings highlighted the necessity for ongoing taxonomic reassessments and molecular investigations to accurately discern and distinguish amoung tick species. *Rhipicephalus* a*franicus n* species has been implicated in the transmission of *Babesia,* a genus of protozoan parasites that infect the red blood cells of animals and human, and *Hepatozoon canis* which is associated with diseases in animals (Giannelli et al., 2017). According to Bakkes et al. (2020), *R. turanicus* previously described from Africa (Afrotropical) has been redescribed as *R. afranicus* n. while *R. turanicus* sensu lato is comprised of two distinct genetic lineages; one of these lineages is found in Southern Europe, while the other is primarily located in the Middle east and Asia (Filippova, 1997).

2. 5. Selected bacterial tick-borne pathogens and Bacterial tick-borne diseases of veterinary importance in South Africa

2.5.1. Selected bacterial tick-borne pathogens

2.5.1.1. *Anaplasma* species

These are gram negative, obligatory intracellular bacteria of haemopoietic cells that are etiological agents of the family *Anaplasma*taceae and of the order Rickettsiales causing disease in both animals and humans (Brouqui & Matsumoto, 2007). The genus *Anaplasma* was first described more than one hundred years ago in the year

1910 by Sir Arnold Theiler in South Africa (Palmer, 2009). The *Anaplasma* species causing anaplasmosis, are of human, veterinary and medical importance. *Anaplasma* species causes diseases by infecting the blood cells of their host. Anaplasmosis is frequently found where competent vectors are native particularly in tropical and sub-tropical regions of the world. The recognised species associated with diseases of economic and veterinary significance are *A. marginale, A. centrale, A. phagocytophilum, A. platys, A. ovis,* and *A. bovis and A. boleense* (Brouqui & Matsumoto, 2007).

2.5.1.1.1. *Anaplasma marginale*

Anaplasma marginale is a widespread bacterium across South Africa with varied incidence in different regions. It is an etiological agent of the disease bovine anaplasmosis which is more prevalent in warmer and more tropical climates simply because the pathogen responsible for the transmission usually thrives in such environments (Tajedin et al., 2016). Bovine anaplasmosis exerts an adverse impact on the economic aspect of cattle farming (Hove et al., 2018), and is transmitted by tick vectors belonging to the *Rhipicephalus species,* for example *R. microplus, R evertsi evertsi, R. decolaratus,* and *Hyalomma* species (Hove et al., 2018). Once the pathogen is introduced in the bloodstream of vulnerable cattle or other ruminants by its vector via a bite during a blood meal, it multiplies and remain in the red blood cells of the host, subsequently causing infections. Infected cattle usually present signs of mild to severe febrile haemolytic anaemia, fever, weight loss, abortion and infertility may also result (Aktas et al., 2011). The severity of bovine anaplasmosis is heightened in animals exceeding the age of two years meanwhile younger animals experience less severe infection simply because they acquire immunity from the mother. This further results in substantial implications for cattle farmers situated across various regions of the globe including South Africa (Kolo, 2023).

2.5.1.1.2. *Anaplasma centrale*

Anaplasma centrale is an intra-erythrocytic tick-borne ricketssia that differs from *A. marginale* in terms of its morphology and aggressiveness. The pathogen *A. centrale* was reported to be discovered in Africa (Theiler, 1912). According to Sir Arnold Theiler's description of *A. centrale*, it is centrally positioned in the erythrocytes of the host animal and is less pathogenic compared to *A. marginale* (Kolo, 2023). Another study also highlighted that *A. centrale* is responsible for inducing a less severe manifestation of anaplasmosis, and it is employed as an attenuated vaccine against *A. marginale* providing long lasting protective immunity against some virulent strains, in numerous African, South American, and the Middle Eastern (Byaruhanga et al., 2018). *Anaplasma centrale* can be transmitted mechanically, through blood inoculation, or biologically through tick vector *R. simus*. It can also induce asymptomatic or paucisymptomatic infections and in most instances, mild anemia is likely to occur, and yet can potentially cause severe diseases. According to Kocan et al. (2010), in regions where *A. marginale* is endemic, cattle are heavily immunized with *A. centrale* against *A. marginale.*

2.5.1.1.3. *Anaplasma phagocytophilum*

Anaplasma phagocytophilum formely known as *Ehrlichia phagocytophilum*, is a Gram-negative bacterium with uncommon infinity for neutrophils. Findings by (Gillespie et al., 2012) revealed that *A. phagocytophilum* has been designated to replace three distinct types of granulocytic bacteria, namely *Ehrlichia phagocytophilia*, *E. equi,* and the causative agent of human granulocytic erhlichiosis. This was a decision taken following the recent restructuring of the *Rickettsiaceae* and *Anaplasmataceae* families within the Rickettsiales order (Gillespie et al., 2012). In humans, the bacterial pathogen is responsible for the zoonotic disease human granulocytic anaplasmosis (HGA), in dogs it is responsible for canine anaplasmosis, and in horses equine anaplasmosis, while causing anaplasmosis which is alternatively known as tick-borne fever (TBF) in domestic animals and livestock, granulocytic anaplasmosis (GA) in wild animals (Woldehiwet, 2010). In ruminants with tick-borne fever neutrophils alongside other granulocytes are impacted. This may lead to a severe bacterial infection, febrile

reaction and leukopenia due to neutropenia, lymphocytopenia and thrombocytopenia which are usually present within a week upon tick bite exposure (Woldehiwet, 2008), therefore, leading to the inhibition of growth rate and milk production. The outbreaks of TBF have been predominantly observed in sheep flocks and also in cattle herds, while infections in goats occur occasionally (Teshale et al., 2018). Further, transmission of *A. phagocytophilum* is through tick vectors which vary depending on the geographical location.

2.5.1.1.4. *Anaplasma platys*

Anaplasma platys is an intracellular bacterial tick-borne pathogen formerly known as *E. platys.* It is an etiological agent of canine infectious cyclic thrombocytopenia and has a world-wide distribution, but is primarily found in tropical and subtropical countries (Selim et al., 2021). Dogs are usually the innate host for *A. platys* where it targets and parasitize platelets which appears as a blue-black cluster when observed within platelets (Grasperge, 2014). *Rhipicephalus sanguineus* sensu lato which is the brown dog tick, is hypothesized to be a possible vector responsible for the transmission of *A. platys* in Africa (Kolo, 2023). Infected canines may exhibit clinical manifestations such tiredness, pyrexia, and a reduction in the number of blood cells involved in clotting. However, (Maggi et al., 2013) and (Arraga-Alvarado et al., 2014) proposed that *A. platys* is a zoonotic agent on the basis of previous investigations that recorded clinical infection in humans. Another study indicated *A. platys* prevalence of 0.2% in South African cattle (Kolo et al., 2020).

2.5.1.1.5*. Anaplasma bovis*

Anaplasma bovis is a Gram-negative obligatory intracellular organism that may also be present in a wide range of cattle and other animals. It is one of the most recently officially described species belonging to the family *Anaplasmataceae* which used to be known as *Ehrlichiaceae* (Park et al., 2018). This well-known tick-borne pathogen is widely distributed in nations practicing cattle and goat farming, as well as those having wild deer populations are mostly impacted. The occurrence of infections by *A. bovis* is

most prominent in Africa, South America, the Middle East, and Japan (Liu et al., 2012). The pathogen can invade and reside in mononuclear cells and macrophages in the blood of its host (domestic and wild ruminants), particularly monocytes, while they rarely cause infections (Jilintai et al., 2009). However, the spread of infections is subject to the influence of seasonality, climate and weather patterns (Estrada-Peña et al., 2005). In the case of cattle, infection caused by *A. bovis* is generally without any apparent symptoms, except for certain scenarios where, symptoms like fever, anaemia, debility, anorexia, swollen lymph nodes, depression and even death may occur (Onyiche et al., 2021). Ticks belonging to the genera *Ixodes*, *Dermacentor, Rhipicephalus* and *Amblyomma* are responsible for the transmission of *A. bovis* from one host to another through sucking of blood meal from an infected cattle host and in the meantime also absorbing the bacterium (Rymaszewska & Grenda, 2008). While vectors are completing their life cycle and targeting a vulnerable host, *A. bovis* can be transmitted to another host (Ueti et al., 2007).

2.5.1.2. *Mycoplasma* species

The genus *Mycoplasma* belongs to the class Mollicutes which lack a cell wall around their cell membrane (Maunsell et al., 2011). Numerous ailments in cattle, including mastitis, arthritis, pneumonia, otitis media and reproductive issues, can all be brought on by the pathogen of the genus *Mycoplasma* (Maunsell et al., 2011). Apart from having several afflicted areas, clinical *Mycoplasma* mastitis is frequently characterised by an inability to respond to treatment. On the other hand, arthritis and pneumonia can also affect adults and calves. Although otitis media is often only present in calves. *Mycoplasma* mastitis in the herd may be present concurrently with any other or all of these clinical symptoms (Radaelli et al., 2011). The two species of veterinary importance are *M. bovis* and *M. wenyonii.*

2.5.1.2.1. *Mycoplasma bovis*

This is a pathogenic bovine *Mycoplasma* that is anaerobic, band is the tiniest living cell known to exist (Razin et al., 1998). The main hosts of *M. bovis* and it has a minor

impact on other agricultural animals. *Mycoplasma bovis* is known to cause a variety of illnesses such as mastitis in dairy cows, arthritis in both cows and calves, pneumonia, and several other illnesses, potentially including late-term miscarriage (Ruffin, 2001). Other organs of the body that may be implicated by *M. bovis* include the eye, the ear or the brain. In cases where some infected cattle shed the pathogen without becoming ill, there are possibilities for the bacterium to spread between farms when healthy animals are relocated (Maunsell et al., 2011).

2.5.1.2.2. *Mycoplasma wenyonii*

This is a hemotrophic epicellular bacterial parasite of cattle formerly known as *Eperythrozoon wenyonni* (Paul et al., 2020). *Mycoplasma wenyonii* is the causative agent of bovine mycoplasmosis, which is a disease that targets cattle and infects the red blood cells, subsequently leading to several clinical conditions, including haemolytic anaemia, reduced milk production and peripheral oedema (Neimark et al., 2002). It is said that apart from ticks there are other vectors responsible for the transmission of the disease, and these are blood-sucking arthropods such as flies with clinical signs inclusive of fever, lethargy, and jaundice (Lefèvre et al., 2010; & Adam, 2019).

2.5.1.3. *Ehrlichia* species

These are obligate intracellular Rickettsiales that are transmitted by ticks to vertebrates. During the earliest stages of infection, *Ehrlichia* species exhibit intercellular transmission facilitated by filopodia of the host cell. However, in the later stages of infection, the pathogens tend to rupture the host cell membrane, resulting in the release of newly formed bacteria into the surrounding tissue, which can then infect neighboring cells and propagate the infection further. The pathogens such as *E. ruminantium* causes disease in ruminants, and is belonging to the genus *Ehrlichia* which is the causal agents of ehrlichiosis (Thomas et al., 2010).

2.5.1.3.1. *Ehrlichia ruminantium*

Ehrilichia ruminantium is a bacterium that is classified as gram-negative and obligate intracellular. It serves as a causative agent for heartwater, a tick-borne disease that can be severe and fatal, primarily affecting ruminants such as cattle, sheep, and goats (Thomas et al., 2010). The pathogen *E. ruminantium* was previously known as *Cowdria ruminantium* and can be transmitted through a bite of an infected tick belonging to the *Amblyomma* genus. Among the ticks responsible for disease transmission is *A. hebraeum* which is considered the most significant vector for transmission (Frutos et al., 2007).

Ehrlichia ruminantium effectively infiltrates and reproduces within the white blood cells of the host, particularly the monocytes and macrophages. This mode of existence within the host's cellular environment enables the bacterium to elude the immune response of the host and establish long-lasting infections (Weese et al., 2019).

According to (Frutos et al., 2007), *E. ruminuntium* is recognized for its extensive range of genetic variation, and the modifications in the composition of proteins located on the outer membrane can undoubtedly contribute to the bacterium's capacity to infiltrate various hosts. Furthermore, the variation in strains has the potential to influence the intensity of illness, the scope of hosts affected, and overall, the degree of pathogenicity.

2.5.1.3.2. *Ehrlichia canis*

Ehrlichia canis are tiny obligatory intracellular gram-negative proteobacterium transmitted by tick-vectors. The pathogen causes canine monocytic ehrlichiosis in dogs (Mavromatis et al., 2006). The pathogen is associated with the brown dog tick *R. sanguineus* serving as a vector which upon feeding through blood engorges on a dog carrying rickettsemia (Bowman et al., 2009). The tick harbours the pathogen within its mid-gut and salivary glands, subsequently transmitting it to the hosts during blood meals. Although the pathogen is commonly associated with dogs, human cases have also been reported (Bowman et al., 2009).

2.5.2. Bacterial tick-borne diseases of veterinary importance in South Africa

Bacterial tick- borne diseases affect the productivity of cattle in various regions of the world. The prevalence of these diseases poses a substantial apprehension for livestock agriculturists in South Africa, as they have the potential to result in financial detriments due to diminished efficiency, high mortality, and the expenses linked to intervention and precautionary measures (Stoffel, 2023). The bacterial tick-borne diseases hold veterinary significance and have the potential to impact livestock and companion animals. These diseases possess the capability to exert an influence on livestock and domesticated animals and are diseases caused b*y Anaplasma* species and *Rickettsia* species for example (Kipp et al., 2023).

2.5.2.1. Anaplasmosis

Anaplasmosis is an infectious disease affecting cattle and other domestic and wild ruminants in tropical and subtropical areas of the world. The disease is brought on by obligate intra-erythrocytic rickettsiae belonging to the genus *Anaplasma* and family *Anaplasmatacea* (Kocan et al., 2004). *Anaplasma marginale* and, to lower degree *A. centrale,* are the major culprits behind anaplasmosis in cattle, often known as gall-sickness (Barré & Uilenberg, 2010). Studies on the disease have been carried out in almost all provinces of South Africa (Marcelino et al., 2012) (Mutshembele et al., 2014). The disease is typically spread by *R. decoloratus, R. microplus, R. simus, and R. evertsi evertsi* tick species. Also, it can be mechanically transmitted by biting flies, infected medical equipment and or through contaminated needles. With ticks, the transmission occurs when an infected tick feeds on naïve cattle, thus introducing the pathogen into the bloodstream. Depending on the severity of the *Anaplasma* strain, the sensitivity of the host, or concurrent infections, the disease can appear clinically in a variety of ways ranging from asymptomatic to lethal. Pyrexia, progressive anaemia, jaundice, anorexia, depression, low milk supply, and sometimes abortion in pregnant animals and mortality are all significant clinical indicators. Treatment is by administration of tetracycline or imidocarb dipropionate.

2.5.2.2. Heartwater

Heartwater also known as ehrlichiosis is rickettsial disease that affects ruminants and is acute, deadly, and non-contagious, and can only be found throughout the year. However, it is frequently common from November to April during rainy seasons. During this time of the year tick activity is at its peak (Marcelino et al., 2012). It is brought on by the bacteria *Ehrlichia ruminantium* which was formerly known as *Cowdria ruminantium* and the vector associated belongs to the genus *Amblyomma*. *Amblyomma hebreum* is the recognized tick vector for the illness in cattle. It is during the feeding process when the pathogen is transmitted to the next available or targeted host. Heartwater is characterised by fever, mouth foam, loss of appetite, constant chewing, lethargy, and odd laying positions.

2.5.2.3. Rickettsiosis

Rickettsiosis is one of the earliest recognized vector-borne infections transmitted by ticks and is caused by obligate intracellular gram-negative bacteria belonging to the spotted fever group (SFG) which is classified as one of the major group within the rickettsial genus (Yssouf et al., 2014). *Rickettsia rickettsii* which causes Rocky Mountain spotted fever belongs to the Rickettsieae family. *Rickettsia africae* is an aetiological agent of African tick-bite fever and is the most prevalent of the rickettsial strains found in the sub-Saharan Africa in ticks and vertebrate animals (Pillay et al., 2022). *Amblyomma variegatum* has been demonstrated to transmit *R. africae* via the trans-ovarial and trans-stadial routes in Uganda (Socolovschi et al., 2007), while in southern Africa *R. africae* is transmitted by *A. hebreum*. *Amblyomma Hebreum* is a vector responsible for the transmission of agents of rickettsiosis (Yssouf et al., 2014). Fever, sadness, conjunctivitis, retinal hemorrhages, soreness in the muscles and joints and coughing are examples of clinical symptoms of the rickettsiosis.

2.5.2.4. Ehrlichiosis

Ehrlichiosis is a bacterial disease carried by ticks, which serve as the major vectors.

Ehrlichiosis is caused by different species *of Ehrlichia* genus. According to findings by (Aziz et al., 2022) these pathogens predominantly invade white blood cells, resulting in a range of symptoms in both animals and humans. The manifestation and severity of the illness vary depending on the species of *Ehrlichia* species involved (Bolling et al., 2022). Ehrlichiosis is commonly referred to as a form of tick fever in animals, while humans, the ailment is known as human monocytic ehrlichiosis (HME) or human granulocytic ehrlichiosis (HGE), conditional upon the specific *Ehrlichia* species implicated. Research has also indicated that dogs appear to have more capacity to contract *E. canis*, *E. ewingii*, and *E. chaffeensis*, with *E. canis* being the most virulent (Bolling et al., 2022).

2.6. Cattle farming and its contribution to the economy

Globally, cattle farming is a crucial industry that supports livelihood and food security. Tick infestation, however, presents a significant problem for cattle farmers because of the potential harm they could do to the health, productivity, and overall profitability of the farm. Ticks and tick-borne diseases pose a threat to cattle farming in most provinces of South Africa leading to economic losses in the livestock industry. These losses threaten food security, global trade, eco-tourism, and livelihoods. The infestation of ticks and manifestation of diseases such as theileriosis, ehrlichiosis, babesiosis and anaplasmosis inhibit production rates and results in substantial economic losses. For example, these diseases can cause severe health issues in cattle, which includes anaemia, fever, weight loss, and decreases immune function. As a result, unhealthy cattle are less productive in terms of milk and meat production. Tick bites and the associated diseases can lead to reduced milk yield due to stress and illness in dairy cattle. Infested and diseases cattle often experience poor growth rates and weight loss, leading to lower meat production and reduced market value. Tick-borne diseases can affect the reproductive health of cattle, leading to lower fertility rates, miscarriages, and poor calf survival, which impacts herd replacement and growth. Managing tick infestations and treating diseases require significant financial investment in veterinary care, medications, and preventative measures, increasing operational costs for farmers. Severe infestations and diseases can lead to high mortality rates in cattle, directly reducing the number of animals available for

production. Infested and sick cattle may not be able to work as efficiently in farming operations, further decreasing overall productivity. Lastly, ticks can damage cattle hides, reducing their quality and market value, which impact the profitability of cattle farming. Therefore, aforementioned factors combined decreases production rates and increase economic losses for cattle farmers (Soneshine & Roe, 2013). Economic losses in South Africa caused by ticks and associated diseases in the livestock industry are estimated to exceed about USD 33 million (approximately ZAR 500 million) per annum, that is (Makwarela et al., 2023).

The tropical and sub-tropical climate in Africa favours the multiplication and maintenance of tick vectors and thus transmission of various pathogens to cattle. The key challenges in controlling tick-borne diseases include acaricide resistance, policy issues, uncontrolled animal movement, and insufficient veterinary services (Mukolwe et al., 2021). Tick-borne pathogens including *Babesia* species, *Theileria* species, *A. marginale*, *Rickettsia* species, *Ehrlichia ruminantium* and *Coxiella burnetii*, have been previously detected in ticks collected from cattle from almost all the provinces of South Africa, except the Northern Cape where there has been few studies conducted on ticks and tick-borne diseases (Guo et al., 2019).

South Africa displays a persistent need for the practice of rearing cattle, both within its borders and on an international scale. The agricultural terrain of South Africa incorporates the practice of cattle farming to a considerable extent. If executed with precision, such an endeavour possesses the potential to yield substantial financial gains and grant a sense of fulfilment. However, the establishment of a cattle farming necessitates a significant capital investment as well as a substantial amount of labour (Kunene-Ngubane et al., 2018).

According to the Agricultural survey published by the Department of Statistics South Africa, Tables 2.1 and 2.2 below give an insight into the importance of agriculture and the impact it has on the economy, and how these two provinces of South Africa, Free State and Kwa-Zulu Natal contribute towards the state of the economy.

The total sales of goods, services, salaries and wages and the total number of employees between the years 2020 and 2001 are shown in Table 2.1. There was an increase in sales of goods and services, an increase in salaries and wages, as well as

an increase in the total number of employees in both Free State and Kwa-Zulu Natal provinces of South Africa. This simply shows how much of an impact these provinces have towards the economic sector.

Table 2.2 is a representation of the importance of agriculture with regards to various activities which plays a role in generating income and expenditure and looking at other related industries. An increase in the total income and expenditure between the years 2020 and 2021 was observed. This was influenced by the activities which were ongoing and off pivotal role towards Agriculture and the economic sector. These activities are inclusive of; farming of animals, growing of crops, market gardening, horticulture, mixed farming - growing of crops combined with farming of animals, agriculture and animal husbandry services, and hunting, trapping and game propagation including related services.

Table 2.1. The total sales of goods, services, salaries and wages and the total number of employees in 2020 and 2021 (adopted from www.stassa.gov.za).

Province	Sales of goods and services		% change	Salaries and wages		% change	Total employees		% change
	2020	2021		2020	2021		2020	2021	
	R'000			R'000			Number		
Western Cape	65 095 293	74 931 914	15,1	11 761 437	12 858 664	9,3	186 553	186 659	0,1
Eastern Cape	29 521 562	31 964 542	8,3	3 895 254	4 403 846	13,1	70 554	74 163	5,1
Northern Cape	16 820 242	17 879 199	6,3	2 577 223	2 880 615	11,8	61 910	66 314	7,1
Free State	42 738 748	51 089 492	19,5	3 201 079	4 065 751	27,0	67 996	73 972	8,8
KwaZulu-Natal	37 184 941	40 404 395	8,7	5 525 008	6 104 887	10,5	97 263	105 076	8,0
North West	40 387 774	45 660 760	13,1	4 402 826	4 221 290	-4,1	66 628	63 115	-5,3
Gauteng	43 820 565	46 393 832	5,9	4 381 468	5 046 480	15,2	50 955	53 703	5,4
Mpumalanga	36 805 241	43 938 541	19,4	4 515 453	5 027 396	11,3	72 046	80 596	11,9
Limpopo	29 268 921	33 596 554	14,8	5 428 709	5 695 447	4,9	106 152	97 905	-7,8
Total	**341 643 287**	**385 859 229**	**12,9**	**45 688 457**	**50 304 376**	**10,1**	**780 057**	**801 503**	**2,7**

Table 2.2. The total income and expenditure by type of activity in the agriculture and related industry, 2020 and 2021 (adopted from www.stassa.gov.za).

Activity	Total income			Current expenditure		
	2020[1]	2021[2]	% change	2020[1]	2021[2]	% change
	R'000			R'000		
Growing of crops; market gardening; horticulture	155 409 058	184 289 674	18,6	144 292 924	168 182 540	16,6
Farming of animals	144 359 856	154 629 020	7,1	138 708 660	149 236 515	7,6
Growing of crops combined with farming of animals (mixed farming)	59 360 449	62 552 690	5,4	57 396 809	57 685 937	0,5
Agricultural and animal husbandry services, except veterinary activities	10 382 628	13 404 011	29,1	8 990 499	11 509 801	28,0
Hunting, trapping and game propagation including related services	3 326 965	2 209 120	-33,6	3 973 055	2 696 197	-32,1
Total	**372 838 956**	**417 084 515**	**11,9**	**353 361 947**	**389 310 990**	**10,2**

2.7. The effect of climate change on tick distribution

The surveys conducted on ticks have revealed that the influence of climate change on tick distribution and the transmission of tick-borne diseases is significant. Alterations in climatic conditions result in the expansion of tick habitats in tropical and subtropical areas, potentially leading to an increase in the occurrence of tick-borne diseases (Lee & Chung, 2023). The high prevalence of tick-borne infections in regions like South Korea has been attributed to climate change, as infections have drastically gone high (McCoy et al., 2023). Surveillance studies on tick populations are of utmost importance, as they evaluate threats posed by tick-borne diseases and assist in formulating control strategies (Cunze et al., 2022). Moreover, understanding the mechanics and the dynamics of tick invasions and their influence on the host populations is imperative for predicting and regulating the consequences of climate change on tick-borne disease as climate change assumes a substantial function in the dispersion, conduct, and transmission of ticks and tick-borne diseases (McCoy et al., 2023).

In a study conducted by (Nyangiwe et al., 2018), which aimed to examine the driving forces responsible for changes in the geographic range of cattle ticks in Africa, the authors highlighted that the current global transformation such as climate change, deforestation, alterations in land utilization, urbanization, amplified trade, and travel exert a significant influence on the cattle industry worldwide by facilitating the introduction of ticks and the diseases they transmit into regions that were previously not reported.

2.8. Tick control methods

Tick management plays a crucial role in the regulation of tick-borne illnesses and the mitigation of the adverse effects that these ectoparasites have on livestock, pets, and human well-being (Stafford III et al., 2017).

A variety of techniques are employed for tick management, with the effectiveness of each method depending on factors such as the specific strategy used, the tick species involved, and the local ecological conditions. Approaches for controlling ticks

encompass the utilization of chemical acaricides (tick control agents), pheromone-assisted control, genetic methods, extensive surveillance for comprehending the species and distribution of ticks, and the development of vaccines, good management practices such as maintaining a tick-free environment and proper animal husbandry (Shaw, 1973; Ginsberg & Stafford III, 2005; Stafford III et al., 2017; Kipp et al., 2023). The main objective of these approaches is to suppress tick infestation, to hinder the transmission of tick-borne diseases, and to further safeguard populations that are highly exposed to risks.

Controlling ticks is an issue of global magnitude and is therefore a top priority for numerous nations and is primarily dependent on the utilization of traditional acaricides such as arsenical chlorinated hydrocarbons, organophosphates, carbamates, formamidines, and synthetic pyrethroids (Rajput et al., 2006; Nchu et al., 2020). The application of these acaricides may take various forms, including topical administration using sprays, dips, or pour-on, as well as the use of impregnated ear tags, and systemic administration using oral medications. When administered with precision, they exhibit efficacy and possess the potential for cost-effectiveness (Willadsen, 2006). There are significant disadvantages with managing tick infestation. These include the presence of chemical remnants in consumables such as milk products and meat, contamination of the environment, the emergence of ticks that are resistant to acaricides, the expenditure incurred in creating novel acaricides, and the obstacles encountered in the breeding of tick-resistant cattle while simultaneously preserving their desired production traits (Bishop et al., 2023). Acaricides have for years been considered as one of the most effective strategies for the control of ticks, however recent evidence has demonstrated that ticks have progressively acquired resistance towards various types of acaricides (Rajput et al., 2006).

Other studies have indicated that some tick species, namely *R. decoloratus* and *R. microplus* have developed resistance to diverse groups of acaricides, encompassing cypermethrin, amitraz, and deltamethrin (Nyangiwe et al., 2018).

There are alternative methods for tick control that have been investigated. This entails the use of predators and parasites for biological control, in addition to this, the advancement of vaccines has also been explored as a means of tick control (Stafford III et al., 2017). Furthermore, integrated management programs that encompass a

range of control strategies have been recommended, while other research aimed at comprehending the mechanisms behind acaricide resistance in ticks and investigating alternative approaches to mitigate resistance. These approaches include the synergistic use of diverse acaricides, the use of plant-derived phytochemicals, the application of fungi as biological control agents, and the development of anti-tick vaccines. With this being the case, it is however imperative to transcend reliance on acaricides and delve into more sustainable and efficacious methods for tick control (Ginsberg & Stafford III, 2005).

2.9. Next-generation sequencing

Next generation sequencing (NGS) plays a significant role in the identification of bacterial tick-borne pathogens due to its capability to detect multiple pathogens and offers a more comprehensive understanding of the ecology and evolution of pathogens (Vayssier-Taussat et al., 2013). Unlike traditional PCR-based methods that rely on known sequences for the design of specific primers, NGS has the advantage of detecting multiple pathogens without any prior information about the targeted pathogens. This characteristic of the NGS technique renders it unbiased and more inclusive, as it can detect coinfections and unravel the complexity of pathogen ecology (Cabezas-Cruz et al., 2018). Moreover, NGS has been extensively utilized to investigate the interactions between pathogenic and non-pathogenic microorganisms associated with ticks, thereby revealing the reciprocal relationship between pathogens and the host microbiota (Chaorattanakawee et al., 2022). However, the analysis of NGS data can be a daunting task, and the application of analytical tools like network analysis can assist in unravelling the structure of microbial communities linked to ticks and various ecosystems (Paulauskas et al., 2008). Consequently, NGS serves as a robust tool for the detection of tick-borne pathogens, providing valuable insights into the pathogen ecology and transmission (Paulauskas et al., 2008).

2.9.1. Assessment of bacterial pathogens using Next Generation Sequencing

Knowledge of ticks as vectors of infectious pathogens has progressed to a stage of identification of emerging tick-borne bacterial diseases that are a threat globally (Andreotti et al., 2011). It is expected that knowledge of several tick-borne pathogens affecting humans and animals will increase as research on tick biology and ecology continues (Andreotti et al., 2011). Cattle ticks have evolved in conjunction with the bovine hosts, and it is more likely that the ecology of their microbiome is influenced by the bovine or host interaction.

Gradually, survey studies of the bacteriome in cattle ticks using non-cultured molecular approaches are gaining momentum. These surveys are advantageous in wide scale of improving and understanding epidemiology of bacterial pathogens, indication of geographic variation in the assemblages of bacteria, identification of new bacterial species and increase in awareness of the tick role in the transmission of pathogenic bacteria (Aivelo et al., 2019).

The sequencing of the bacterial pathogens using NGS, targets most of the 16S rRNA gene. The hypervariable regions V1 to V9 differ between species, therefore sequencing of these regions allows the classification of bacterial pathogens at the family, genus and even species level (Papa et al., 2020). Additionally, the bacteriome assessment includes a variety of symbionts. Therefore the NGS technology enables a broader revelation of tick-microbiome-host interactions which plays a critical role in tick survival, vectorial capacity, pathogen transmissibility and host susceptibility to tick-borne infections (Papa et al., 2020).

In a recent study by (Mtshali et al., 2022), bacterial communities present in bovine milk, faeces and blood were explored and compared using 16S metagenomics sequencing. The amplification of V3 and V4 hypervariable regions of the 16 rRNA gene, yielded novel insights into the structure and composition of microorganisms present in bovine milk, faeces and blood. Furthermore, (Mtshali et al., 2022) highlighted that conducting a comprehensive study of microorganisms on a large scale is valuable in identifying potential harmful bacteria populations, which in turn can provide guidance in conducting more specific sampling and detection methods to identify both harmful and harmless bacteria in various parts of the body.

Another study conducted by (Khasapane et al., 2023) focused on the characterisation of milk microbiota from subclinical mastitis and apparent healthy dairy cattle in the Free State province of South Africa. A 16S meta-barcoding technique was employed to characterise the microbial communities from milk, from dairy cows affected by subclinical mastitis (SCM) in comparison to milk from non-subclinical mastitis dairy cows. The findings indicated that the composition of the microbial communities in SCM and non-SCM cows differs significantly. The researchers further concluded that comprehensive epidemiological investigations are recommended to better manage bovine mammary gland health. Additionally, these investigations can provide supplementary insights into the ecology of raw milk microbiota and the identification of fastidious bacteria and poly-microbial diseases.

CHAPTER 3

MATERIALS AND METHODS

3.1. Ethical approval

The study was approved by the University of Pretoria, Faculty of Veterinary Science Animal Ethics Committee (AEC), Research Ethics Committee (REC) with ethics reference number REC029-21, and the collection of cattle samples was approved by the Department of Agriculture, Land Reform and Rural Development under section 20 of the Animal Diseases Act of 1984 with reference 12/11/1/1/MG.

3.2. Study area and sample collection

3.2.1. Study sites

The study was conducted in three neighbouring towns in South Africa, two in the Free State province (Harrismith and Phuthaditjhaba) and one in KwaZulu-Natal province (Bergville) from 29 October to 01 November 2021 (Figure 3.1). The three sites are rural and demarcated by Drakensberg mountains and there is a constant uncontrolled movement of cattle among the three areas for traditional practices and trade.

In Harrismith, the temperature typically varies from -1°C to 26°C. The warm season is from October to March and the cold season is from May to August. The rainy period is from August to May, with a sliding 31-day rainfall of at least 12.5 mm. Average temperatures in October and November are 16.3°C and 17.3°C, while average rainfall is 94 mm and 128 mm, respectively (https://en.climate-data.org/africa/south-africa/free-state/harrismith-12770/).

The climate in Phuthaditjhaba is mild, and generally warm and temperate. The temperature averages 13.4°C (monthly average range 7°C to 17.8°C). The summer months are December, January, February and March. Average temperatures in October and November are 15.2°C and 16.4°C, while average rainfall is 109 mm and 135 mm, respectively (https://en.climate-data.org/africa/south-africa/free-state/phuthaditjhaba-55825/).

The climate in Bergville is warm and temperate, with temperature varying from 3°C to 28°C (average 15°C). January is the warmest month of the year, with average temperature of 20.1°C. The summers are much rainier than the winters and the annual rainfall is 1657 mm. The months of summer are December, January, February and March. The driest month is June with 19 mm and the greatest amount of precipitation occurs in January, with an average of 312 mm. Average temperatures in October and November are 17.1°C and 18.5°C, while average rainfall is 148 mm and 196 mm, respectively (https://en.climate-data.org/africa/south-africa/kwazulu-natal/bergville-14872/).

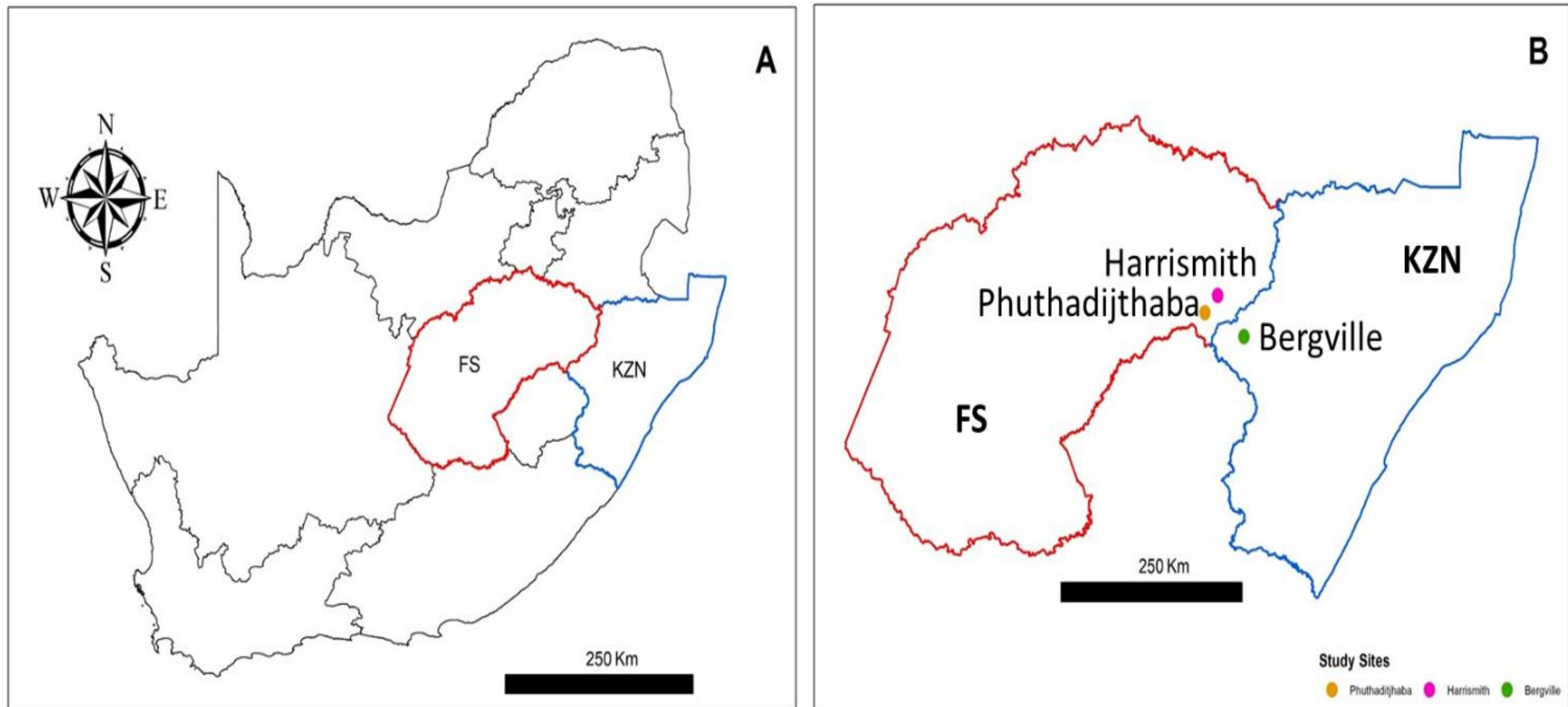

Figure 3.1: (A) Map of South Africa showing the two provinces Free State (FS) and KwaZulu Natal (KZN). **(B)** Map showing the three study sites/neighbouring towns. Maps were constructed using ArcGIS Desktop 10.8.2 (Esri ArcMap).

In Harrismith, the Makholokoeng village was the area of interest. The farmers assembled their cattle. The cattle appeared physically fit but had tick infestations, hides presented with wounds which were not properly treated and some of the animals appeared under conditioned. The source of food is grass shared with other livestock: sheep and goats. The control of ticks is through Taktic® Cattle Spray and Terramycin ® LA Injectable Solution, the application of these two products is done sporadically. In Phuthaditjhaba, cattle belonging to farmers at Thabo Mofutsanyane with geographical coordinates of 28,46964°S, 28,81456°E were assembled for the purpose of blood and tick collection. The cattle at this site were predominantly female Brahman, with poor body condition, restless and highly infested with ticks and blood sucking arthropods like flies. Cattle grazed in a mountainous grass land. A close interaction between dogs and cattle was observed as the herders are always accompanied by their dogs to the field. Drastic Deadline Pour On is used to control tick infestations for a period of three to four months.

On the other hand, Woodford in Bergville situated at 28,68725°S, 29,29705°E was comprised of mixed breed with more female cattle. Cattle graze in mountainous land and interact with cattle belonging to other famers is a daily practice. Cattle at this site were highly infested. Farmers indicated to use Drastic Deadline Pour On for any other ectoparasites.

3.3. Sample collection

Blood and tick samples were collected from cattle as stipulated in sections 3.3.1 and 3.3.2, respectively. Figure 3.2 shows the schematic workflow of the study design from sample collection to sequence analysis and taxonomic classification.

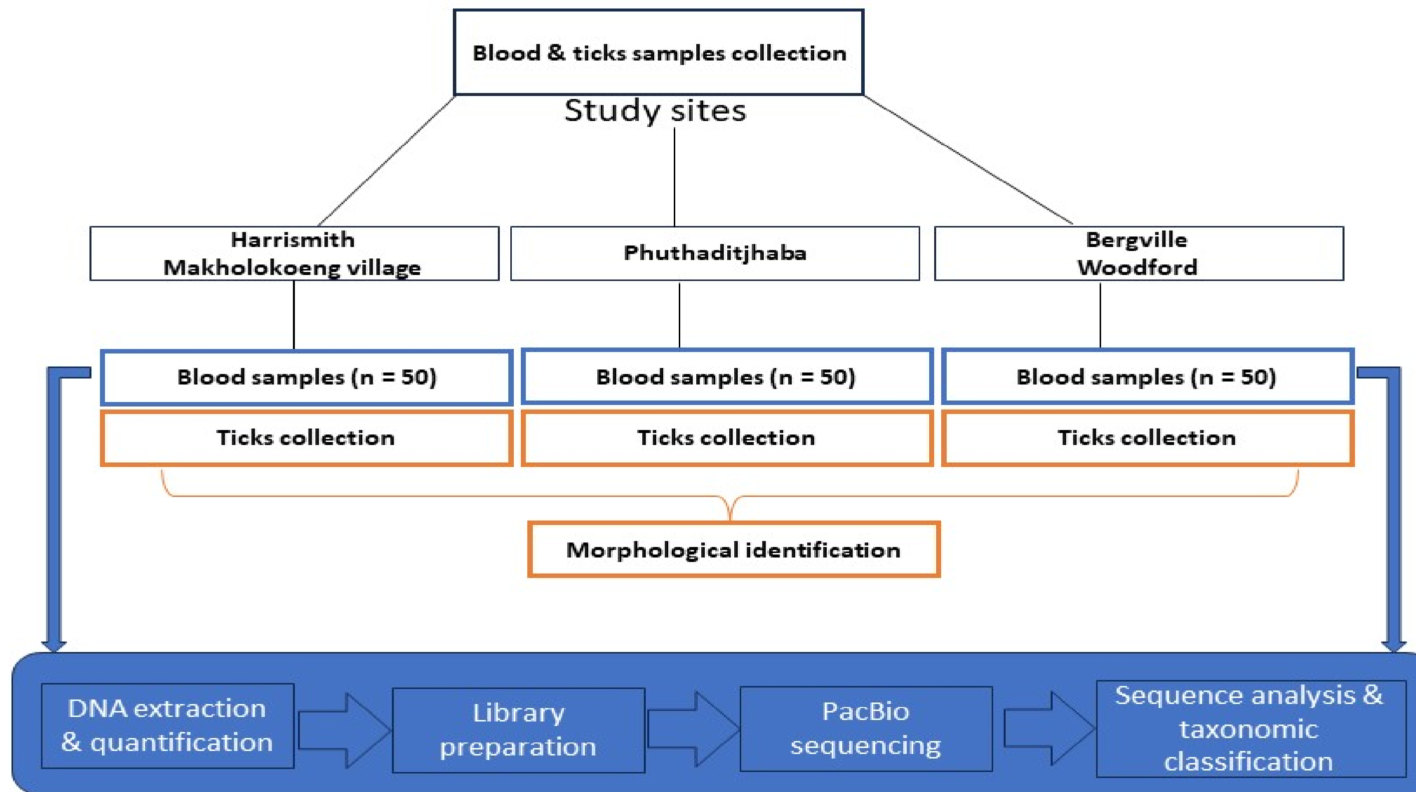

Figure 3.2. Schematic workflow of the study design from sample collections to analysis

3.3.1. Blood sample collection

In total N=150 blood samples were collected from cattle, comprised of fifty samples from each study site. Blood sample collection was performed by certified Animal Health Technicians from the Department of Agriculture Land Reform and Rural Development (DALRRD). Cattle were individually restrained, and blood was collected from the tail targeting the coccygeal vein into 6 mL Ethylenediaminetetraacetic acid (EDTA) coated vacutainer blood collection tubes with sterile needles following precautionary measures and safety guidelines. Each EDTA tube was assigned an identification number with Harrismith denoted as sample 1-50, Phuthaditjhaba denoted as sample 51-100 and Bergville denoted as sample 101-150. Samples were placed in tube racks and transported in a cooler box with icepacks until arrival in the laboratory where they were stored at -20°C at the University of Pretoria, Ondersterpoort campus prior to processing.

3.3.2. Tick sample collection

Ticks were collected from all the sampled cattle (n=150) targeting predilection sites such as the legs, the abdomen, neck, back and anal regions. Ticks were carefully handpicked with gloves on and placed into 30 mL specimen containers with cotton wool and addition of water droplets. The containers were labelled according to the collection sites (Harrismith, Phuthaditjhaba and Bergville) and transported to the University of Pretoria.

3.4. Tick Samples

3.4.1. Morphological identification

Prior to microscopic examination, engorged ticks were excluded while others were washed with 10% (w/v) Tween 20 for approximately an hour then rinsed with 70% ethanol followed by rinsing with double distilled water for thorough elimination of dirt and other environmental contaminants and dried on paper towel. The number of ticks collected per site was recorded to a total of 418 with Harrismith (n=126),

Phuthaditjhaba (n=160) and Bergville (n=132). Tick species were identified using a stereomicroscope (C-W10Xa/22 Nikon Japan) following literature key guides as suggested by Walker, (2003) and expert advice by Dr Nyangiwe who is an Acting Director: Livestock Research at Döhne Agricultural Development Institute in the Eastern Cape Province of South Africa was employed. The key features used during microscopic examination were the anal groove, size of the ticks, colour or pattern of the enamel, palps, eyes, festoons, legs and adanal plates and most importantly the mouthparts which are very much helpful in differentiating similar species such as *R. microplus* and *R. decoloratus*. After identification, ticks were recorded and grouped according to the study site and species.

3.5. Cattle blood samples

3.5.1. Characterisation of microbiome present in cattle

3.5.1.1. DNA extraction from blood of cattle

Cattle genomic DNA was extracted using the QIAmp® Blood Mini Kit (cat. nos. 51105 and 511060 following the manufacturer's guide, all centrifugation steps were done at room temperature (15 - 25°C) with lysis buffer (AL) warmed at 56°C to dissolve any precipitates using a heating block. Ethanol was added to both buffers AW1 and AW2 concentrates as per package insert instructions. Protease solvent was added to the lyophilized QIAGEN Protease thus, samples were equilibrated at room temperature (15 - 25°C) and the heating block was preheated to 56°C.

QIAGEN Protease (20 µL) was pipetted into a 1.5 mL micro-centrifuge tube containing 200 µL of whole blood. An amount of 200 µL of buffer AL was added to the tube and mixed by vortexing. The solution was incubated at 56°C for 10 minutes and centrifuged briefly for complete removal of possible drops from the lids of the micro-centrifuge tubes. A volume of 200 µL of ethanol was added and vortexed thoroughly followed by centrifugation. The solution was then transferred to a 2 mL QIAamp Mini spin column and centrifuged at 6000 x g (8000 rpm), discarding the flow-through and the collection tube. The QIAamp Mini spin columns were placed in a new set of collection tubes with an addition of 500 µL buffer AW1 and centrifuged for a minute at 6000 x g (8000 rpm),

after spinning the flow-through and collection tubes were discarded. Another set of 2 mL collection tubes were used to place the QIAamp Mini spin column with 500 µL of buffer AW2 added onto it. Centrifugation at full speed (20,000 x g; 14 000 rpm) followed for 3 minutes, discarding the flow-through and collection tubes right after. For thorough elimination of possible buffer AW2 carryover, a new set of 2 mL collection tubes were used to place the QIAamp Mini spin column and centrifuged at full speed for 1 minute thereafter, placing the QIAamp Mini spin columns in 1.5mL micro-centrifuge tubes with an addition of 200 µL buffer AE which was then incubated at room temperature (15 - 25°C) for a minute then centrifuged at 6000 x g (8000 rpm) for a minute for DNA elution.

3.5.1.2. Genomic DNA quantification using Qubit Assay

Genomic DNA was quantified using a double stranded Qubit assay and the concentrations were recorded. This was done to determine the concentration and purity of the DNA prior further processing. The Invitrogen by Thermo Fisher Scientific Qubit™1X dsDNA HS Assay Kit was used to measure the integrity of genomic DNA from cattle blood. A total of N=150 gDNA samples were assembled and allowed to thaw while preparations of master mix for the 150 samples with 2 standards (standard #1 and standard #2) and additional of 10 pipetting inaccuracies were done (150+2+10=162) reactions. In preparations for buffer, total reactions (162) were multiplied by the given value (199 µL) (adopted from the Qubit assay manual), thus 162 *199=32238 µL. the master mix consisted of 32238 µL buffer, 162 µL qubit reagent, DNA samples. 32238 µL buffer was pipetted into the cylindrical tube followed with 162 µL reactions of the qubit reagent added to the cylindrical tubes then mixed well by vortexing. 199 µL of the master mix was added to the reactions/ assay tubes for the samples to be tested and 190 µL of the master mix added to two tubes specifically labelled for standards #1 and standard #2. 10 µL of standard #1 and standard #2 were pipetted into the respective tubes consisting of the 190 µL master mix. A microliter (1 µL) of DNA was added to each reaction tube then mixed by vortexing and centrifugation.

The Qubit assay machine was activated configured with accurate setting suitable for measuring DNA concentration. Subsequently, standard #1 and standard #2 were inserted into the designated slots of the sample/assay tubes, enabling the machine to analyse them sequentially before processing the actual DNA sample.Immediately after removing standard #2 the first assay tube was put in the assay tube slot and a read sample option was selected, followed by volume selection (1 µL) which was the actual volume of DNA added to the master mix. After reading the first sample, an option to read the next sample was selected then the tube with known DNA concentration was then removed and the next tube inserted. This was done until the last tube ID 150.

3.5.1.3. Amplification of 16S rRNA gene from DNA

The diversity of bacterial communities present in cattle DNA samples from the three study sites were examined using single molecule real-time PacBio sequencing technology (Pacific Biosciences, Menlo Park, CA, USA).

Amplification of the full-length 16S rRNA gene (V1-V8) from the cattle gDNA was done using bacterial specific primers 27F (5'-AGA GTT TGAT CMT GGC TCA G-3') and 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3'). Target-specific primers were tailed with universal M13 forward (5'-TGT AAA ACG ACG GCC AGT-3') and M13 reverse (5'-GGA AAC AGC TAT GAC CAT G-3') sequences for multiplexing of amplicons. Second round PCR utilized barcoded M13 forward and reverse primers. Following the aforementioned, 5'-block (5'-NH4-C6) was added to the 16S specific primers to make sure there were no ligation of carryover amplicons from the first PCR round to the SMRTbell adapters. To construct PacBio sequencing-ready amplicons from the 16S rRNA target sequence flanked by M13 universal overhangs, a set of five barcoded M13 forward and five barcoded M13 reverse primers were designed. Primers were generated and purified through High-Performance Liquid Chromatography (HPLC) in accordance with PacBio's SMRT sequencing recommendation provided by Integrated DNA Technology (San Jose, CA, USA).

During the first round of PCR, M13 tagged forward and reverse primers specific to the 16S gene were used, with final volumes of 25 µL of NEB Q5 HotStart Master mix which constituted 12.5 µL Q5 High-Fidelity 2X Master mix, 1.25 µL of each 10 µM forward and reverse primers, 3 µL of the DNA template and 7 µL of nuclease-free water. For each sample, three technical replicates were used with the same sample-specific barcoded primer set. The Microbial DNA Positive Control (pool of synthetic DNA template) was used as a positive control and PCR grade water was used as a no template negative control. The thermal cycling conditions were; pre-incubation at 98˚C for 2 minutes, 10 cycles of denaturation at 98˚C for 30 seconds followed, annealing stage at 66˚C for 15 seconds, elongation at 72˚C for 45 seconds, 10 cycles of denaturation at 98˚C for 30 seconds, annealing at 68˚C for 15 seconds, elongation at 72˚C for 45 seconds and with final extension stage at 72˚C for 5 minutes.

3.5.1.4. Visualisation of PCR products through agarose gel

After running the first PCR, products were loaded on an agarose gel to visualize and identify positive PCR products to use as input in the second PCR, and the same procedure was applied to the second PCR. This was done using the Agilent 2100 Bio-analyser System. After successful rounds of PCR (first and second), we obtained two sets of barcoded 16S amplicons. The amplicons were further purified using Agencourt AMPure XP magnetic beads (Beckman Coulter, Indianapolis, IN, USA) followed by quantification using a Qubit 2.0 Fluorometer and a Qubit dsDNA BR Assay Kit (Thermo fisher Scientific, Waltman, MA, USA). Prior purification, the amplicons were further pooled (pool 1 and pool 2) in equimolar amounts for SMARTbell library constructions.

The two sets of pools each constituting 75 samples were submitted to the Sequencing Core Facility at the National Institute for Communicable Diseases (NICD), South Africa for 16S rRNA next-generation sequencing based on PacBio sequencing platform.

3.5.1.5. 16S rRNA next-generation sequencing on PacBio sequencing platform

The diversity of bacterial communities in blood samples from the three study sites were analysed using single-molecule real-time (SMRT) PacBio sequencing technology (Pacific Biosciences, Menlo Park, CA, USA). Single-molecule real-time bell libraries were created using SMRTbell™ Template Prep Kit 1.0 (Pacific Biosciences, CA, USA) following instructions in the protocol "Procedure & Checklist – Amplicon Template Preparation and Sequencing" (part number 100-801-600-04).

Prior to purification of the pooled amplicons, the concentration and integrity of DNA was measured through Qubit® Fluorometer (Invitrogen, Carlsbad, USA) and through the NanoPhotometer® NP80 (Implen GmbH, Munich, Germany) respectively. The amplicons with equimolar concentrations were pooled and purified using AMPure® PB beads (Pacific Biosciences, CA, USA). Quality control mainly looking at the integrity of the DNA, the purity and confirmation of base pair size was done on all the purified pooled amplicons using the Qubit® Fluorometer (Invitrogen, Carlsbad, USA) and the NanoPhotometer® NP80 (Implen GmbH, Munich, Germany), and for the size selection?? using the Agilent 4200 Tapestation (Agilent Technologies, CA, USA) respectively. Sequencing was done using the Sequel® Sequencing Kit 2.1 (Pacific Biosciences, CA, USA) with an on-plate loading concentration of 4 pM. The SMRTbell adapters were ligated onto barcoded PCR products - before sequencing of the libraries on a PacBio RII system using the P6-C4 polymerase and chemistry with a 360 minutes' movie time.

3.6. Sequence analysis

3.6.1. Bacterial 16S rRNA profiling analysis summary

Raw reads were quality controlled and filtered (Q>20 and length > 50 bp) using fastqc (v0.11.8) and trimGalore (v0.6.4_dev; https://github.com/FelixKrueger/TrimGalore), respectively. In addition, trimGalore was also used for adapter removal. Krona charts for interactive visualisation of the data were generated using kraken2 (Wood et al., 2019) and krona (Ondov et al., 2011). All the downstream analyses were performed in R (v3.6.1), including classification, abundance estimations, statistical analysis and

visualisation. Clean reads were preprocessed using the DADA2 package (v1.12.1) (Callahan et al., 2016), including quality inspection, trimming, de-replication, merging paired-end reads and removal of chimeric sequences. Taxonomy was assigned to the obtained amplicon sequence variants (ASVs) and the ASV abundance estimates were determined using training sequence sets based on the SILVA reference database (v138;https://zenodo.org/record/1172783#.XvCmtkUzY2w). Ordinations for Beta diversity, abundance bar plots, Alpha diversity and richness estimates, and heatmaps were generated using the phyloseq package (v1.28.0) (McMurdie & Holmes, 2013), ggplot2 (v3.2.1) and AmpVis2 package (v2.6.4) (Andersen et al., 2018). Data clustering in MDS and PCoA were assessed using PERMANOVA (permutation test with pseudo-F ratios) as implemented in the adonis function in the vegan package (https://github.com/vegandevs/vegan). Venn diagrams were generated using VennDiagram (v1.6.20) and UpsetR (v1.4.0) (Conway et al., 2017). Differential abundance analysis between samples was performed using DESeq2 (v1.24.0) (Wood et al., 2019).

3.6.2. Sequence analysis using Divisive Amplicon Denoising Algorithm 2 (DADA2) workflow

The DADA2 package (v1.12.1) analysis workflow implemented in the R software package (v3.6.1) was used to analyse raw amplicon sequencing data resulting from the PacBio Sequel System (Pacific Biosciences, CA, USA). To deduce amplicon sequence variants, error-model learning, and chimera removal were performed on the filtered reads using default DADA2 parameters. Taxonomic allocations were based on the curated SILVA 16S rRNA database (Henderson et al., 2019). Taxa and abundance tables generated by DADA2 were imported into the phyloseq package v1.28.0 (McMurdie & Holmes, 2013) for downstream analysis and visualisation, including estimation of richness and visualisation of the alpha-diversity, as well as visualisation of differences in taxa abundance between the three study sites.

The significant differences amongst the study sites were inferred using Kruskal-Wallis test, with statistical significance at $P < 0,05$. Effect sizes of the differences between the three study sites were calculated using Cohen’s D measure using the effsize

package in R (https://github.com/mtorchiano/effsize). Alpha diversity was based on Chao1 and Shannon diversity indices. Ordinations for Beta diversity between study sites were assessed using Principal Coordinate Analysis (PCoA) based on weighted-UniFrac distance and Non-Metric Multidimensional Scale (NMDS) using Bray distance metric as implemented in the plot_ordination and amp_ordinate functions in PhyloSeq and the ampvis2 package (https://madsalbertsen.github.io/ampvis2/articles/ampvis2.html), respectively. Permutational Multivariate Analysis of Variance (PERMANOVA) using a permutation test with pseudo-F ratios as implemented in the Adonis function of the Vegan R package (https://github.com/vegandevs/vegan) was used to determine the significance for sample clustering on ordination plots.

The project was registered in GenBank under the BioProject accession number PRJNA1031221.

CHAPTER 4

RESULTS

4.1. Tick species identification from the three study sites

During a once-off visit to study sites, ticks were discovered on various areas of cattle such as their legs, back, neck, anal region, and abdomen. These ticks were collected and transported to the Faculty of Veterinary Science, University of Pretoria. After the blood meal was properly digested, a total of 418 collected ticks were sorted based on the collection site. The sites included Harrismith (n=126), Phuthaditjhaba (n=160), and Bergville (n=132). Phuthaditjhaba had the highest number of ticks identified, a phenomenon that can be linked to the physical appearance of the cattle Most of the cattle were very slim, indicating an unhealthy ill state. Additionally, their hides were damaged due to tick infestations, leading to a higher number of ticks found on the site.

Ticks that were found infesting cattle in the study sites were identified as belonging to hard ticks. These ticks were morphologically identified based on published keys (Walker, 2003) as species from two genera, namely *Rhipicephalus* and *Hyalomma*. In Harrismith these ticks included *R. decoloratus* (n=4), *R. evertsi everts*i (n=113), *R. microplus* (n=3), *H. rufipes* (n=4) and *H. truncatum* (n=2). In Phuthaditjhaba, the identified tick species were *R. appendiculatus* (n=48), *R. evertsi evertsi* (n=89), *R. simus* (n=11), *R. afranicus n. sp (formerly known as R. turanicus)* (n=11), *H. rufipes* (n=1). Meanwhile, in Bergville, only *R. appendi*culatus (n=1), *R. evertsi evertsi* (n=130), and *H. truncantum* (n=1) were identified (Table 4.1).

The most abundant tick species were *R. evertsi evertsi* (79.4%), *R. appendiculatus* (11.7%), followed by *R. simus* and *R. afranicus n. sp* (2.6%). *Rhipicephalus evertsi evertsi* was present in all study sites while other species were present in one or two of the sites. These species are *R. simus* and *R. afranicus n. sp* both present in Phuthatjhaba and not in Harrismith and Bergville. On the other hand, *R. microplus* and *R. decoloratus* were present only in Harrismith (Table 4.1).

Table 4.1. The proportion of tick species collected from the study sites.

	Number of ticks collected from the three study sites			
	Total (proportion)	**Harrismith**	**Phuthaditjhaba**	**Bergville**
Tick species	**418**	**126**	**160**	**132**
Hyalomma rufipes	5 (1.2%)	4 (3.2%)	1 (0.6%)	0
Hyalomma truncatum	3 (0.7%)	2 (1.6%)	0	1 (0.8%)
Rhipicephalus decoloratus	4 (1.0 %)	4 (3.2%)	0	0
Rhipicephalus evertsi evertsi	332 (79.4%)	113 (89.7%)	89 (54.3%)	130 (98.5%)
Rhipicephalus microplus	3 (0.7%)	3 (2.4%)	0	0
Rhipicephalus appendiculatus	49 (11.7%)	0	48 (29.3%)	1 (0.8%)
Rhipicephalus simus	11 (2.6%)	0	11 (6.7%)	0
Rhipicephalus afranicus n. sp	11 (2.6%)	0	11 (6.7%)	0

4.2. Blood microbial composition of cattle at the three study sites

The mean species diversity of bacterial populations detected in the cattle blood samples plotted using the rarefaction curves showed that the sequencing depth was adequate to capture the existing microbial diversity (Figure 4.1).

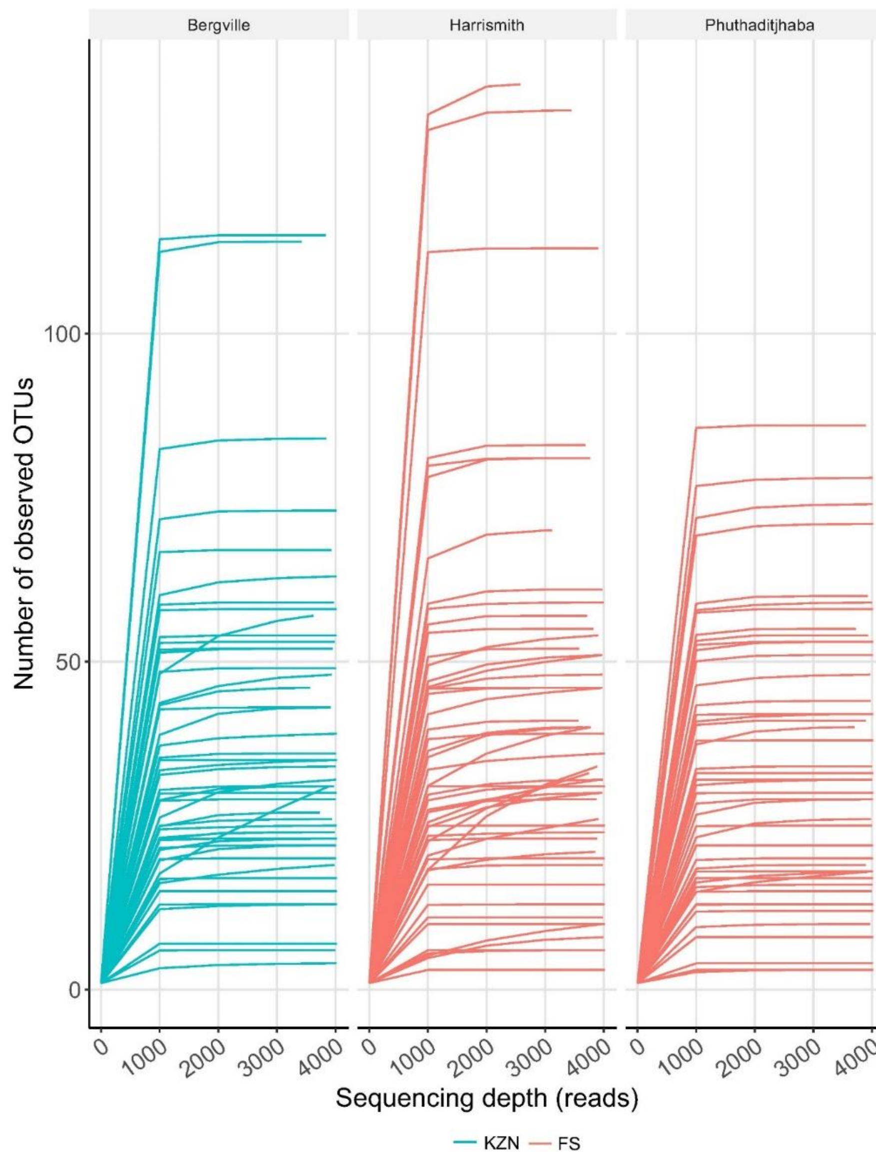

Figure 4.1. Rarefaction curves indicating the effect of sequencing depth (read numbers per sample, X axis) on species richness (Y axis) in bovine blood samples.

4.2.1. Alpha and Beta diversity

The alpha diversities were estimated through Chao1 index for species richness, and the Shannon index which caters for richness and evenness (Hoque et al., 2020). The microbial diversity of samples from Harrismith indicated significantly higher species richness than Bergville (p = 0.0064) and Phuthaditjhaba (p = 0.00025) samples as determined using the Chao1 index estimator (Kruskal-Wallis: p = 0.00065) (Figure 4.2). However, the differences in species diversity between the three study sites were insignificant as per the Shannon estimator (Kruskal-Wallis: p = 0,48) (Figure 4.3). Using the pairwise analysis, the Alpha diversity varied significantly in microbial communities between Bergville and Harrismith and between Harrismith and Phuthadijthaba based on Chao 1 index estimator (p = 0.0064 and p = 0,00025, respectively). In contrast, there was no significant difference between Bergville and Phuthaditjhaba microbial communities based on Chao 1 index estimator (p = 0.31)

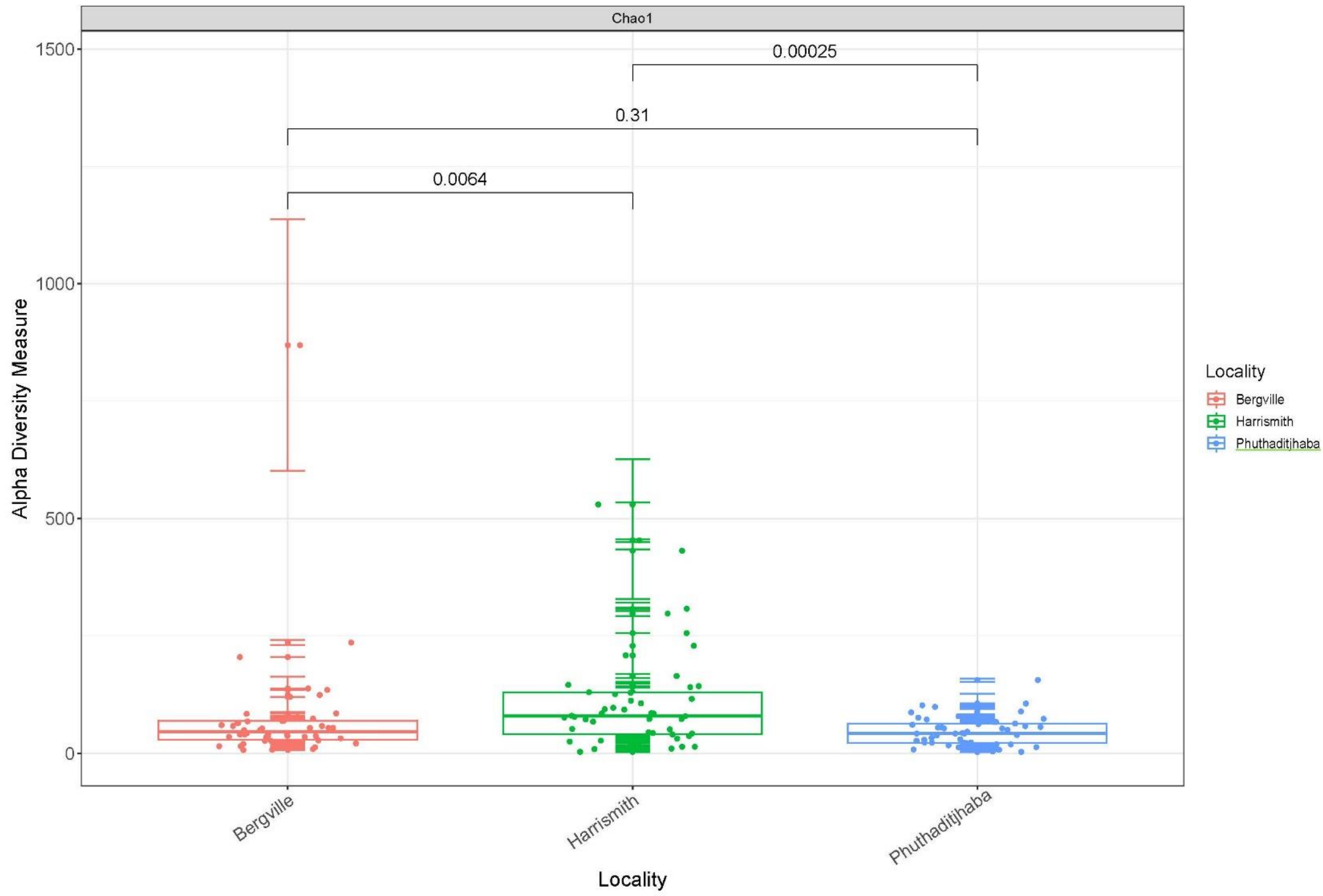

Figure 4.2. Boxplots showing Alpha diversity of bacterial composition estimated through Chao1 index.

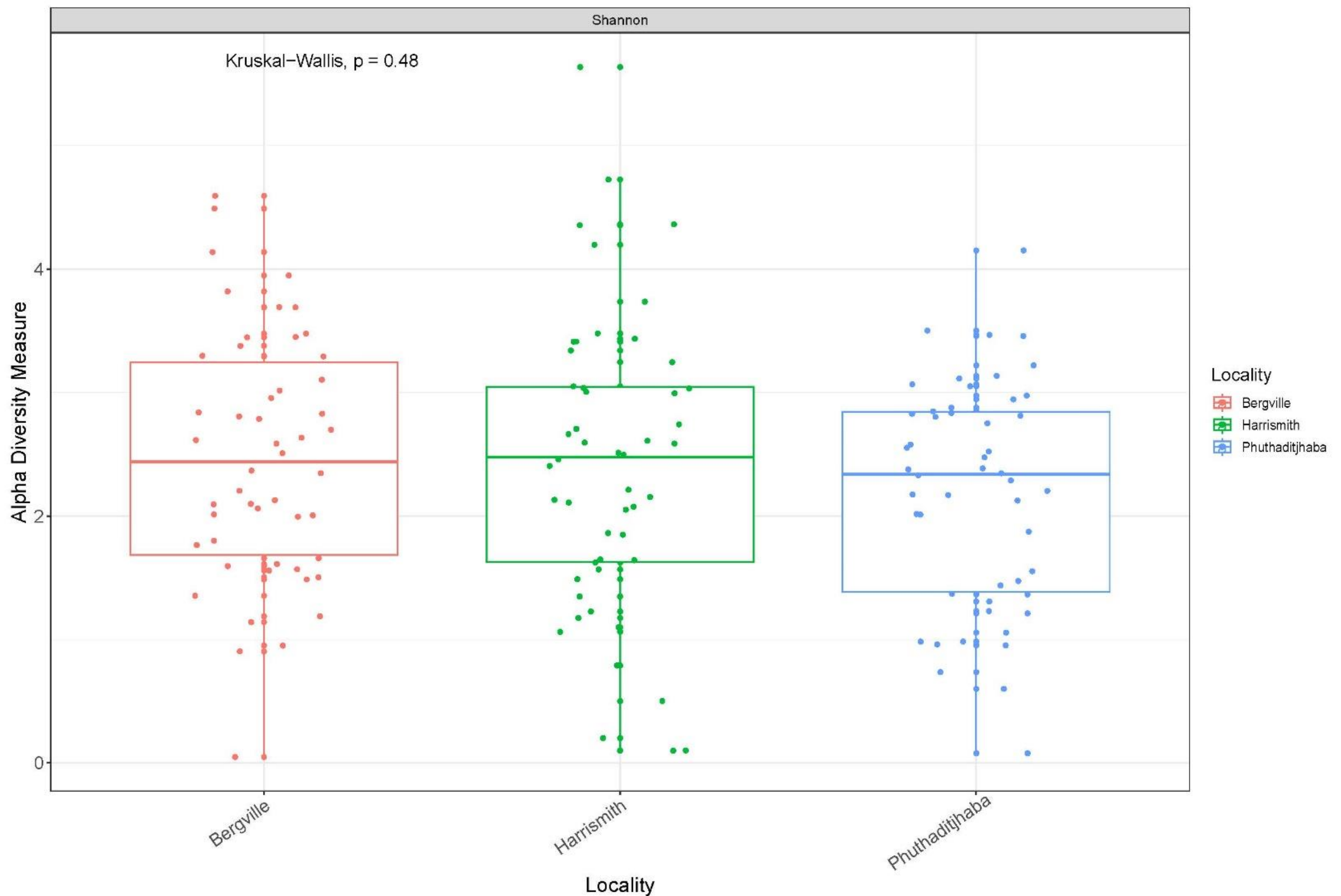

Figure 4.3 Boxplots showing Alpha diversity of bacterial composition estimated through Shannon index.

Furthermore, microbial composition dissimilarities between the three study sites were analysed through PCoA plots. The plots showed one cluster of microbial communities using the weighted UniFrac distance metric on PCoA, which considers abundance and the phylogenetic distance between ASVs. Three clusters indicative of the study sites could be observed (PCoA persanova). The clusters in Bergville had unique bacterial communities than the clusters in Harrismith and Phuthaditjhaba. This is the reason

Bergville sample composition was divergent from the Harrismith and Phuthaditjhaba. However, the Phuthaditjhaba and Harrismith groups clustered closely (Figure 4.4).

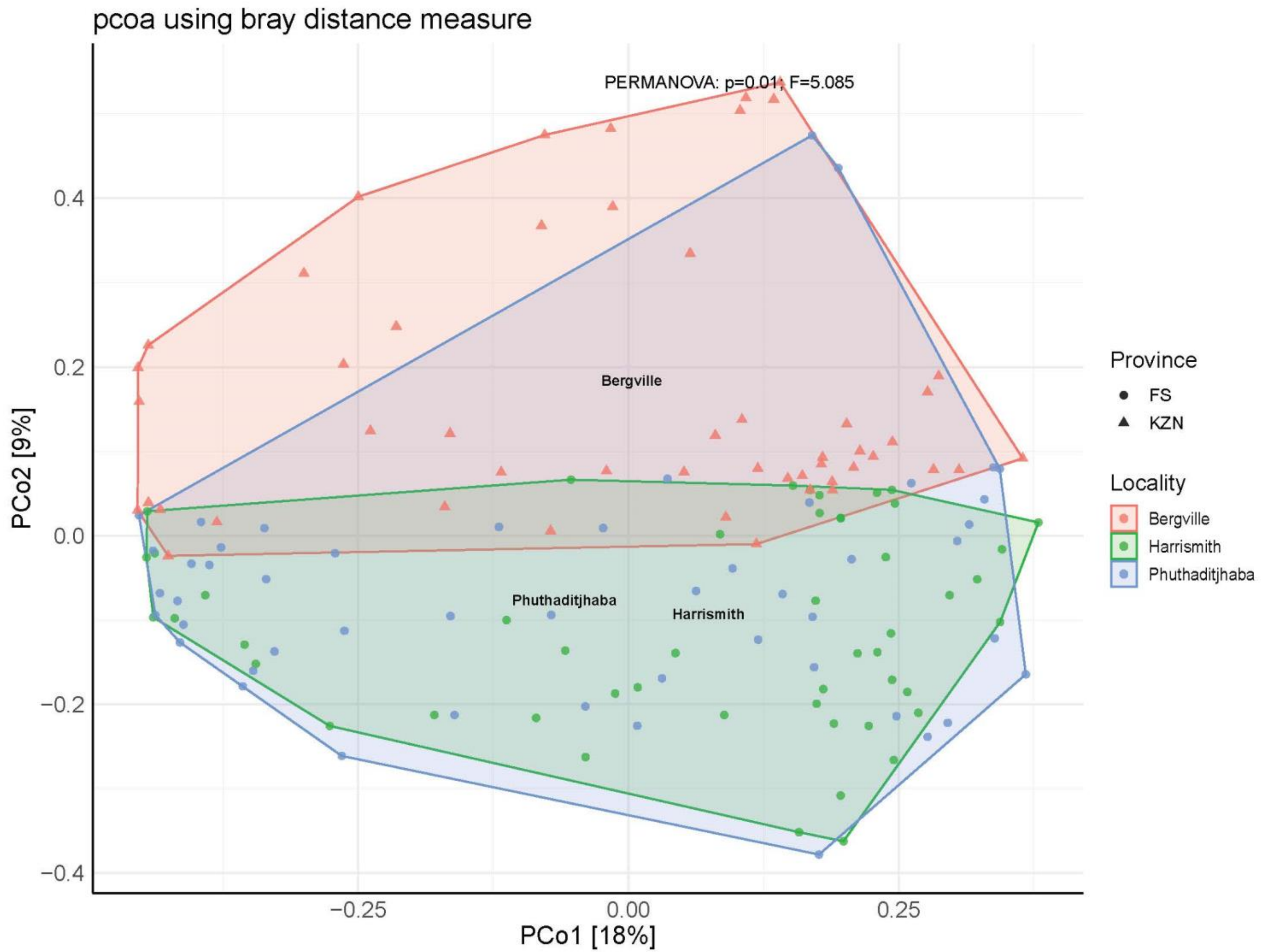

Figure 4.4. Ordination Plots showing clustering of bacterial diversity at the three study sites estimated using PCoa through Bray distance matrix.

4.2.2. Relative abundances of taxa at three study sites

Overall, 16 phyla, 30 classes, 71 order, 114 families, 159 genera and 71 species were identified from the three sites (Appendix 1). At the phylum level, the composition of bovine blood microbiomes was dominated by Proteobacteria with a relative abundance of 67.2% (Bergville), 73.8% (Harrismith) and 84.8% (Phuthaditjhaba), followed by Firmicutes at 9.6% (Phuthaditjhaba), 18.9% (Bergville) and 19.6% (Harrismith) (Figure 4.5, Appendix 1). Bacteroidota was the third most abundant at 4% (Phuthaditjhaba), 5.3% (Harrismith) and 10.2% (Bergville) (Figure 4.5, Appendix 1). The relative abundance of the Proteobacteria, Firmicutes and Bacteroidota remained higher in Phuthaditjhaba, Harrismith and Bergville, respectively (Appendix 1). The phyla Fusobacteriota and Spirochaetota were only found in Bergville, while Deinococcota was only found in Phuthaditjhaba (Appendix 1). About half of the detected phyla had a relative abundance of ≥0.1% in the various microbiomes (Bergville=7/13, Harrismith=5/11, Phuthaditjhaba=8/13).

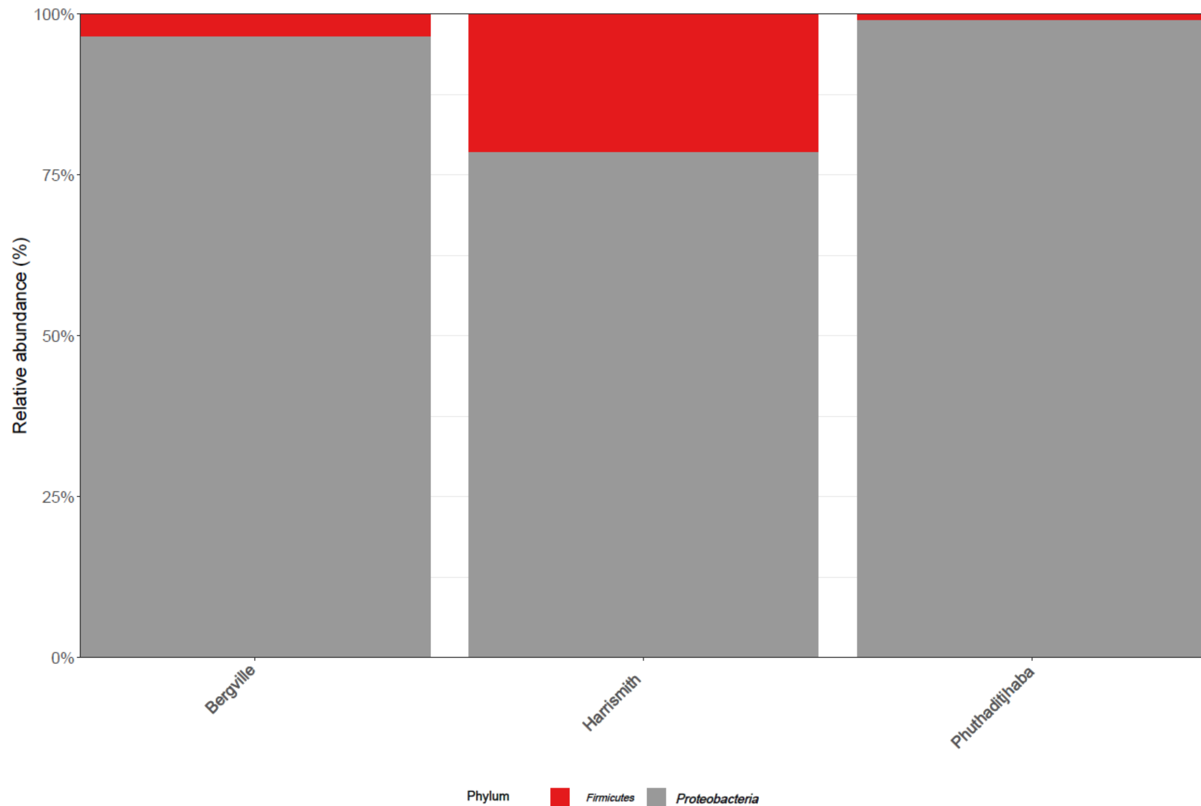

Figure 4.5. Barplots showing the relative abundances of the two most abundant phyla across the three study sites.

The most dominant classes in the Bergville, Harrismith and Phuthaditjhaba microbiomes were Alphaproteobacteria (64.8, 72.4%, 84.4%), Bacilli (14.3%, 17.5%, 8.2%) and Bacteroidia (10.2%, 5.3%, 4%) (Figure 4.6, Appendix 1). Class Spirochaetia was unique to the Bergville microbiome, while classes Blastocatellia, Cynobacteria, Deinococei and Phycisphaerae were unique to the Phuthaditjhaba microbiome (Appendix 1). About half of the detected classes had a relative abundance of ≥0.1% in Bergville (11/20), Harrismith (8/20) and Phuthaditjhaba (12/23) (Appendix 1).

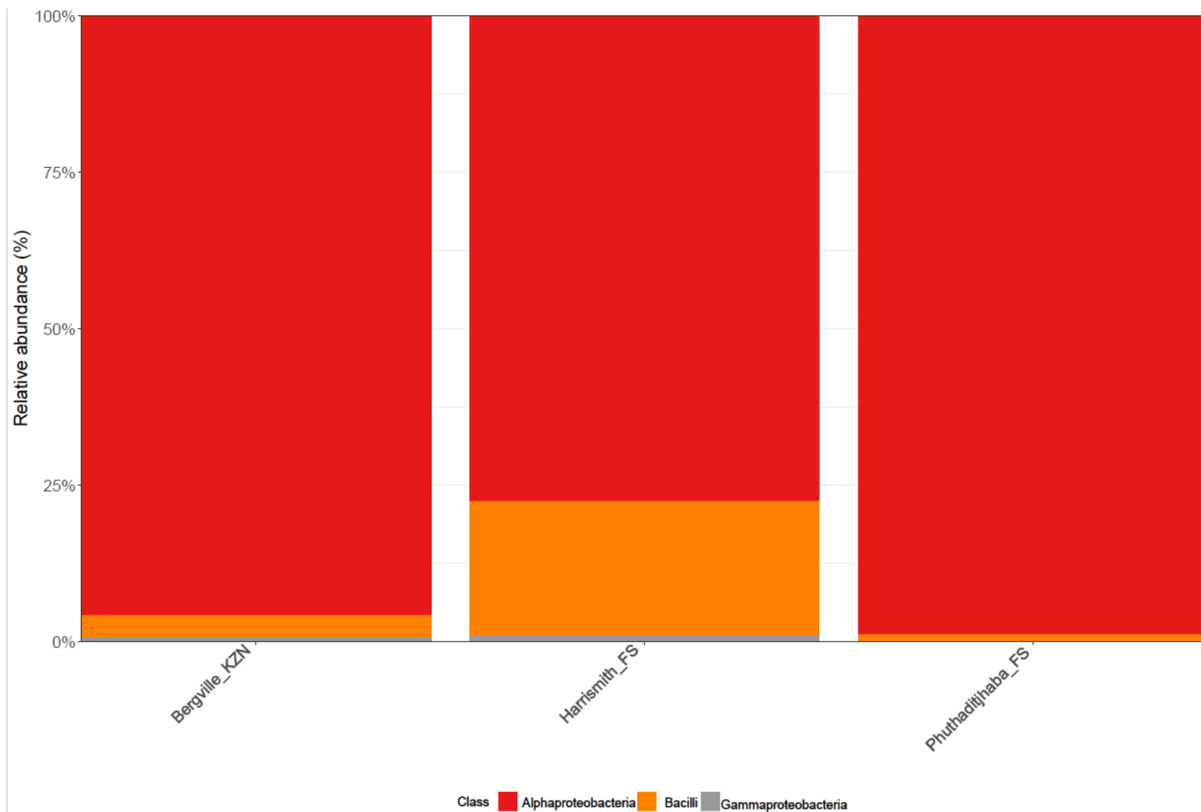

Figure 4.6 Barplots showing the relative abundances of the three most abundant classes across the three study sites.

The composition of bovine blood microbiomes at the order level in Bergville, Harrismith and Phuthaditjhaba was numerically dominated by Rickettsiales (64.7%, 72%, 84.3%), respectively, followed by Mycoplasmatales (11%, 17.3%, 8.1%) and Bacteroidales (10.2%, 5%, 4%) (Figure 4.7, Appendix 1). There was a significant difference in the relative abundance among the three study sites (Kruskal Wallis test, p=0.0281). At least 46 orders of bacteria were identified in each of the three microbiomes, of which 13.0% (6/46), 16.0% (8/50) and 19.6% (10/51) were exclusively unique in the Bergville, Harrismith and Phuthaditjhaba microbiome, respectively (Appendix 1). There were more orders with relative percent abundance ≥0.5% in the Bergville microbiome (12/46) than in the Harrismith (6/50) and Phuthaditjhaba (5/51) (Appendix 1).

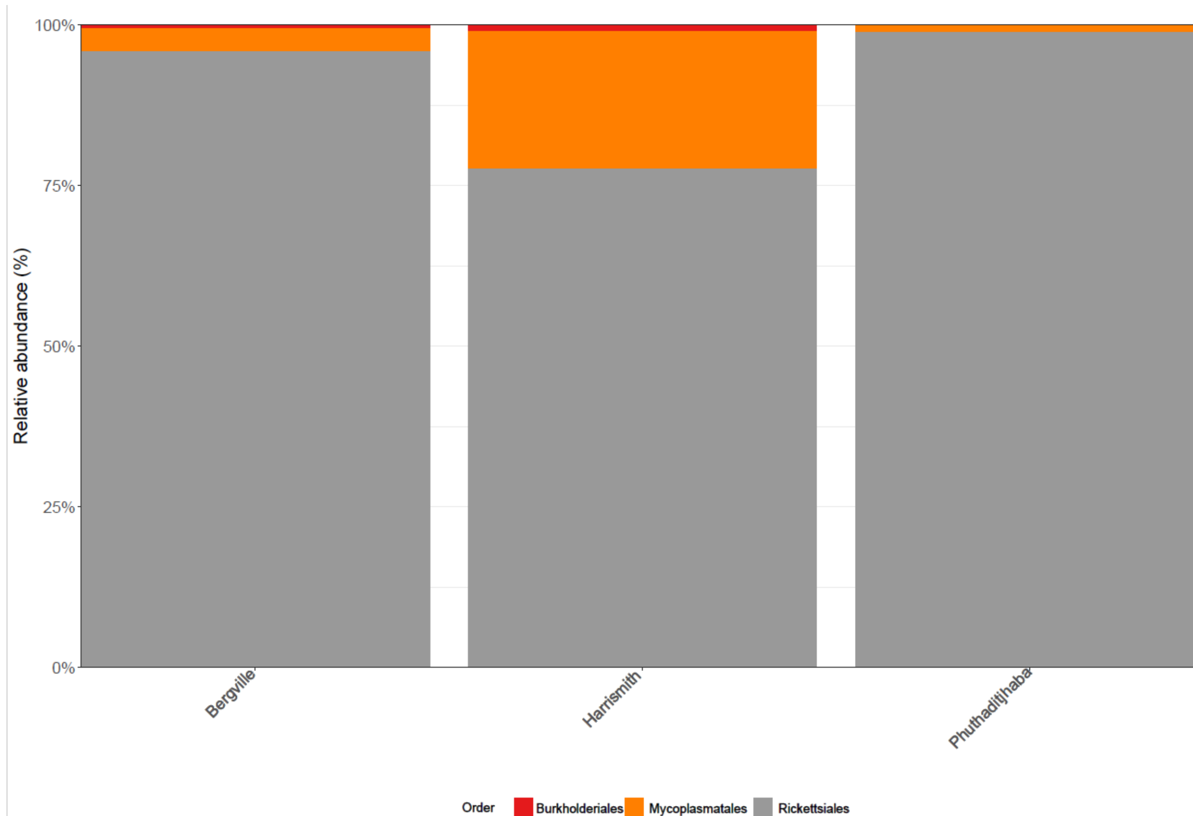

Figure 4.7 Barplots showing the relative abundances of the three most abundant orders across the three study sites.

The unique and shared distribution of bacterial families, genera and species found in the Phuthaditjhaba, Harrismith and Bergville samples are shown by comprehensive Venn diagrams (Figures 4.8- A, B, C). We detected 114 bacterial families, of which 69, 77 and 81 were found in Phuthaditjhaba, Bergville and Harrismith microbiomes, respectively (Figure 4.8A, Appendix 1). Moreover, 41 bacterial families were shared across the three study sites (Figure 4.8A). The Phuthaditjhaba microbiome had an exclusively unique association with 13 bacterial families, while 12 and 17 bacterial families were uniquely present in Bergville and Harrismith bovine blood samples (Figure 4.8A, Appendix 1). There was substantial variation in the relative percent abundance (Kruskal-Walli's test, p = 0.0045) of the bacteria at the family level among the three study sites. More than 80% of the bacterial microbiome in Bergville, Harrismith and Phuthaditjhaba was largely

dominated by members of three families: *Anaplasmataceae* (65.0%, 72.1%, 84.7%, respectively), *Mycoplasmataceae* (11.1%, 17.4%, 8.1%) and *Rikenellaceae* (6.6%, 4.3%, 2.7%). There were more families with relative percent abundance ≥0.5% in the Bergville microbiome (14/77) than in the Harrismith (7/81) and Phuthaditjhaba (4/69).

Overall, we detected 159 microbiome genera, of which 94, 108 and 82 were found in Bergville, Harrismith and Phuthaditjhaba, respectively. Forty-two genera were shared across the three study sites), while 23, 31 and 22 exclusively associated microbiome genera were found in the Bergville, Harrismith and Phuthaditjhaba, respectively (Figure 4.8B, Appendix 1). There were significant differences in the relative percent abundance (Kruskal-Wallis test, P=0.00042) of the bacteria at the genus level in the locality microbiome. *Anaplasma* was the most abundant bacterial genus in the Phuthaditjhaba, Harrismith and Bergville samples, with a relative abundance of 85.4%, 72.4% and 67.1%, respectively. This was followed by *Mycoplasma* (8.2%, 17.6% and 11.4%, respectively) (Appendix 1). The Phuthaditjhaba microbiome was also predominated with *Rikenellaceae RC9 gut group* (2.3%). Other abundant genera in the Bergville blood microbiome were *Rikenellaceae RC9 gut group* (5.2%), *Aerococcus* (2.1%), *Akkermansia* (1.9%), *Alistipes* (1.4%), *UCG-005* (1.4%), *Planomicrobium* (0.9%), *Corynebacterium* (0.7%), *Acinetobacter* (0.6%), *Porphyromonas* (0.5%) and *Pseudomonas* (0.5%). Other top abundant genera in the Harrismith microbiome were *Pelomonas* (0.7%), *Cutibacterium* (0.7%), *UCG-005* (0.6%) and *Ehrlichia* (0.6%) (Appendix 1). The rest of the genera (82, 102, 79) had <0.5% relative abundance in the three study sites (Bergville, Harrismith, Phuthaditjhaba, respectively) (Appendix 1).

We investigated the species-level differences of microbial communities across the three study sites, which showed no significant differences (Kruskal Wallis test, p-0.081) in the microbiome relevant abundance (Appendix 1). Of the 71 detected species (159 genera), 12.67%, 22.54% and 21.13% had a sole association with Phuthaditjhaba, Bergville and Harrismith study sites respectively. *Anaplasma marginale* (relative abundance 56.2%, 43.5%, 54.2%, respectively) was the most abundant bacterial pathogen in the Bergville, Harrismith and Phuthaditjhaba samples, followed by *A. platys* (22.6%, 31.5%, 32.9%) and *Mycoplasma wenyonii* (14%, 19.6%, 7.8%) (Appendix 1). Other predominant species in

Bergville bovine blood microbiome were *Aerococcus vaginalis* (2.6%), *Acinetobacter lwoffii* (0.6%), *Pseudomonas poae* (0.5%) and *Yersinia pestis* (0.5%). In contrast, *Anaplasma bovis* (1.3%), *Anaplasma centrale* (0.9%), *Anaplasma ovis* (0.8%), *Cutibacterium namnetense* (0.8%) and *Ehrlichia canis* (0.6%) were the other relatively predominant species in Harrismith. Moreover, *Anaplasma bovis* (3.0%) and *Mycoplasma haemobos* (1.0%) were the other predominant bacterial species in Phuthaditjhaba (Appendix 1). The rest of the species identified in these samples had relative abundance lower than 0.5% (Appendix 1). The presence of few predominating bacterial genera in the three study sites could have suggested that these differences might also occur in composition at the species level, but instead most of the genera identified in each microbiome were represented on average by two species.

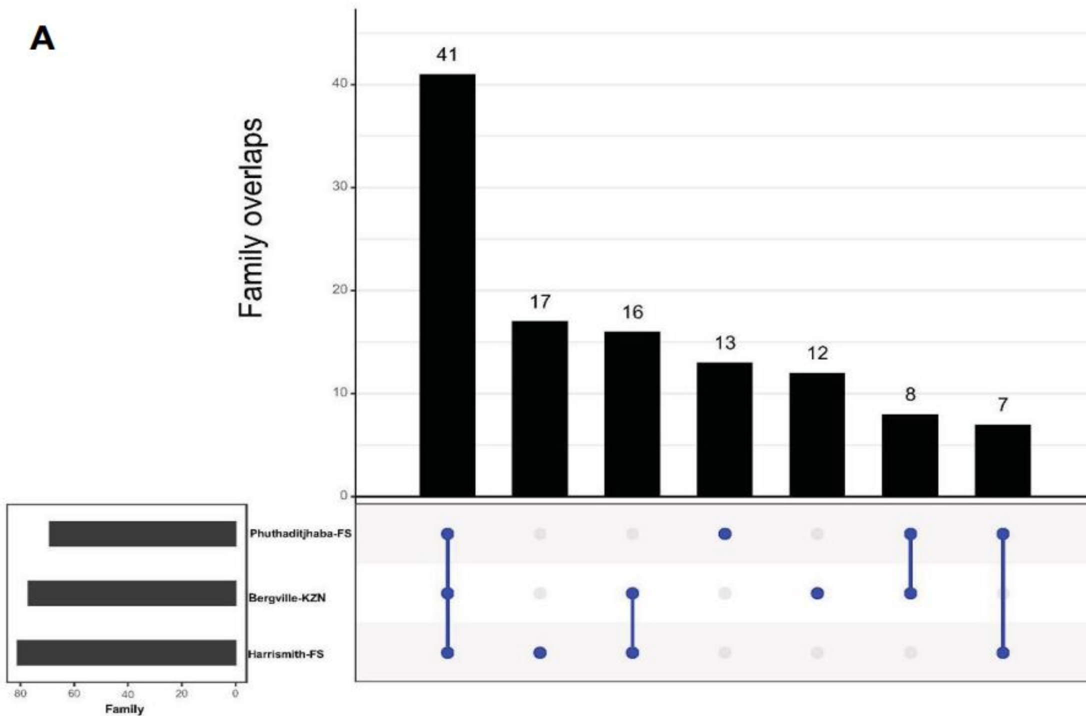

Figure 4.8A Taxonomic composition of microbiomes from three locations in South Africa. Venn diagrams representing the unique and shared microbiomes in the Phuthaditjhaba, Bergville and Harrismith bovine blood samples. Venn diagram representing unique and

shared bacterial families. Microbiome uniqueness or sharing among the locations is symbolised by blue dots or lines.

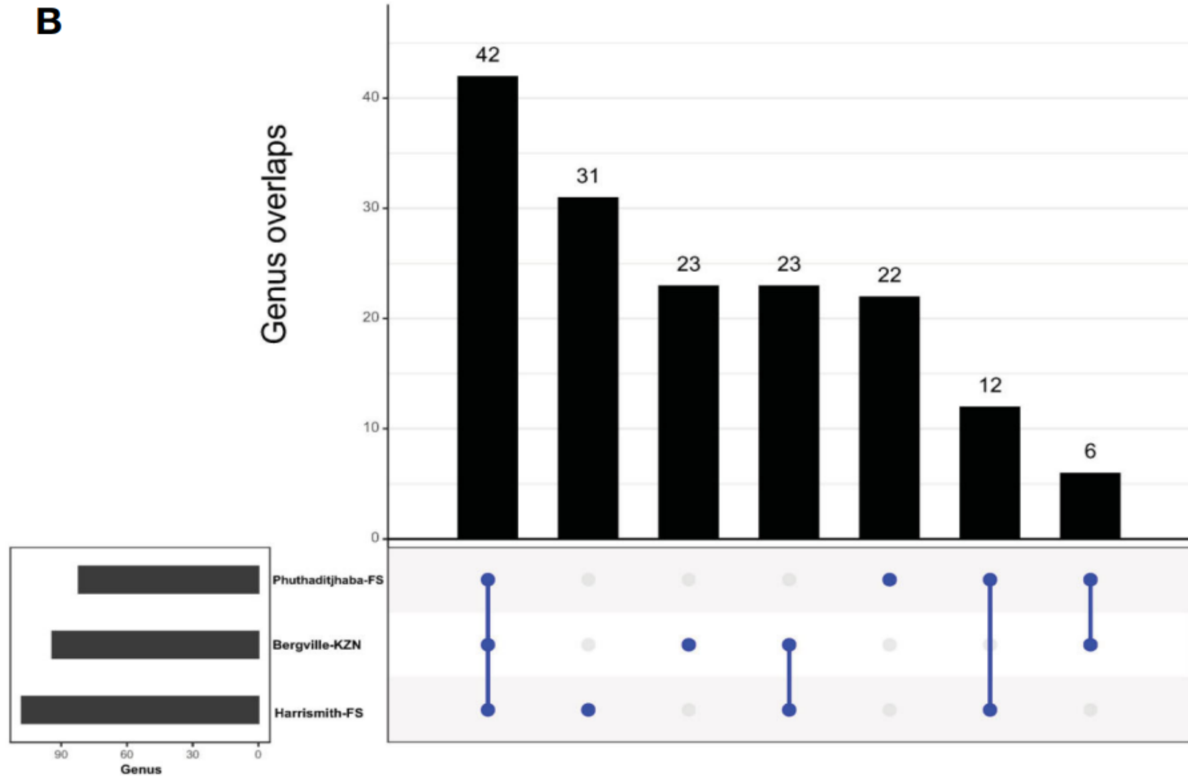

Figure 4.8B Taxonomic composition of microbiomes from three locations in South Africa. Venn diagrams representing the unique and shared microbiomes in the Phuthaditjhaba, Bergville and Harrismith bovine blood samples. Venn diagram comparison of bacteria at genus level by MR analysis. Microbiome uniqueness or sharing among the locations is symbolised by blue dots or lines.

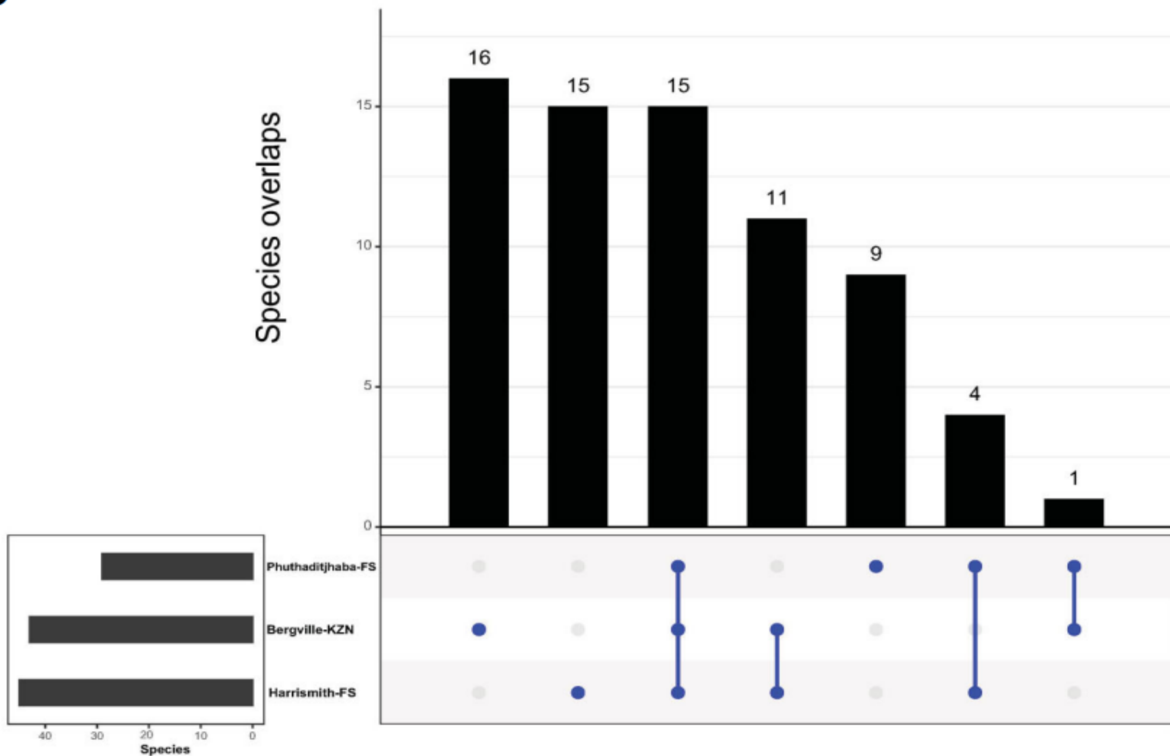

Figure 4.8C Taxonomic composition of microbiomes from three locations in South Africa. Venn diagrams representing the unique and shared microbiomes in the Phuthaditjhaba, Bergville and Harrismith bovine blood samples. Venn diagram showing unique and shared bacterial species in blood samples as determined using the MR pipeline. Microbiome uniqueness or sharing among the locations is symbolised by blue dots or lines.

CHAPTER 5

DISCUSSION AND CONCLUSION

5.1. Discussion

Briefly, in this study 150 cattle at three study sites were sampled for blood and ticks. Morphological identification keys by Walker, (2003), along with the assistance from Prof Nkululeko Nyangiwe were employed in the identification of ticks infesting cattle at the three study sites. A total of 418 ticks were collected and identified as *Rhipicephalus* and *Hyalomma* species. DNA extracted from blood of the cattle was amplified and analysed for the presence of bacterial pathogens targeting the full-length 16S rRNA gene, and resulting amplicons were sequenced on the PacBio platform. *Anaplasma* species were the most abundant relative to other identified bacterial species.

5.1.1. Identified tick species from bovine cattle at three study sites

Overall, a total of 418 ticks were collected and identified from the three study sites in Free State and KwaZulu-Natal provinces of South Africa. Phuthaditjhaba had the most ticks (38.8%) collected than the other two sites Bergville (31.6%) and Harrismith (30.1%). The most abundant tick species was *R. evertsi evertsi* (79.4%) also known to be the most widespread rhipicephalid in South Africa. Numerous studies, including those by (Tsotetsi & Mbati, 2003; Spickett et al., 2011; Mtshali et al., 2015; Khumalo et al., 2016, 2017) have confirmed a high abundance of this tick species in various provinces. Previous studies conducted in the Free State Province identified *R. evertsi evertsi* to be the second prevalent tick species in cattle after *R. decoloratus*. In the eastern Free State, the previous of *R. evertsi evertsi* was found to be 6.86% (Dreyer et al., 1998), from 1998 to 2000, *R. evertsi evertsi* was 44.7% (Mbati et al., 2003). Interestingly, this tick species has been identified from wildlife species in the Free State, it was found to be 100% in Gemsbok, black wildebeest, greater kudu, roan and plain zebra, and 84.5% in common eland, 66.5% in eland, 30% in springbok and 20% in red hartebeest (Tonetti et al., 2009). In KwaZulu Natal at the same study site at

Bergville between 2015 - 2016, *R. evertsi evertsi* was the most prevalent tick collected with a prevalence of 55.5% (Khumalo, 2017). The distribution of this tick species in this study agrees with the previous studies and does seem to be the most prevalent tick amongst other tick species. This tick species has also been reported to transmit several pathogens including protozoans and bacteria, such as *A. marginale*, *A. centrale*, *A. ovis*, *Babesia bigemina*, *B. bovis*, *Theileria parva*, *Coxiella burnetii* and *E. ruminantium,* causing diseases that could have an impact on livestock and people's health (Tonetti et al., 2009).

The second most abundant tick species was *R. appendiculatus* with a total proportion of 11.7%. It was reported to be more prevalent in Phuthaditjhaba (29.3%), and in Bergville (0.8%) and was not reported in Harrismith. This tick was intensively controlled since it is a vector of *T. parva,* however, it has been identified in wildlife species in four game reserves in the Free State province (Tonetti et al., 2009). In 2015, Horak confirmed that this tick was established in the Free State as all the developmental stages were collected from cattle and vegetation on two commercial farms alongside a buffalo ranch (Horak et al., 2017). In a study conducted in Bergville in 2016, at the same dip tank as the present study, *R. appendiculatus* was present at 15.2% (Khumalo, 2017). *Rhipicephalus appendiculatus* is a biological vector of the protozoan parasite *T. parva* - the causative agent of East Coast Fever (ECF) in East Africa and Corridor disease in South Africa which is a disease that poses a serious economic risk to the cattle farming industry in endemic areas (Perry et al., 1991; Maboko et al., 2001; Estrada-Peña, 2015). There were no historical records of its occurrence in the eastern Free State during a three-year long survey on ticks according to (Hlatshwayo et al., 2000) and during the following years (Tonetti et al., 2009; Horak et al., 2015), respectively.

The third most abundant tick species were *R. simus* (2.6%) and *R. afranicus* n. sp. (2.6%), and both occurred in Phuthaditjhaba. *Rhipicephalus simus* is responsible for the transmission of sub-acute bovine anaplasmosis which is caused by *A. centrale* (Jongejan & Uilenberg, 2004). According to our knowledge, there are no historical occurrences of this tick species in the three study sites. This is the first study to identify its presence in Phuthaditjhaba.

Rhipicephalus afranicus n species is a recently described species in South Africa (Bakkes et al., 2020) and was subsequently identified in Uganda (Balinandi et al., 2020). The genetic divergence between the Ugandan and Southern African sequences is 2.4%, demonstrating that two different populations of this species may be occurring between Southern and East Africa (Balinandi et al., 2020). According to Bakkes et al., (2020), all ticks that were previously identified as *R. turanicus* from South Africa, are *R. afranicus*. In our knowledge, this study is the first to report the presence *Rhipicephalus afranicus* n sp. in Phuthaditjhaba. This then calls for successive tick survey studies that will record the distribution of this tick species in South Africa, and further determine its vectorial capacity. Such studies are important in informing ticks and ticks borne disease control programs.

Rhipicephalus microplus and *R. decoloratus* were only present in Harrismith. *Rhipicephalus microplus* is an invasive tick species, it was introduced to the African continent, East and South Africa by the exportation of infected cattle from Asia via Madagascar (Berggoetz, 2013). This tick has been previously recovered in the Free State province from a few sites including wild animals (Tonetti et al., 2009; Horak et al., 2015; Nyangiwe et al., 2017). *Rhipicephalus microplus* was introduced to West Africa and Guinea through livestock trading with Mali and the Ivory Coast. The spread of this tick species was perpetuated by uncontrolled livestock translocation in Cameroon, where it was noted to infest a variety of hosts inclusive of humans (Makenov et al., 2021). In Ivory Coast, native tick species have been displaced by introduced tick species which culminated in an adverse effect on cattle productivity and manifested invasive traits (Adakal et al., 2013; Ali et al., 2019; Lontsi-Demano et al., 2020; Makenov et al., 2021). In consideration of the high *R. microplus* dispersal rate as reported in South-East Africa (Nyangiwe et al., 2017), the presence of *R. microplus* in this study, warrants further investigation about its distribution in the Free State.

Interestingly, this study reports the concurrent occurrence of *R. microplus* and *R. decoloratus* which may suggest that the invasive tick has not completely displaced the native tick *R. decoloratus* in the Free State, or it may also mean that *R. microplus* has just been introduced in the Free State and is continually expanding its geographic distribution and host preference. In fact, there are records that have identified this tick

species in sheep (Nyangiwe et al., 2017) goats (Jongejan et al., 2020) and wildlife species (Tonetti et al., 2009; Horak et al., 2015).

The tick burden recordings from the previous years indicates that the current control systems and movement of animals need to be reconsidered and regulated. Integrated approaches for tick control such as incorporating veld rotation, vegetation burning, tick endemicity status control, community outreaches and proper translocation procedures may aid in controlling tick burdens and tickborne diseases. In Harrismith, farmers indicated that there are no proper communal dipping programs provided by the local Department of Agriculture, and dipping last took place in winter in the year 2021, this was before 29 September 2021 when the collection of samples took place. Those with little resources were able to opt for other methods and used TAKTIC and Terramycin for tick control; however, ticks were still observed from their cattle. This could indicate improper dipping, thus promoting tick resistance. In Phuthaditjhaba dipping method through pour-on dipping which takes place within a period of 3-4 months, farmers indicated to use “Deadline” for controlling external parasites, however high tick infestations were observed. Bergville, Woodford dip tank has been sampled in 2015, 2016 and 2021 with intensive dipping, practised twice a month. Nevertheless, there haven’t been any changes in the tick burden as animals were highly infested with ticks. In view of the above, there is a necessity to undertake studies of this kind in order to continuously update the status and distribution of cattle ticks in this community.

5.1.2. Bacterial tick-borne pathogens detected from bovine cattle blood

In the three study sites, bacterial tick-borne pathogens of the following phyla, and others were detected; Phylum; Proteobacteria, class; Alphaproteobacteria, order; Rickettsiales, family; *Anaplasmataceae*, genus; *Anaplasma*. Phylum; Fermicutes, class; Bacilli, order; Mycoplasmatales, family; *Mycoplasmataceae*, genus; *Mycoplasma*. Phylum; Bacteroidota, class; Bacteroidia, order; Bacteroidales, family; *Rickenellaceae*, genus; *Rickenellaceae* RC9 gut group. Phylum; Verrucomicrobiota, class; verrucomicrobiae, order; Verrucomicrobiales, family; *Akkermansiaceae,* genus; *Akkermansia*.

The three most abundant families were *Anaplasmataceae*, *Mycoplasmataceae* and *Commamodacea*. *Anaplasma* genus was the most detected bacterial pathogen, which correlates with the presence of its tick vectors, namely *R. decoloratus, R. evertsi evertsi, R. microplus, R. appendiculatus, R. simus and R. afranicus* n. *Anaplasma marginale* and *A. platys* were the most abundant bacterial pathogens in Harrismith, Phuthaditjhaba and Bergville. *Anaplasma marginale* with relative abundance of (43.5%), (54.2%), (56.2%) and *A. platys* with relative abundance of (31.5%), (32.9%) and 22.6%) respectively. In addition, *Anaplasma ovis*, *A. bovis*, *A. boleense*, *A. centrale* and *A. phagocytophilum* species were also identified. This suggests a high potential risk of anaplasmosis infections in cattle, dogs, and humans. *Anaplasma* species are widespread in South Africa, with a wide host range. A recent 16S microbiome analysis study revealed the presence of both previously described species and novel species in nine different free-roaming wildlife species (African buffalo, impala, kudu, zebra, warthog, hyena, leopard, lion, and elephant) in the Kruger National Park and surroundings game reserves (Makgabo et al., 2023).

The unexpected occurrence of *A. platys* in the genetic material of bovines invites a reassessment of the conventional comprehension of host specificity pertaining to this bacterial pathogen. The intimate association between bovine and canines emerges as a possible elucidation, underscoring the necessity for interdisciplinary investigation to decipher the intricacies of pathogen dynamics within varied ecosystems. There could be a potential for cross-species transmission and the implications for zoonotic risk, however this warrants further investigation.

The One Health approach does emphasize the interrelationship between the health of humans, animals and the environment they live in. Therefore, it is important to conduct additional investigations, surveillance, and monitoring in order to evaluate the zoonotic capacity of *A. platys* and gain insight into its effects on both animal and human well-being.

Anaplasma bovis is the causative-agent of bovine anaplasmosis in cattle. This pathogen is not well studied in South Africa, however there are speculations that it could be transmitted by *R. afranicus* n sp. reported to have been isolated from the same tick species in a study conducted in Israel (Peleg et al., 2010). In South Africa, most collections of adult ticks have been from scrub hares, followed by domestic and

wild carnivores (Horak et al. 1993, 1995, 2001, 2010). The presence of this pathogen is no surprise as the potential vector *R. afranicus* n sp. was identified in this study.

Mycoplasma wenyonii was the only *Mycoplasma* species found to be present from the three study sites. Cattle are more susceptible to infections by this species. However, information on its method of transmission is lacking. The transmission of *M. wenyonii* is assumed to be through blood-sucking arthropods (Sasaoka et al., 2015) (Thongmeesee et al., 2022). Blood-sucking arthropods have been found to contain *M. wenyonii* DNA, which is an indication that they may serve as mechanical vectors for the spread of this pathogen and other possible bovine hemoplasmas (Song et al., 2012). Another possible route of infection in cattle is vertical transmission which is when the pathogen is passed from dams to cattle, particularly to neonatal calves (Quiroz-Castañeda et al., 2018). Many cattle infected with the pathogen do not exhibit any clinical illness, unless when there is concurrent illness which may ultimately lead to immunosuppression. Overall, although *M. wenyonii* ‘s precise method of transmission is still unclear, possible tick vectors need to be investigated to comprehend the dynamics of this pathogen.

The bacterial tick-borne pathogens identified in this study correlate with the identified tick species, however there is a need for further studies that will shed light on vectors that are associated with the transmission of *M. wenyonii*.

5.2. Conclusion

In summary, the study highlights the expansion in the geographic range of tick species *R. decoloraus* and *R. microplus*, emphasizing their potential role as vectors of economically significant diseases impacting both human and livestock health. The consequences of heightened tick burdens present lower livestock productivity and the potential for zoonotic implications, warranting urgent and effective tick control measures. The integration of advanced molecular approaches such as tick-blocking vaccines or blocking pathogen transmission vaccines, coupled with farmer outreach programs focusing on fundamental skills like proper reading of packet inserts instructions, appears as a crucial component in addressing improper vaccinations and dipping methods, thereby contributing to the mitigation of tick resistance to acaracides.

However, the looming factor of uncontrollable animal movement due to traditional practices poses a substantial risk, potentially introducing new tick species and highly pathogenic agents. It is therefore imperative to adhere to regulations set by the South African Veterinary Council to ensure proper translocation of animals.

The study also sheds light on the impact of climate change, indicating that certain tick species turn to adapt to different environments and hosts for survival. This underlines the need for ongoing research to document the distribution and vectorial capacity of ticks in response to changing climatic conditions.

The presence of *R. microplus* in Harrismith raises concerns about the potential presence of protozoan pathogens, in addition to the detected BTBDs, posing a threat to naïve cattle herds in the Free State province.

In conclusion, the study underlines the adverse effects of improper tick control measures, acaricide resistance, unregulated animal translocations, and climate changes effect. It calls for collaborative efforts in tick and tick-borne pathogen surveillance studies to inform effective control strategies and enhance our understanding of tickborne pathogens and transmission dynamics. Such One Health collaborative approaches are essential for the development of comprehensive and informed strategies to tackle the multifaceted challenges posed by ticks and tickborne diseases.

5.3. Research outcomes

5.3.1. Presentations

The findings of the study have been presented at the Faculty of Veterinary Science and Parasitological Society of Southern Africa (PARSA) conference as follows:

Khoza B, Oosthuizen MC & Khumalo ZTH. 2002. Detection of bacterial tick-borne pathogens in two provinces of South Africa using a microbiome sequencing approach. Faculty Day. Faculty of Veterinary Science. Faculty Day 20 October 2022.

Khoza B, Byaruhanga C, Makgabo SM, Oosthuizen MC & Khumalo ZTH. 2003. Detection of Bacterial Tick-Borne Pathogens from Cattle in Two Provinces of South Africa Using a Microbiome Sequencing Approach. 51st Annual PARSA Conference September 17 - September 20, 2023.

5.3.2. Publication

Bongekile L. Khoza, Charles Byaruhanga, S Marcus Makgabo, Nkululeko Nyangiwe, Themba Mnisi, Samukelo Nxumalo, Marinda C. Oosthuizen, Zamantungwa T.H. Mnisi. Tick distribution and Comparative analysis of the bovine blood microbiome in two provinces of South Africa using 16S rRNA PacBio sequencing approach. (Appendix 2).

5.4. Recommendations

The study underlines the importance of further research in the related field. Below are several recommendations.

- **Tick distribution studies should be conducted in a longitudinal approach and in collaboration with Ecologists:** This approach will provide a more holistic understanding of the effects of climate change in tick distribution over time thus contributing to comprehensive strategies for tick management.

- **Expand the number of target genes for pathogen detection:** While the use of whole 16S rRNA is commendable for bacterial pathogens, it is essential to acknowledge the significance of protozoan pathogens. Future studies should consider targeting additional genes specific to protozoans and other pathogens. This approach will enhance the accuracy of detecting and understanding the diversity of pathogenic agents carried by various tick species, thereby improving our knowledge of vectorial capacity.

CHAPTER 6

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APPENDICES

Appendix 1: Relative Abundances per rank per each locality.

LOCALITY	PHYLUM	ABUNDANCE	PERCENT
BERGVILLE	Acidobacteriota	4	0
BERGVILLE	Actinobacteriota	2800	1.4
BERGVILLE	Bacteroidota	20191	10.2
BERGVILLE	Chloroflexi	56	0
BERGVILLE	Cyanobacteria	28	0
BERGVILLE	Desulfobacterota	11	0
BERGVILLE	Firmicutes	37201	18.9
BERGVILLE	Fusobacteriota	219	0.1
BERGVILLE	Gemmatimonadota	17	0
BERGVILLE	Planctomycetota	58	0
BERGVILLE	Proteobacteria	132559	67.2
BERGVILLE	Spirochaetota	395	0.2
BERGVILLE	Verrucomicrobiota	3661	1.9
LOCALITY	**Class**	**Abundance**	**Percent**
BERGVILLE	Acidimicrobiia	155	0.1
BERGVILLE	Acidobacteriae	4	0
BERGVILLE	Actinobacteria	2626	1.3
BERGVILLE	Alphaproteobacteria	127720	64.8
BERGVILLE	Anaerolineae	13	0
BERGVILLE	Bacilli	28247	14.3
BERGVILLE	Bacteroidia	20191	10.2
BERGVILLE	Chloroflexia	43	0
BERGVILLE	Clostridia	8447	4.3
BERGVILLE	Desulfovibrionia	11	0
BERGVILLE	Fusobacteriia	219	0.1
BERGVILLE	Gammaproteobacteria	4839	2.5
BERGVILLE	Gemmatimonadetes	17	0
BERGVILLE	Lentisphaeria	57	0
BERGVILLE	Negativicutes	507	0.3
BERGVILLE	Planctomycetes	58	0
BERGVILLE	Spirochaetia	395	0.2
BERGVILLE	Thermoleophilia	19	0
BERGVILLE	Vampirivibrionia	28	0
BERGVILLE	Verrucomicrobiae	3604	1.8
LOCALITY	**Order**	**Abundance**	**Percent**
BERGVILLE	Acidaminococcales	507	0.3
BERGVILLE	Actinomycetales	243	0.1
BERGVILLE	Bacillales	1956	1
BERGVILLE	Bacteroidales	20171	10.2
BERGVILLE	Bryobacterales	4	0
BERGVILLE	Burkholderiales	1042	0.5
BERGVILLE	Caulobacterales	1	0

BERGVILLE	Christensenellales	155	0.1
BERGVILLE	Clostridia UCG-014	46	0
BERGVILLE	Clostridia vadinBB60 group	163	0.1
BERGVILLE	Corynebacteriales	1410	0.7
BERGVILLE	Cytophagales	17	0
BERGVILLE	Defluviicoccales	83	0
BERGVILLE	Desulfovibrionales	11	0
BERGVILLE	Enterobacterales	1567	0.8
BERGVILLE	Fusobacteriales	219	0.1
BERGVILLE	Gastranaerophilales	28	0
BERGVILLE	Gemmatimonadales	17	0
BERGVILLE	Isosphaerales	35	0
BERGVILLE	Lachnospirales	360	0.2
BERGVILLE	Lactobacillales	4388	2.2
BERGVILLE	Micrococcales	421	0.2
BERGVILLE	Micromonosporales	18	0
BERGVILLE	Microtrichales	155	0.1
BERGVILLE	Monoglobales	211	0.1
BERGVILLE	Mycoplasmatales	21688	11
BERGVILLE	Oscillospirales	5596	2.8
BERGVILLE	Peptostreptococcales-Tissierellales	1916	1
BERGVILLE	Pirellulales	23	0
BERGVILLE	Propionibacteriales	101	0.1
BERGVILLE	Pseudomonadales	2199	1.1
BERGVILLE	Pseudonocardiales	423	0.2
BERGVILLE	RF39	131	0.1
BERGVILLE	Rhizobiales	141	0.1
BERGVILLE	Rickettsiales	127494	64.7
BERGVILLE	SBR1031	13	0
BERGVILLE	Solirubrobacterales	19	0
BERGVILLE	Sphingobacteriales	3	0
BERGVILLE	Sphingomonadales	1	0
BERGVILLE	Spirochaetales	395	0.2
BERGVILLE	Staphylococcales	84	0
BERGVILLE	Streptomycetales	10	0
BERGVILLE	Thermomicrobiales	43	0
BERGVILLE	Verrucomicrobiales	3604	1.8
BERGVILLE	Victivallales	57	0
BERGVILLE	Xanthomonadales	31	0

LOCALITY	**Family**	**Abundance**	**Percent**
BERGVILLE	*[Eubacterium] coprostanoligenes group*	837	0.4
BERGVILLE	*67-14*	19	0
BERGVILLE	*A4b*	4	0
BERGVILLE	*Acidaminococcaceae*	507	0.3
BERGVILLE	*Actinomycetaceae*	243	0.1
BERGVILLE	*Aerococcaceae*	4068	2.1

BERGVILLE	*Akkermansiaceae*	3604	1.8
BERGVILLE	*AKYG1722*	43	0
BERGVILLE	*Alcaligenaceae*	221	0.1
BERGVILLE	*Anaerovoracaceae*	683	0.3
BERGVILLE	*Anaplasmataceae*	127494	65
BERGVILLE	*Bacillaceae*	25	0
BERGVILLE	*Bacteroidaceae*	779	0.4
BERGVILLE	*Bacteroidales RF16 group*	778	0.4
BERGVILLE	*Barnesiellaceae*	56	0
BERGVILLE	*Beijerinckiaceae*	29	0
BERGVILLE	*Bryobacteraceae*	4	0
BERGVILLE	*Butyricicoccaceae*	68	0
BERGVILLE	*Caulobacteraceae*	1	0
BERGVILLE	*Cellulomonadaceae*	13	0
BERGVILLE	*Cellvibrionaceae*	12	0
BERGVILLE	*Christensenellaceae*	155	0.1
BERGVILLE	*Comamonadaceae*	821	0.4
BERGVILLE	*Corynebacteriaceae*	1303	0.7
BERGVILLE	*Cyclobacteriaceae*	17	0
BERGVILLE	*Defluviicoccaceae*	83	0
BERGVILLE	*Dermabacteraceae*	121	0.1
BERGVILLE	*Desulfovibrionaceae*	11	0
BERGVILLE	*Devosiaceae*	28	0
BERGVILLE	*Dietziaceae*	107	0.1
BERGVILLE	*Enterobacteriaceae*	273	0.1
BERGVILLE	*F082*	1561	0.8
BERGVILLE	*Family XI*	849	0.4
BERGVILLE	*Fusobacteriaceae*	219	0.1
BERGVILLE	*Gemmatimonadaceae*	17	0
BERGVILLE	*Hungateiclostridiaceae*	78	0
BERGVILLE	*Ilumatobacteraceae*	81	0
BERGVILLE	*Intrasporangiaceae*	154	0.1
BERGVILLE	*Isosphaeraceae*	35	0
BERGVILLE	*Lachnospiraceae*	360	0.2
BERGVILLE	*Microbacteriaceae*	10	0
BERGVILLE	*Micrococcaceae*	111	0.1
BERGVILLE	*Micromonosporaceae*	18	0
BERGVILLE	*Monoglobaceae*	211	0.1
BERGVILLE	*Moraxellaceae*	1254	0.6
BERGVILLE	*Muribaculaceae*	338	0.2
BERGVILLE	*Mycoplasmataceae*	21688	11.1
BERGVILLE	*Nocardioidaceae*	101	0.1
BERGVILLE	*Oscillospiraceae*	3056	1.6
BERGVILLE	*p-251-o5*	32	0
BERGVILLE	*p-2534-18B5 gut group*	343	0.2
BERGVILLE	*Paludibacteraceae*	202	0.1
BERGVILLE	*Pasteurellaceae*	87	0
BERGVILLE	*Peptostreptococcaceae*	384	0.2

BERGVILLE	*Pirellulaceae*	23	0
BERGVILLE	*Planococcaceae*	1931	1
BERGVILLE	*Porphyromonadaceae*	960	0.5
BERGVILLE	*Prevotellaceae*	1529	0.8
BERGVILLE	*Promicromonosporaceae*	7	0
BERGVILLE	*Pseudomonadaceae*	933	0.5
BERGVILLE	*Pseudonocardiaceae*	423	0.2
BERGVILLE	*Rhizobiaceae*	84	0
BERGVILLE	*Rikenellaceae*	13011	6.6
BERGVILLE	*Ruminococcaceae*	222	0.1
BERGVILLE	*Sanguibacteraceae*	5	0
BERGVILLE	*Sphingobacteriaceae*	3	0
BERGVILLE	*Sphingomonadaceae*	1	0
BERGVILLE	*Spirochaetaceae*	395	0.2
BERGVILLE	*Staphylococcaceae*	84	0
BERGVILLE	*Streptococcaceae*	320	0.2
BERGVILLE	*Streptomycetaceae*	10	0
BERGVILLE	*Succinivibrionaceae*	274	0.1
BERGVILLE	*UCG-010*	1335	0.7
BERGVILLE	*vadinBE97*	41	0
BERGVILLE	*Victivallaceae*	16	0
BERGVILLE	*Xanthomonadaceae*	31	0
BERGVILLE	*Yersiniaceae*	933	0.5

LOCALITY	Genus	Abundance	Percent
BERGVILLE	*[Eubacterium] nodatum group*	623	0.3
BERGVILLE	*Acinetobacter*	1097	0.6
BERGVILLE	*Actinoplanes*	18	0
BERGVILLE	*Actinotalea*	3	0
BERGVILLE	*Aerococcus*	4068	2.1
BERGVILLE	*Aeromicrobium*	10	0
BERGVILLE	*Akkermansia*	3604	1.9
BERGVILLE	*Algoriphagus*	2	0
BERGVILLE	*Alistipes*	2642	1.4
BERGVILLE	*Alloprevotella*	332	0.2
BERGVILLE	*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*	20	0
BERGVILLE	*Altererythrobacter*	1	0
BERGVILLE	*Anaerococcus*	719	0.4
BERGVILLE	*Anaerostignum*	36	0
BERGVILLE	*Anaplasma*	127465	67.1
BERGVILLE	*Aquabacterium*	102	0.1
BERGVILLE	*Arcanobacterium*	243	0.1
BERGVILLE	*Arthrobacter*	12	0
BERGVILLE	*Aurantimonas*	64	0
BERGVILLE	*Bacteroides*	779	0.4
BERGVILLE	*Bilophila*	11	0
BERGVILLE	*Brachybacterium*	121	0.1
BERGVILLE	*Brevundimonas*	1	0

BERGVILLE	*Bryobacter*	4	0
BERGVILLE	*Candidatus Soleaferrea*	44	0
BERGVILLE	*Cellulomonas*	10	0
BERGVILLE	*Christensenellaceae R-7 group*	155	0.1
BERGVILLE	*Chryseomicrobium*	16	0
BERGVILLE	*Citricoccus*	99	0.1
BERGVILLE	*Colidextribacter*	32	0
BERGVILLE	*Coprococcus*	19	0
BERGVILLE	*Corynebacterium*	1303	0.7
BERGVILLE	*Defluviicoccus*	83	0
BERGVILLE	*Devosia*	28	0
BERGVILLE	*dgA-11 gut group*	470	0.2
BERGVILLE	*Dietzia*	107	0.1
BERGVILLE	*Dorea*	27	0
BERGVILLE	*Ehrlichia*	29	0
BERGVILLE	*Enhydrobacter*	157	0.1
BERGVILLE	*Escherichia-Shigella*	273	0.1
BERGVILLE	*Family XIII AD3011 group*	60	0
BERGVILLE	*Finegoldia*	14	0
BERGVILLE	*Fusobacterium*	219	0.1
BERGVILLE	*Gallibacterium*	87	0
BERGVILLE	*Helcococcus*	116	0.1
BERGVILLE	*Isoptericola*	7	0
BERGVILLE	*Janibacter*	42	0
BERGVILLE	*Lachnoclostridium*	122	0.1
BERGVILLE	*Lachnospiraceae AC2044 group*	12	0
BERGVILLE	*Lachnospiraceae FCS020 group*	12	0
BERGVILLE	*Luteimonas*	31	0
BERGVILLE	*Marmoricola*	37	0
BERGVILLE	*Methylobacterium-Methylorubrum*	29	0
BERGVILLE	*Monoglobus*	211	0.1
BERGVILLE	*Mycoplasma*	21688	11.4
BERGVILLE	*Negativibacillus*	23	0
BERGVILLE	*NK4A214 group*	98	0.1
BERGVILLE	*Nocardioides*	54	0
BERGVILLE	*OLB13*	3	0
BERGVILLE	*Ornithinicoccus*	90	0
BERGVILLE	*Ornithinimicrobium*	19	0
BERGVILLE	*Oscillibacter*	87	0
BERGVILLE	*p-1088-a5 gut group*	23	0
BERGVILLE	*Paeniclostridium*	122	0.1
BERGVILLE	*Pedobacter*	3	0
BERGVILLE	*Pelistega*	221	0.1
BERGVILLE	*Pelomonas*	683	0.4
BERGVILLE	*Phascolarctobacterium*	507	0.3
BERGVILLE	*Planococcus*	230	0.1

BERGVILLE	*Planomicrobium*	1672	0.9
BERGVILLE	*Porphyromonas*	960	0.5
BERGVILLE	*Prauserella*	423	0.2
BERGVILLE	*Prevotellaceae UCG-001*	165	0.1
BERGVILLE	*Prevotellaceae UCG-003*	435	0.2
BERGVILLE	*Prevotellaceae UCG-004*	597	0.3
BERGVILLE	*Pseudomonas*	933	0.5
BERGVILLE	*Rhodoferax*	32	0
BERGVILLE	*Rikenellaceae RC9 gut group*	9899	5.2
BERGVILLE	*Romboutsia*	251	0.1
BERGVILLE	*Ruminiclostridium*	78	0
BERGVILLE	*Ruminobacter*	274	0.1
BERGVILLE	*Salinibacterium*	10	0
BERGVILLE	*Sanguibacter-Flavimobilis*	5	0
BERGVILLE	*Staphylococcus*	84	0
BERGVILLE	*Streptococcus*	320	0.2
BERGVILLE	*Streptomyces*	10	0
BERGVILLE	*Terrisporobacter*	11	0
BERGVILLE	*Tetrasphaera*	3	0
BERGVILLE	*Treponema*	395	0.2
BERGVILLE	*UCG-002*	83	0
BERGVILLE	*UCG-005*	2697	1.4
BERGVILLE	*UCG-009*	68	0
BERGVILLE	*Variovorax*	4	0
BERGVILLE	*Yersinia*	933	0.5

LOCALITY	Species	Abundance	Percent
BERGVILLE	*Acinetobacter_lwoffii*	980	0.6
BERGVILLE	*Aerococcus_vaginalis*	4068	2.6
BERGVILLE	*Anaplasma_boleense*	200	0.1
BERGVILLE	*Anaplasma_bovis*	548	0.4
BERGVILLE	*Anaplasma_centrale*	274	0.2
BERGVILLE	*Anaplasma_marginale*	87015	56.2
BERGVILLE	*Anaplasma_phagocytophilum*	40	0
BERGVILLE	*Anaplasma_platys*	35068	22.6
BERGVILLE	*Arthrobacter_luteolus*	12	0
BERGVILLE	*Bacteroides_thetaiotaomicron*	108	0.1
BERGVILLE	*Brevundimonas_vesicularis*	1	0
BERGVILLE	*Cellulomonas_flavigena*	10	0
BERGVILLE	*Corynebacterium_efficiens*	27	0
BERGVILLE	*Corynebacterium_falsenii*	74	0
BERGVILLE	*Corynebacterium_lactis*	558	0.4
BERGVILLE	*Corynebacterium_resistens*	519	0.3
BERGVILLE	*Corynebacterium_vitaeruminis*	96	0.1
BERGVILLE	*Dietzia_maris*	85	0.1
BERGVILLE	*Ehrlichia_canis*	16	0
BERGVILLE	*Escherichia-Shigella_coli*	196	0.1
BERGVILLE	*Escherichia-Shigella_flexneri*	77	0
BERGVILLE	*Finegoldia_magna*	14	0

BERGVILLE	*Fusobacterium_periodonticum*	219	0.1
BERGVILLE	*Helcococcus_sueciensis*	84	0.1
BERGVILLE	*Methylobacterium-Methylorubrum_fujisawaense*	15	0
BERGVILLE	*Methylobacterium-Methylorubrum_jeotgali*	14	0
BERGVILLE	*Mycoplasma_haemobos*	64	0
BERGVILLE	*Mycoplasma_wenyonii*	21624	14
BERGVILLE	*Nocardioides_albus*	11	0
BERGVILLE	*Ornithinimicrobium_humiphilum*	17	0
BERGVILLE	*Pedobacter_bauzanensis*	3	0
BERGVILLE	*Planococcus_psychrotoleratus*	60	0
BERGVILLE	*Porphyromonas_somerae*	265	0.2
BERGVILLE	*Prauserella_isguenensis*	423	0.3
BERGVILLE	*Pseudomonas_antarctica*	81	0.1
BERGVILLE	*Pseudomonas_monteilii*	14	0
BERGVILLE	*Pseudomonas_poae*	783	0.5
BERGVILLE	*Pseudomonas_yamanorum*	2	0
BERGVILLE	*Sanguibacter-Flavimobilis_marinus*	5	0
BERGVILLE	*Staphylococcus_chromogenes*	46	0
BERGVILLE	*Streptococcus_oralis*	292	0.2
BERGVILLE	*Streptococcus_pluranimalium*	28	0
BERGVILLE	*Yersinia_pestis*	801	0.5

LOCALITY	PHYLUM	ABUNDANCE	PERCENT
HARRISMITH	Actinobacteriota	2214	1.2
HARRISMITH	Bacteroidota	10228	5.3
HARRISMITH	Chloroflexi	72	0
HARRISMITH	Desulfobacterota	28	0
HARRISMITH	Fibrobacterota	35	0
HARRISMITH	Firmicutes	37522	19.6
HARRISMITH	Gemmatimonadota	4	0
HARRISMITH	Myxococcota	25	0
HARRISMITH	Planctomycetota	27	0
HARRISMITH	Proteobacteria	141478	73.8
HARRISMITH	Verrucomicrobiota	176	0.1
LOCALITY	**CLASS**	**ABUNDANCE**	**PERCENT**
HARRISMITH	Acidimicrobiia	54	0
HARRISMITH	Actinobacteria	2157	1.1
HARRISMITH	Alphaproteobacteria	138943	72.4
HARRISMITH	Anaerolineae	71	0
HARRISMITH	Bacilli	33654	17.5
HARRISMITH	Bacteroidia	10224	5.3
HARRISMITH	Chloroflexia	1	0
HARRISMITH	Clostridia	3703	1.9
HARRISMITH	Desulfuromonadia	28	0
HARRISMITH	Fibrobacteria	35	0
HARRISMITH	Gammaproteobacteria	2535	1.3
HARRISMITH	Kiritimatiellae	20	0
HARRISMITH	Lentisphaeria	41	0
HARRISMITH	Longimicrobia	4	0
HARRISMITH	Negativicutes	165	0.1
HARRISMITH	Planctomycetes	27	0
HARRISMITH	Polyangia	25	0
HARRISMITH	Rhodothermia	4	0
HARRISMITH	Thermoleophilia	3	0
HARRISMITH	Verrucomicrobiae	115	0.1
LOCALITY	**ORDER**	**ABUNDANCE**	**PERCENT**
HARRISMITH	Acidaminococcales	165	0.1
HARRISMITH	Ardenticatenales	14	0
HARRISMITH	Bacillales	267	0.1
HARRISMITH	Bacteroidales	9623	5
HARRISMITH	Bradymonadales	28	0
HARRISMITH	Burkholderiales	1661	0.9
HARRISMITH	Caulobacterales	25	0
HARRISMITH	Chitinophagales	65	0
HARRISMITH	Christensenellales	109	0.1
HARRISMITH	Clostridia UCG-014	19	0
HARRISMITH	Clostridia vadinBB60 group	63	0
HARRISMITH	Clostridiales	16	0
HARRISMITH	Corynebacteriales	61	0

HARRISMITH	Cytophagales	225	0.1
HARRISMITH	Enterobacterales	12	0
HARRISMITH	Fibrobacterales	35	0
HARRISMITH	Flavobacteriales	262	0.1
HARRISMITH	Haliangiales	12	0
HARRISMITH	Lachnospirales	83	0
HARRISMITH	Lactobacillales	65	0
HARRISMITH	Longimicrobiales	4	0
HARRISMITH	Micavibrionales	14	0
HARRISMITH	Micrococcales	588	0.3
HARRISMITH	Microtrichales	27	0
HARRISMITH	Monoglobales	11	0
HARRISMITH	Mycoplasmatales	33265	17.3
HARRISMITH	Opitutales	2	0
HARRISMITH	Oscillospirales	2597	1.4
HARRISMITH	Peptostreptococcales-Tissierellales	805	0.4
HARRISMITH	Pirellulales	27	0
HARRISMITH	Polyangiales	13	0
HARRISMITH	Propionibacteriales	1470	0.8
HARRISMITH	Pseudomonadales	484	0.3
HARRISMITH	Pseudonocardiales	33	0
HARRISMITH	R7C24	13	0
HARRISMITH	Rhizobiales	659	0.3
HARRISMITH	Rhodothermales	4	0
HARRISMITH	Rickettsiales	137987	72
HARRISMITH	SBR1031	57	0
HARRISMITH	Solirubrobacterales	3	0
HARRISMITH	Sphingobacteriales	49	0
HARRISMITH	Sphingomonadales	258	0.1
HARRISMITH	Staphylococcales	57	0
HARRISMITH	Steroidobacterales	27	0
HARRISMITH	Streptomycetales	5	0
HARRISMITH	Thermomicrobiales	1	0
HARRISMITH	Verrucomicrobiales	113	0.1
HARRISMITH	Victivallales	41	0
HARRISMITH	WCHB1-41	20	0
HARRISMITH	Xanthomonadales	326	0.2

LOCALITY	**Family**	**Abundance**	**Percent**
HARRISMITH	*[Eubacterium] coprostanoligenes group*	194	0.1
HARRISMITH	*211ds20*	12	0
HARRISMITH	*67-14*	3	0
HARRISMITH	*A4b*	14	0
HARRISMITH	*Acidaminococcaceae*	165	0.1
HARRISMITH	*Aerococcaceae*	54	0
HARRISMITH	*Akkermansiaceae*	113	0.1
HARRISMITH	*AKYG1722*	1	0
HARRISMITH	*Anaerovoracaceae*	50	0
HARRISMITH	*Anaplasmataceae*	137987	72.1

HARRISMITH	*Bacillaceae*	19	0
HARRISMITH	*Bacteroidaceae*	167	0.1
HARRISMITH	*Bacteroidales RF16 group*	250	0.1
HARRISMITH	*Bacteroidales UCG-001*	32	0
HARRISMITH	*Beijerinckiaceae*	57	0
HARRISMITH	*BIrii41*	13	0
HARRISMITH	*Burkholderiaceae*	77	0
HARRISMITH	*Carnobacteriaceae*	11	0
HARRISMITH	*Caulobacteraceae*	25	0
HARRISMITH	*Cellulomonadaceae*	82	0
HARRISMITH	*Cellvibrionaceae*	305	0.2
HARRISMITH	*Chitinophagaceae*	65	0
HARRISMITH	*Christensenellaceae*	109	0.1
HARRISMITH	*Clostridiaceae*	16	0
HARRISMITH	*Comamonadaceae*	1553	0.8
HARRISMITH	*Corynebacteriaceae*	32	0
HARRISMITH	*Cyclobacteriaceae*	87	0
HARRISMITH	*Cytophagaceae*	36	0
HARRISMITH	*Demequinaceae*	44	0
HARRISMITH	*Dermabacteraceae*	32	0
HARRISMITH	*Devosiaceae*	399	0.2
HARRISMITH	*Dietziaceae*	29	0
HARRISMITH	*F082*	146	0.1
HARRISMITH	*Fibrobacteraceae*	35	0
HARRISMITH	*Flavobacteriaceae*	248	0.1
HARRISMITH	*Haliangiaceae*	12	0
HARRISMITH	*Hungateiclostridiaceae*	19	0
HARRISMITH	*Ilumatobacteraceae*	4	0
HARRISMITH	*Intrasporangiaceae*	264	0.1
HARRISMITH	*Lachnospiraceae*	83	0
HARRISMITH	*Longimicrobiaceae*	4	0
HARRISMITH	*Micavibrionaceae*	14	0
HARRISMITH	*Microbacteriaceae*	30	0
HARRISMITH	*Micrococcaceae*	103	0.1
HARRISMITH	*Monoglobaceae*	11	0
HARRISMITH	*Moraxellaceae*	3	0
HARRISMITH	*Muribaculaceae*	38	0
HARRISMITH	*MWH-CFBk5*	102	0.1
HARRISMITH	*Mycoplasmataceae*	33265	17.4
HARRISMITH	*Nocardioidaceae*	114	0.1
HARRISMITH	*Opitutaceae*	2	0
HARRISMITH	*Oscillospiraceae*	1457	0.8
HARRISMITH	*Oxalobacteraceae*	31	0
HARRISMITH	*p-2534-18B5 gut group*	194	0.1
HARRISMITH	*Paludibacteraceae*	56	0
HARRISMITH	*Peptostreptococcaceae*	755	0.4
HARRISMITH	*Pirellulaceae*	27	0
HARRISMITH	*Planococcaceae*	235	0.1

HARRISMITH	*Prevotellaceae*	366	0.2
HARRISMITH	*Promicromonosporaceae*	10	0
HARRISMITH	*Propionibacteriaceae*	1356	0.7
HARRISMITH	*Pseudohongiellaceae*	11	0
HARRISMITH	*Pseudomonadaceae*	153	0.1
HARRISMITH	*Pseudonocardiaceae*	33	0
HARRISMITH	*Rhizobiaceae*	142	0.1
HARRISMITH	*Rhodothermaceae*	4	0
HARRISMITH	*Rikenellaceae*	8268	4.3
HARRISMITH	*Ruminococcaceae*	181	0.1
HARRISMITH	*Sanguibacteraceae*	6	0
HARRISMITH	*Sphingobacteriaceae*	49	0
HARRISMITH	*Sphingomonadaceae*	258	0.1
HARRISMITH	*Sporolactobacillaceae*	13	0
HARRISMITH	*Staphylococcaceae*	57	0
HARRISMITH	*Steroidobacteraceae*	27	0
HARRISMITH	*Streptomycetaceae*	5	0
HARRISMITH	*Succinivibrionaceae*	12	0
HARRISMITH	*UCG-010*	731	0.4
HARRISMITH	*Victivallaceae*	41	0
HARRISMITH	*Weeksellaceae*	14	0
HARRISMITH	*Xanthobacteraceae*	61	0
HARRISMITH	*Xanthomonadaceae*	326	0.2

LOCALITY	Genus	Abundance	Percent
HARRISMITH	*[Eubacterium] nodatum group*	20	0
HARRISMITH	*[Ruminococcus] torques group*	12	0
HARRISMITH	*Acinetobacter*	3	0
HARRISMITH	*Actinotalea*	45	0
HARRISMITH	*Aerococcus*	54	0
HARRISMITH	*Aeromicrobium*	34	0
HARRISMITH	*Akkermansia*	113	0.1
HARRISMITH	*Algoriphagus*	63	0
HARRISMITH	*Alistipes*	477	0.3
HARRISMITH	*Alloprevotella*	91	0
HARRISMITH	*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*	67	0
HARRISMITH	*Altererythrobacter*	216	0.1
HARRISMITH	*Aminobacter*	75	0
HARRISMITH	*Anaerostignum*	11	0
HARRISMITH	*Anaplasma*	136787	72.3
HARRISMITH	*Antarcticibacterium*	88	0
HARRISMITH	*Aquabacterium*	95	0.1
HARRISMITH	*Arsenicitalea*	16	0
HARRISMITH	*Arthrobacter*	80	0
HARRISMITH	*Bacillus*	19	0
HARRISMITH	*Bacteroides*	167	0.1
HARRISMITH	*Blyi10*	11	0
HARRISMITH	*Brachybacterium*	32	0

HARRISMITH	*Brevundimonas*	14	0
HARRISMITH	*Candidatus Soleaferrea*	115	0.1
HARRISMITH	*Cellulomonas*	37	0
HARRISMITH	*Cellvibrio*	60	0
HARRISMITH	*Christensenellaceae R-7 group*	109	0.1
HARRISMITH	*Citricoccus*	23	0
HARRISMITH	*Clostridium sensu stricto 1*	16	0
HARRISMITH	*Comamonas*	58	0
HARRISMITH	*Coprococcus*	18	0
HARRISMITH	*Corynebacterium*	32	0
HARRISMITH	*Cutibacterium*	1339	0.7
HARRISMITH	*Demequina*	44	0
HARRISMITH	*Devosia*	349	0.2
HARRISMITH	*dgA-11 gut group*	317	0.2
HARRISMITH	*Dietzia*	29	0
HARRISMITH	*Ehrlichia*	1200	0.6
HARRISMITH	*Ellin6055*	13	0
HARRISMITH	*Family XIII AD3011 group*	30	0
HARRISMITH	*Fibrobacter*	22	0
HARRISMITH	*Flaviaesturariibacter*	11	0
HARRISMITH	*Flavisolibacter*	35	0
HARRISMITH	*Flavobacterium*	61	0
HARRISMITH	*Haliangium*	12	0
HARRISMITH	*Isoptericola*	10	0
HARRISMITH	*Janibacter*	57	0
HARRISMITH	*Jeotgalibaca*	11	0
HARRISMITH	*Lachnospiraceae NK4A136 group*	38	0
HARRISMITH	*Leptothrix*	17	0
HARRISMITH	*Limnobacter*	77	0
HARRISMITH	*Luteimonas*	291	0.2
HARRISMITH	*Luteococcus*	17	0
HARRISMITH	*Lysinibacillus*	19	0
HARRISMITH	*Lysobacter*	35	0
HARRISMITH	*Marmoricola*	13	0
HARRISMITH	*Methylobacterium-Methylorubrum*	57	0
HARRISMITH	*Moheibacter*	14	0
HARRISMITH	*Monoglobus*	11	0
HARRISMITH	*Mumia*	2	0
HARRISMITH	*Mycoplasma*	33265	17.6
HARRISMITH	*Negativibacillus*	35	0
HARRISMITH	*NK4A214 group*	34	0
HARRISMITH	*Nocardioides*	65	0
HARRISMITH	*Noviherbaspirillum*	31	0
HARRISMITH	*OLB13*	14	0
HARRISMITH	*Opitutus*	2	0
HARRISMITH	*Ornithinicoccus*	156	0.1
HARRISMITH	*Ornithinimicrobium*	34	0
HARRISMITH	*Oscillibacter*	47	0

HARRISMITH	*Paeniclostridium*	91	0
HARRISMITH	*Pedobacter*	49	0
HARRISMITH	*Pelagibacterium*	34	0
HARRISMITH	*Pelomonas*	1367	0.7
HARRISMITH	*Phascolarctobacterium*	165	0.1
HARRISMITH	*Pir4 lineage*	24	0
HARRISMITH	*Planococcus*	24	0
HARRISMITH	*Planomicrobium*	192	0.1
HARRISMITH	*Prauserella*	33	0
HARRISMITH	*Prevotellaceae Ga6A1 group*	13	0
HARRISMITH	*Prevotellaceae UCG-001*	12	0
HARRISMITH	*Prevotellaceae UCG-003*	47	0
HARRISMITH	*Prevotellaceae UCG-004*	203	0.1
HARRISMITH	*Pseudoclavibacter*	24	0
HARRISMITH	*Pseudomonas*	153	0.1
HARRISMITH	*Rhodopseudomonas*	61	0
HARRISMITH	*Rikenellaceae RC9 gut group*	7474	4
HARRISMITH	*Romboutsia*	507	0.3
HARRISMITH	*Rubrivivax*	12	0
HARRISMITH	*Ruminococcus*	31	0
HARRISMITH	*Saccharofermentans*	19	0
HARRISMITH	*Salinibacterium*	6	0
HARRISMITH	*Salinimicrobium*	27	0
HARRISMITH	*Sanguibacter-Flavimobilis*	6	0
HARRISMITH	*Segetibacter*	19	0
HARRISMITH	*Sphingomonas*	14	0
HARRISMITH	*Sphingopyxis*	15	0
HARRISMITH	*Staphylococcus*	57	0
HARRISMITH	*Steroidobacter*	27	0
HARRISMITH	*Streptomyces*	5	0
HARRISMITH	*Succinivibrio*	12	0
HARRISMITH	*Terrisporobacter*	157	0.1
HARRISMITH	*Tetrasphaera*	17	0
HARRISMITH	*UCG-002*	77	0
HARRISMITH	*UCG-005*	1226	0.6
HARRISMITH	*Variovorax*	4	0
HARRISMITH	*Vitellibacter*	60	0
LOCALITY	**Species**	**Abundance**	**Percent**
HARRISMITH	*Acinetobacter_lwoffii*	3	0
HARRISMITH	*Aerococcus_vaginalis*	54	0
HARRISMITH	*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium_flavum*	39	0
HARRISMITH	*Anaplasma_boleense*	479	0.3
HARRISMITH	*Anaplasma_bovis*	2268	1.3
HARRISMITH	*Anaplasma_centrale*	1566	0.9
HARRISMITH	*Anaplasma_marginale*	73744	43.5
HARRISMITH	*Anaplasma_ovis*	1339	0.8
HARRISMITH	*Anaplasma_phagocytophilum*	161	0.1

HARRISMITH	*Anaplasma_platys*	53403	31.5
HARRISMITH	*Arthrobacter_gandavensis*	13	0
HARRISMITH	*Arthrobacter_luteolus*	67	0
HARRISMITH	*Brevundimonas_abyssalis*	11	0
HARRISMITH	*Brevundimonas_vesicularis*	3	0
HARRISMITH	*Cellulomonas_flavigena*	37	0
HARRISMITH	*Comamonas_jiangduensis*	36	0
HARRISMITH	*Corynebacterium_lactis*	12	0
HARRISMITH	*Corynebacterium_resistens*	20	0
HARRISMITH	*Cutibacterium_namnetense*	1339	0.8
HARRISMITH	*Devosia_riboflavina*	54	0
HARRISMITH	*Dietzia_maris*	29	0
HARRISMITH	*Ehrlichia_canis*	1061	0.6
HARRISMITH	*Limnobacter_thiooxidans*	77	0
HARRISMITH	*Lysinibacillus_odysseyi*	19	0
HARRISMITH	*Lysobacter_spongiicola*	15	0
HARRISMITH	*Methylobacterium-Methylorubrum_fujisawaense*	4	0
HARRISMITH	*Methylobacterium-Methylorubrum_jeotgali*	34	0
HARRISMITH	*Methylobacterium-Methylorubrum_persicinum*	19	0
HARRISMITH	*Mycoplasma_wenyonii*	33265	19.6
HARRISMITH	*Nocardioides_albus*	25	0
HARRISMITH	*Pedobacter_bauzanensis*	49	0
HARRISMITH	*Pelomonas_aquatica*	13	0
HARRISMITH	*Planomicrobium_glaciei*	19	0
HARRISMITH	*Prauserella_isguenensis*	33	0
HARRISMITH	*Pseudoclavibacter_faecalis*	24	0
HARRISMITH	*Pseudomonas_antarctica*	78	0
HARRISMITH	*Pseudomonas_monteilii*	26	0
HARRISMITH	*Pseudomonas_poae*	2	0
HARRISMITH	*Pseudomonas_sabulinigri*	15	0
HARRISMITH	*Pseudomonas_yamanorum*	8	0
HARRISMITH	*Rhodopseudomonas_palustris*	61	0
HARRISMITH	*Sanguibacter-Flavimobilis_marinus*	6	0
HARRISMITH	*Sphingomonas_panaciterrae*	14	0
HARRISMITH	*Staphylococcus_epidermidis*	4	0
HARRISMITH	*Staphylococcus_hominis*	31	0

LOCALITY	PHYLUM	ABUNDANCE	PERCENT
PHUTHADITJHABA	Acidobacteriota	52	0
PHUTHADITJHABA	Actinobacteriota	1948	1
PHUTHADITJHABA	Bacteroidota	7963	4
PHUTHADITJHABA	Chloroflexi	131	0.1
PHUTHADITJHABA	Cyanobacteria	142	0.1
PHUTHADITJHABA	Deinococcota	31	0
PHUTHADITJHABA	Fibrobacterota	27	0
PHUTHADITJHABA	Firmicutes	19134	9.6
PHUTHADITJHABA	Gemmatimonadota	81	0
PHUTHADITJHABA	Myxococcota	12	0
PHUTHADITJHABA	Planctomycetota	212	0.1
PHUTHADITJHABA	Proteobacteria	168903	84.8
PHUTHADITJHABA	Verrucomicrobiota	527	0.3
LOCALITY	**Class**	**Abundance**	**Percent**
PHUTHADITJHABA	Acidimicrobiia	282	0.1
PHUTHADITJHABA	Acidobacteriae	15	0
PHUTHADITJHABA	Actinobacteria	1666	0.8
PHUTHADITJHABA	Alphaproteobacteria	168105	84.4
PHUTHADITJHABA	Anaerolineae	131	0.1
PHUTHADITJHABA	Bacilli	16265	8.2
PHUTHADITJHABA	Bacteroidia	7954	4
PHUTHADITJHABA	Blastocatellia	37	0
PHUTHADITJHABA	Clostridia	2713	1.4
PHUTHADITJHABA	Cyanobacteriia	127	0.1
PHUTHADITJHABA	Deinococci	31	0
PHUTHADITJHABA	Fibrobacteria	27	0
PHUTHADITJHABA	Gammaproteobacteria	798	0.4
PHUTHADITJHABA	Gemmatimonadetes	25	0
PHUTHADITJHABA	Kiritimatiellae	11	0
PHUTHADITJHABA	Longimicrobia	56	0
PHUTHADITJHABA	Negativicutes	156	0.1
PHUTHADITJHABA	Phycisphaerae	15	0
PHUTHADITJHABA	Planctomycetes	197	0.1
PHUTHADITJHABA	Polyangia	12	0
PHUTHADITJHABA	Rhodothermia	9	0
PHUTHADITJHABA	Vampirivibrionia	15	0
PHUTHADITJHABA	Verrucomicrobiae	516	0.3
LOCALITY	**Order**	**Abundance**	**Percent**
PHUTHADITJHABA	Acidaminococcales	156	0.1
PHUTHADITJHABA	Ardenticatenales	43	0
PHUTHADITJHABA	Bacillales	52	0
PHUTHADITJHABA	Bacteroidales	7880	4
PHUTHADITJHABA	Blastocatellales	37	0
PHUTHADITJHABA	Bryobacterales	15	0
PHUTHADITJHABA	Burkholderiales	517	0.3
PHUTHADITJHABA	Caulobacterales	42	0
PHUTHADITJHABA	Chloroplast	127	0.1

PHUTHADITJHABA	Christensenellales	58	0
PHUTHADITJHABA	Clostridia vadinBB60 group	26	0
PHUTHADITJHABA	Corynebacteriales	164	0.1
PHUTHADITJHABA	Cytophagales	13	0
PHUTHADITJHABA	Deinococcales	31	0
PHUTHADITJHABA	Enterobacterales	192	0.1
PHUTHADITJHABA	Entomoplasmatales	11	0
PHUTHADITJHABA	Fibrobacterales	27	0
PHUTHADITJHABA	Gastranaerophilales	15	0
PHUTHADITJHABA	Gemmatimonadales	25	0
PHUTHADITJHABA	Haliangiales	12	0
PHUTHADITJHABA	Isosphaerales	160	0.1
PHUTHADITJHABA	Kiloniellales	39	0
PHUTHADITJHABA	Lachnospirales	416	0.2
PHUTHADITJHABA	Lactobacillales	44	0
PHUTHADITJHABA	Longimicrobiales	56	0
PHUTHADITJHABA	Micrococcales	913	0.5
PHUTHADITJHABA	Microtrichales	282	0.1
PHUTHADITJHABA	Monoglobales	45	0
PHUTHADITJHABA	Mycoplasmatales	16058	8.1
PHUTHADITJHABA	Opitutales	9	0
PHUTHADITJHABA	Oscillospirales	1707	0.9
PHUTHADITJHABA	Pedosphaerales	34	0
PHUTHADITJHABA	Peptococcales	27	0
PHUTHADITJHABA	Peptostreptococcales-Tissierellales	434	0.2
PHUTHADITJHABA	Phycisphaerales	15	0
PHUTHADITJHABA	Pirellulales	37	0
PHUTHADITJHABA	Propionibacteriales	502	0.3
PHUTHADITJHABA	Pseudomonadales	53	0
PHUTHADITJHABA	Pseudonocardiales	37	0
PHUTHADITJHABA	RF39	11	0
PHUTHADITJHABA	Rhizobiales	56	0
PHUTHADITJHABA	Rhodospirillales	11	0
PHUTHADITJHABA	Rhodothermales	9	0
PHUTHADITJHABA	Rickettsiales	167957	84.3
PHUTHADITJHABA	SBR1031	88	0
PHUTHADITJHABA	Sphingobacteriales	61	0
PHUTHADITJHABA	Staphylococcales	89	0
PHUTHADITJHABA	Streptosporangiales	50	0
PHUTHADITJHABA	Verrucomicrobiales	473	0.2
PHUTHADITJHABA	WCHB1-41	11	0
PHUTHADITJHABA	Xanthomonadales	36	0

LOCALITY	**Family**	**Abundance**	**Percent**
PHUTHADITJHABA	*[Eubacterium] coprostanoligenes group*	171	0.1
PHUTHADITJHABA	*A4b*	61	0
PHUTHADITJHABA	*Acidaminococcaceae*	156	0.1
PHUTHADITJHABA	*Aerococcaceae*	44	0

PHUTHADITJHABA	*Akkermansiaceae*	473	0.2
PHUTHADITJHABA	*Alteromonadaceae*	29	0
PHUTHADITJHABA	*Anaerovoracaceae*	133	0.1
PHUTHADITJHABA	*Anaplasmataceae*	167957	84.7
PHUTHADITJHABA	*Bacteroidaceae*	396	0.2
PHUTHADITJHABA	*Bacteroidales RF16 group*	141	0.1
PHUTHADITJHABA	*Blastocatellaceae*	37	0
PHUTHADITJHABA	*Bryobacteraceae*	15	0
PHUTHADITJHABA	*Burkholderiaceae*	22	0
PHUTHADITJHABA	*Butyricicoccaceae*	151	0.1
PHUTHADITJHABA	*Caulobacteraceae*	42	0
PHUTHADITJHABA	*Cellulomonadaceae*	742	0.4
PHUTHADITJHABA	*Christensenellaceae*	58	0
PHUTHADITJHABA	*Comamonadaceae*	130	0.1
PHUTHADITJHABA	*Corynebacteriaceae*	134	0.1
PHUTHADITJHABA	*Devosiaceae*	4	0
PHUTHADITJHABA	*Dietziaceae*	30	0
PHUTHADITJHABA	*Enterobacteriaceae*	49	0
PHUTHADITJHABA	*F082*	213	0.1
PHUTHADITJHABA	*Fibrobacteraceae*	27	0
PHUTHADITJHABA	*Gemmatimonadaceae*	25	0
PHUTHADITJHABA	*Haliangiaceae*	12	0
PHUTHADITJHABA	*Hymenobacteraceae*	13	0
PHUTHADITJHABA	*Intrasporangiaceae*	31	0
PHUTHADITJHABA	*Isosphaeraceae*	160	0.1
PHUTHADITJHABA	*Kiloniellaceae*	39	0
PHUTHADITJHABA	*Lachnospiraceae*	416	0.2
PHUTHADITJHABA	*Longimicrobiaceae*	56	0
PHUTHADITJHABA	*Micrococcaceae*	52	0
PHUTHADITJHABA	*Monoglobaceae*	45	0
PHUTHADITJHABA	*Muribaculaceae*	11	0
PHUTHADITJHABA	*Mycoplasmataceae*	16058	8.1
PHUTHADITJHABA	*Neisseriaceae*	11	0
PHUTHADITJHABA	*Nocardioidaceae*	502	0.3
PHUTHADITJHABA	*Opitutaceae*	9	0
PHUTHADITJHABA	*Oscillospiraceae*	716	0.4
PHUTHADITJHABA	*Oxalobacteraceae*	354	0.2
PHUTHADITJHABA	*p-2534-18B5 gut group*	107	0.1
PHUTHADITJHABA	*Paludibacteraceae*	69	0
PHUTHADITJHABA	*Pasteurellaceae*	9	0
PHUTHADITJHABA	*Pedosphaeraceae*	34	0
PHUTHADITJHABA	*Peptococcaceae*	27	0
PHUTHADITJHABA	*Peptostreptococcaceae*	301	0.2
PHUTHADITJHABA	*Phycisphaeraceae*	15	0
PHUTHADITJHABA	*Pirellulaceae*	37	0
PHUTHADITJHABA	*Planococcaceae*	52	0
PHUTHADITJHABA	*Porphyromonadaceae*	305	0.2
PHUTHADITJHABA	*Prevotellaceae*	1065	0.5

PHUTHADITJHABA	*Promicromonosporaceae*	73	0
PHUTHADITJHABA	*Pseudomonadaceae*	53	0
PHUTHADITJHABA	*Pseudonocardiaceae*	37	0
PHUTHADITJHABA	*Rhizobiaceae*	34	0
PHUTHADITJHABA	*Rhodanobacteraceae*	12	0
PHUTHADITJHABA	*Rhodomicrobiaceae*	18	0
PHUTHADITJHABA	*Rhodothermaceae*	9	0
PHUTHADITJHABA	*Rikenellaceae*	5285	2.7
PHUTHADITJHABA	*Ruminococcaceae*	29	0
PHUTHADITJHABA	*Sphingobacteriaceae*	61	0
PHUTHADITJHABA	*Spiroplasmataceae*	11	0
PHUTHADITJHABA	*Staphylococcaceae*	89	0
PHUTHADITJHABA	*Thermomonosporaceae*	50	0
PHUTHADITJHABA	*Trueperaceae*	31	0
PHUTHADITJHABA	*UCG-010*	640	0.3
PHUTHADITJHABA	*Xanthomonadaceae*	24	0
PHUTHADITJHABA	*Yersiniaceae*	105	0.1

LOCALITY	Genus	Abundance	Percent
PHUTHADITJHABA	*[Eubacterium] brachy group*	18	0
PHUTHADITJHABA	*[Eubacterium] nodatum group*	23	0
PHUTHADITJHABA	*[Ruminococcus] torques group*	47	0
PHUTHADITJHABA	*Actinomadura*	50	0
PHUTHADITJHABA	*Aerococcus*	44	0
PHUTHADITJHABA	*Akkermansia*	473	0.2
PHUTHADITJHABA	*Alistipes*	404	0.2
PHUTHADITJHABA	*Alloprevotella*	291	0.1
PHUTHADITJHABA	*Aminobacter*	22	0
PHUTHADITJHABA	*Anaplasma*	167810	85.4
PHUTHADITJHABA	*Aquabacterium*	1	0
PHUTHADITJHABA	*Arthrobacter*	44	0
PHUTHADITJHABA	*Bacteroides*	396	0.2
PHUTHADITJHABA	*Blastopirellula*	13	0
PHUTHADITJHABA	*Brevundimonas*	42	0
PHUTHADITJHABA	*Bryobacter*	15	0
PHUTHADITJHABA	*Candidatus Soleaferrea*	3	0
PHUTHADITJHABA	*Cellulomonas*	742	0.4
PHUTHADITJHABA	*Christensenellaceae R-7 group*	58	0
PHUTHADITJHABA	*Citricoccus*	8	0
PHUTHADITJHABA	*Corynebacterium*	134	0.1
PHUTHADITJHABA	*dgA-11 gut group*	417	0.2
PHUTHADITJHABA	*Dietzia*	30	0
PHUTHADITJHABA	*Dokdonella*	12	0
PHUTHADITJHABA	*Duganella*	277	0.1
PHUTHADITJHABA	*Ehrlichia*	147	0.1
PHUTHADITJHABA	*Escherichia-Shigella*	49	0
PHUTHADITJHABA	*Family XIII AD3011 group*	92	0
PHUTHADITJHABA	*Fibrobacter*	27	0
PHUTHADITJHABA	*Gallibacterium*	9	0

PHUTHADITJHABA	*Gemmatimonas*	25	0
PHUTHADITJHABA	*Haliangium*	12	0
PHUTHADITJHABA	*Incertae Sedis*	22	0
PHUTHADITJHABA	*Isoptericola*	73	0
PHUTHADITJHABA	*Janthinobacterium*	77	0
PHUTHADITJHABA	*Lachnospiraceae FCS020 group*	153	0.1
PHUTHADITJHABA	*Lachnospiraceae NK4A136 group*	34	0
PHUTHADITJHABA	*Limnobacter*	22	0
PHUTHADITJHABA	*Luteimonas*	2	0
PHUTHADITJHABA	*Lysobacter*	22	0
PHUTHADITJHABA	*Monoglobus*	45	0
PHUTHADITJHABA	*Mumia*	502	0.3
PHUTHADITJHABA	*Mycoplasma*	16058	8.2
PHUTHADITJHABA	*Oikopleura*	34	0
PHUTHADITJHABA	*Opitutus*	9	0
PHUTHADITJHABA	*Ornithinicoccus*	1	0
PHUTHADITJHABA	*Ornithinimicrobium*	16	0
PHUTHADITJHABA	*Oscillibacter*	15	0
PHUTHADITJHABA	*Paeniclostridium*	88	0
PHUTHADITJHABA	*Paludisphaera*	49	0
PHUTHADITJHABA	*Pedobacter*	61	0
PHUTHADITJHABA	*Pelagibacterium*	4	0
PHUTHADITJHABA	*Pelomonas*	64	0
PHUTHADITJHABA	*Phascolarctobacterium*	156	0.1
PHUTHADITJHABA	*Pir4 lineage*	9	0
PHUTHADITJHABA	*Planococcus*	11	0
PHUTHADITJHABA	*Planomicrobium*	41	0
PHUTHADITJHABA	*Pontibacter*	13	0
PHUTHADITJHABA	*Porphyromonas*	305	0.2
PHUTHADITJHABA	*Prauserella*	37	0
PHUTHADITJHABA	*Prevotella*	64	0
PHUTHADITJHABA	*Prevotellaceae Ga6A1 group*	27	0
PHUTHADITJHABA	*Prevotellaceae UCG-003*	518	0.3
PHUTHADITJHABA	*Prevotellaceae UCG-004*	165	0.1
PHUTHADITJHABA	*Pseudomonas*	53	0
PHUTHADITJHABA	*Rheinheimera*	29	0
PHUTHADITJHABA	*Rhodomicrobium*	18	0
PHUTHADITJHABA	*Rikenellaceae RC9 gut group*	4464	2.3
PHUTHADITJHABA	*Romboutsia*	213	0.1
PHUTHADITJHABA	*Serratia*	105	0.1
PHUTHADITJHABA	*SM1A02*	15	0
PHUTHADITJHABA	*Spiroplasma*	11	0
PHUTHADITJHABA	*Staphylococcus*	89	0
PHUTHADITJHABA	*Stenotrophobacter*	25	0
PHUTHADITJHABA	*Tetrasphaera*	14	0
PHUTHADITJHABA	*Tistlia*	39	0
PHUTHADITJHABA	*Truepera*	31	0
PHUTHADITJHABA	*Tundrisphaera*	15	0

PHUTHADITJHABA	*UCG-005*	673	0.3
PHUTHADITJHABA	*UCG-007*	17	0
PHUTHADITJHABA	*UCG-009*	151	0.1
PHUTHADITJHABA	*Variovorax*	65	0
PHUTHADITJHABA	*Alistipes_obesi*	22	0
PHUTHADITJHABA	*Anaplasma_boleense*	193	0.1
PHUTHADITJHABA	*Anaplasma_bovis*	5535	3
PHUTHADITJHABA	*Anaplasma_centrale*	11	0
PHUTHADITJHABA	*Anaplasma_marginale*	98610	54.2
PHUTHADITJHABA	*Anaplasma_ovis*	103	0.1
PHUTHADITJHABA	*Anaplasma_phagocytophilum*	27	0
PHUTHADITJHABA	*Anaplasma_platys*	59742	32.9
PHUTHADITJHABA	*Arthrobacter_citreus*	22	0
PHUTHADITJHABA	*Arthrobacter_gandavensis*	22	0
PHUTHADITJHABA	*Brevundimonas_vesicularis*	42	0
PHUTHADITJHABA	*Cellulomonas_cellasea*	614	0.3
PHUTHADITJHABA	*Cellulomonas_flavigena*	128	0.1
PHUTHADITJHABA	*Corynebacterium_resistens*	123	0.1
PHUTHADITJHABA	*Corynebacterium_tuberculostearicum*	11	0
PHUTHADITJHABA	*Dietzia_maris*	16	0
PHUTHADITJHABA	*Ehrlichia_canis*	97	0.1
PHUTHADITJHABA	*Janthinobacterium_lividum*	65	0
PHUTHADITJHABA	*Lysobacter_spongiicola*	22	0
PHUTHADITJHABA	*Mycoplasma_haemobos*	1890	1
PHUTHADITJHABA	*Mycoplasma_wenyonii*	14168	7.8
PHUTHADITJHABA	*Paludisphaera_borealis*	49	0
PHUTHADITJHABA	*Prauserella_isguenensis*	37	0
PHUTHADITJHABA	*Pseudomonas_monteilii*	5	0
PHUTHADITJHABA	*Pseudomonas_yamanorum*	48	0
PHUTHADITJHABA	*Serratia_liquefaciens*	105	0.1
PHUTHADITJHABA	*Spiroplasma_platyhelix*	11	0
PHUTHADITJHABA	*Staphylococcus_epidermidis*	89	0
PHUTHADITJHABA	*Tistlia_consotensis*	39	0

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Tick distribution and comparative analysis of bovine blood microbiome in two provinces of South Africa using 16S rRNA PacBio sequencing approach

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Introduction: Ticks are obligate ectoparasites recognized worldwide as major vectors of several disease-causing pathogens and are good indicators of disease distribution and epidemiology. Recent years have seen a growing concern regarding emerging and re-emerging of economically important tick-borne pathogens of livestock and humans worldwide. The overall objective of the study was to give an insight into current tick distribution and associated bacterial pathogens that may pose a threat to cattle in the sampled study sites.

Methods: A total of 150 cattle were randomly selected from three study sites, Harrismith and Phuthaditjhaba in Free State Province and Bergville in KwaZulu Natal Province, South Africa. Blood samples were collected from the cattle and DNA was subjected to the 16S rRNA gene microbiome sequencing on the circular consensus PacBio sequencing platform. Ticks were also collected from various predilection sites of the sampled animals.

Results: A total of eight tick species were identified and *Rhipicephalus evertsi evertsi* (79.4%) was the most abundant followed by *R. appendiculatus* (11.7%), *R. afranicus* (2.6%), *R. simus* (2.6%), *Hyalomma rufipes* (1.2%), *R. decoloratus* (1.0%), *H. truncatum* (0.7%) and *R. microplus* (0.7%). The bacterial microbiome sequence analysis revealed up to 16 phyla and 30 classes in the three study sites. Proteobacteria was the most dominant bacterial phyla with a relative abundance of 67.2% (Bergville), 73.8% (Harrismith) and 84.8% (Phuthaditjhaba), followed by Firmicutes at 9.6% (Phuthaditjhaba), 18.9% (Bergville) and 19.6% (Harrismith).

Conclusion and perspectives: The Chao 1 index estimator revealed significant differences in the α-diversity of microbial communities among three study sites. This study expands the knowledge on tick fauna and microbial communities in the three study sites.

KEYWORDS

PacBio, *Anaplasma*, Bergville, Harrismith, Phuthaditjhaba, ticks, 16S rRNA, South Africa

1 Introduction

Ticks are good indicators of disease distribution and epidemiology (1) and are widely distributed particularly in tropical and subtropical areas. Their distribution is dependent on the environment and host for growth and survival (2). Various studies have shown that cattle and other animal species are mostly infested by ticks, transmitting several pathogens (3–10). Ticks transmit obligate intracellular Gram-negative bacterial species of animal and human importance in the order *Rickettsiales* such as *Ehrlichia chaffeensis*, *E. ruminantium* the causative agent of heartwater in ruminants (11), *Anaplasma phagocytophilum* that causes human granulocytic anaplasmosis, and *A. marginale* and *A. centrale* that cause anaplasmosis (gallsickness) in ruminants (12). The other pathogens are *Borrelia duttonii* which causes tick-borne relapsing fever and *Rickettsia* species responsible of causing rickettsioses (13), while *Coxiella burnetii* causes Query fever (Q-fever) in humans or coxiellosis in domestic animals (14). Apart from pathogen transmission, ticks can damage hides, cause blood loss, severe allergic reactions, immune-depression, irritation, and chronic stress (15).

There is a growing concern of emerging and re-emerging tick-borne pathogens of public health concern and of economic significance amongst livestock, but data from African ecosystems is still scanty. The most affected areas are resource-poor, where factors such as poor veterinary services, inability of the farmers to purchase veterinary medicines, uncontrolled translocation of livestock, the wildlife-livestock interface, and burning of grazing land promote emergence and re-emergence of tick-borne pathogens in an environment where climate change is notable (16). Among the few microbiome studies conducted in South Africa, Kolo et al. (17) investigated possible causes of acute febrile illness and found *A. phagocytophilum*, *A. platys* and *Anaplasma* sp. SA/ZAM dog DNA in blood of rodents, dogs, cattle, and humans. In another study, Makgabo et al. (18) revealed the presence of nine novel *Anaplasma* 16S rRNA genotypes in 11 wild animal species, highlighting the importance of emerging tick-borne pathogens among communities in South Africa.

Global warming has effected the change in the climate and disrupted the ecosystems in agriculture. Consequently, tick distribution and the pathogens they transmit. The other factor is the competence of invasive tick species. The invasive ticks, such as the one-host tick, *R. microplus* and the three-host tick, *A. variegatum* have high adaptability and capacity to transmit several pathogens. In South Africa, *R. microplus*, is the vector of *Babesia bigemina*, *B. bovis* and *A. marginale*. This tick has been reported to displace the indigenous tick, *R. decoloratus*, in South Africa (7, 19). *Amblyomma variegatum* is prevalent in various regions within Africa and is considered as the second most invasive tick species after *R. microplus* (20). It is the vector of *E. ruminantium*.

Tick distribution has been used as an indication of tick-borne disease distribution, thus demonstrating the presence of various pathogens. However, the aforementioned factors such as climate change, the adaptation of invasive tick species and translocation of animals indicate that the tick distribution might not be a concise marker of the distribution of disease. For instance, the vector may be present, but the pathogen might either not have been reported or the vector might have been displaced by invasive tick species, while other tick-borne pathogens might be concurrently transmitted by other vectors such as biting flies or through contaminated veterinary instruments (21, 22).

In this study, three neighboring small towns: Bergville in KwaZulu Natal Province, Harrismith and Phuthaditjhaba in the Free State Province, South Africa were targeted for the surveillance of ticks and tick-borne pathogens. These towns are separated by the Drakensberg Mountains that serve as the grazing areas for the livestock. Between the three towns, there is a constant movement of livestock for cultural practices, breeding for beef production and barter trade. Tick distribution studies on cattle have previously been carried out to catalog ticks and tick-borne pathogens in Phuthaditjhaba and Harrismith (9, 14, 23, 24). In Bergville, however, only one study has been carried out thus far (25). The studies were conducted eight years ago in the Free State and five years ago in KwaZulu-Natal.

In view of the above, this study sought to determine whether there have been alterations in tick distribution due to factors such as climate change, livestock translocation, and vector displacement and to further determine associated bacterial pathogens and conduct a comparative analysis of cattle blood microbiome present at the three study sites.

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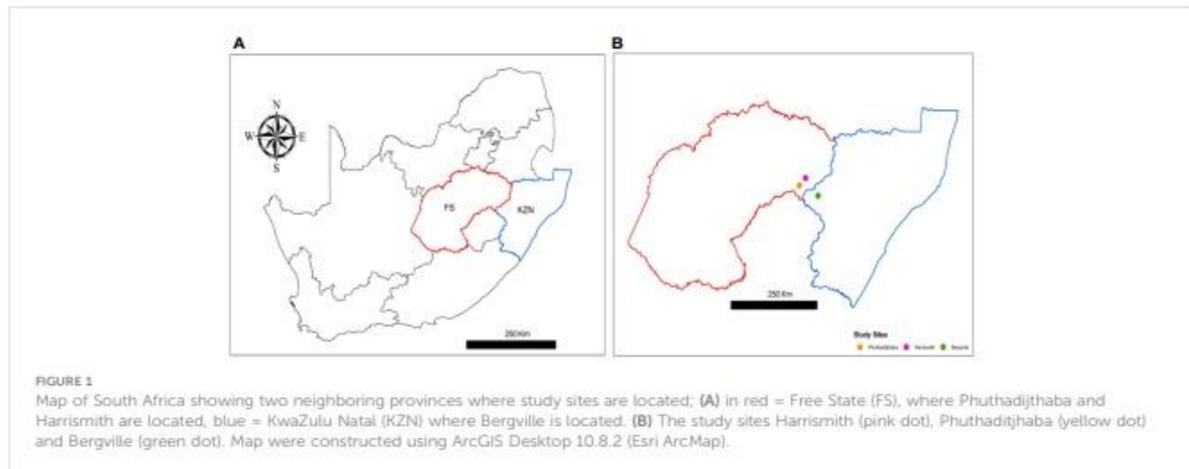

FIGURE 1
Map of South Africa showing two neighboring provinces where study sites are located; **(A)** in red = Free State (FS), where Phuthadijthaba and Harrismith are located, blue = KwaZulu Natal (KZN) where Bergville is located. **(B)** The study sites Harrismith (pink dot), Phuthaditjhaba (yellow dot) and Bergville (green dot). Map were constructed using ArcGIS Desktop 10.8.2 (Esri ArcMap).

2 Materials and methods

2.1 Ethics approval

The study was approved by the Animal Ethics Committee of the University of Pretoria, South Africa (REC029–21), and collection of cattle samples was approved by the Department of Agriculture, Land Reform and Rural Development under section 20 of the *Animal Disease Act 35* of 1984 with reference 12/11/1/1/MG.

2.2 Study area

The study was conducted in three neighboring sites in South Africa, one site in KwaZulu-Natal Province (Bergville) and two in the Free State Province (Harrismith, Phuthaditjhaba), from 29 October through 01 November 2021 (Figure 1). The three sites are rural and demarcated by Drakensberg mountains.

There is regular movement of cattle among the three areas. The climate in Bergville is warm and temperate, with temperature varying from 3°C to 28°C (average 15°C). January is the warmest month of the year, with an average temperature of 20.1°C. The summer months (December to March) are wetter than the winters and the annual rainfall is 1657 mm. The driest month is June with 19 mm and the greatest amount of precipitation occurs in January, with an average of 312 mm. Average temperatures in October and November are 17.1°C and 18.5°C, while average rainfall is 148 mm and 196 mm, respectively (https://en.climate-data.org/africa/south-africa/kwazulu-natal/bergville-14872/).

In Harrismith, the temperature typically varies from -1°C to 26°C. The warm season is from October through March and the cold season is from May through August. The rainy period is from August through May, with a sliding 31-day rainfall of at least 12.5 mm. Average temperatures in October and November are 16.3°C and 17.3°C, while average rainfall is 94 mm and 128 mm, respectively (https://en.climate-data.org/africa/south-africa/free-state/harrismith-12770/).

The climate in Phuthaditjhaba is mild, and generally warm and temperate. The temperature averages 13.4°C (monthly average range 7°C to 17.8°C). Precipitation is about 1020 mm (monthly average range 12 mm to 184 mm). The summer months are December, January, February and March. Average temperatures in October and November are 15.2°C and 16.4°C, while average rainfall is 109 mm and 135 mm, respectively (https://en.climate-data.org/africa/south-africa/free-state/phuthaditjhaba-55825/).

2.3 Collection and identification of adult ticks

Representative adult tick samples were collected from the predilection sites: ears, dewlap, tail switch, back, groin, udder, around anus, lower abdomen of the 150 cattle from the three study sites. The ticks were kept in airtight containers with moist cotton wool prior to identification using a stereoscopic microscope at the Ectoparasitology laboratory in the Department of Veterinary Tropical Diseases, University of Pretoria, South Africa, and using morphological keys described by Walker (26).

2.4 Blood sample collection

A total of 150 cattle from the three sites were enrolled for the study. A herd of fifty cattle were randomly selected from each of the three sampling sites, at their respective communal sites or dip tanks. Cattle were apparently healthy but infested with ticks, predominantly adults of both sexes and of Nguni breed (Bergville and Harrismith) or Brahman breed (Phuthaditjhaba). In Harrismith, communal dipping programs are lacking, with the last dipping conducted four months preceding this study (June 2021). However, some farmers, despite limited resources, resorted to alternative methods such as cattle sprays and injectable solutions for tick control. In Phuthaditjhaba, pour-on dipping is the method used every 3–4 months. In Bergville, dipping takes place twice a month at the Woodford dip tank.

Blood samples were collected from the coccygeal vein of individual animals into 10-ml sterile EDTA vacutainer tubes. The blood was stored at 4°C until further analysis.

2.5 DNA extraction, 16S rRNA gene amplification, and PacBio sequencing

Genomic DNA was extracted from 200 µl of blood using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The presence of bacterial communities in the blood samples was analyzed using single molecule real-time PacBio sequencing technology (Pacific Biosciences, Menlo Park, CA, USA). The full-length 16S rRNA gene was amplified from genomic DNA using bacterial-specific primer 27F 5'-AGA GTT TGA TCM TGG CTC AG-3' and 1492R 5'-TAC GGY TAC CTT GTT ACG ACT T-3'. For multiplexing of amplicons, the 5`-ends of the 16S rRNA forward and reverse primers were tagged with the universal M13F (TGTAAAACGACGGCCAGT) and M13R (GGAAACAGCTATGACCATG) sequences respectively. Two amplification reactions contained water as a template were used as negative controls. SMRTbell libraries were created using SMRTbell™ Template Prep Kit 1.0 (Pacific Biosciences, CA, USA) following instructions in the protocol "Procedure & Checklist – Amplicon Template Preparation and Sequencing" (part number 100–801-600–04). Sequencing was done using the Sequel® Sequencing Kit 2.1 (Pacific Biosciences, CA, USA) with on-plate loading concentration of 4 pM.

2.6 Sequence analysis using divisive amplicon denoising algorithm 2 workflow

The DADA2 package (version 1.12.1) analysis workflow implemented in R statistical software (v3.6.1) was used to analyze raw amplicon sequencing data generated using the PacBio Sequel System (Pacific Biosciences, CA, USA). To infer amplicon sequence variants, error-model learning, and chimera removal were performed on the filtered reads using default DADA2 parameters. Taxonomic assignments were made based on the curated SILVA 16S rRNA database (27). Taxa and abundance tables generated by DADA2 were imported into the phyloseq package v1.28.0 (28) for downstream analysis and visualization, including estimation of richness and visualization of the alpha-diversity, as well as visualization of differences in taxa abundance between the three study sites.

2.7 Statistical analysis

Effect sizes of the differences among the three study sites were calculated using the Cohen's D measure using the effsize package in R (https://github.com/mtorchiano/effsize), based on Shannon diversity indices. Ordinations for β-diversity between study sites were estimated using Principal Coordinate Analysis (PCoA) based on weighted-UniFrac distance and Non-Metric Multidimensional Scale (NMDS) using Bray distance metric as implemented in the plot_ordination and amp_ordinate functions in PhyloSeq and the ampvis2 package (https://madsalbertsen.github.io/ampvis2/articles/ampvis2.html), respectively. Permutational Multivariate Analysis of Variance (PERMANOVA) using permutation test with pseudo-F ratios as implemented in the Adonis function of the Vegan R package (https://github.com/vegandevs/vegan) was used to determine significance for sample clustering on ordination plots.

We assessed the difference in relative abundance by family, genus, order and species among the three localities using the Kruskal-Wallis test. Analyses were performed in R statistical software (29) at a significant level of 0.05.

3 Results

3.1 Distribution of tick species among cattle in the three study sites

A total of 418 ticks were collected from 150 cattle in the three study sites (Harrismith, Phuthaditjhaba, Bergville). Eight tick species were identified, and the most abundant species in all study sites was *R. evertsi evertsi* (79.4%), followed by *R. appendiculatus* (11.7%), *R. simus* (2.6%) and *R. afranicus n.* sp. (formerly *R. turanicus*) (2.6%) (Table 1). *Rhipicephalus simus* and *R. afranicus* were found in Phuthaditjhaba

TABLE 1 Identification and distribution of ticks collected from cattle in three study sites of South Africa.

	Number of ticks collected from the three study sites			
Tick species	Total (proportion) 418	Harrismith 126	Phuthaditjhaba 160	Bergville 132
Hyalomma rufipes	5 (1.2%)	4 (3.2%)	1 (0.6%)	0
Hyalomma truncatum	3 (0.7%)	2 (1.6%)	0	1 (0.8%)
Rhipicephalus decoloratus	4 (1.0%)	4 (3.2%)	0	0
Rhipicephalus evertsi evertsi	332 (79.4%)	113 (89.7%)	89 (54.3%)	130 (98.5%)
Rhipicephalus microplus	3 (0.7%)	3 (2.4%)	0	0
Rhipicephalus appendiculatus	49 (11.7%)	0	48 (29.3%)	1 (0.8%)
Rhipicephalus simus	11 (2.6%)	0	11 (6.7%)	0
Rhipicephalus afranicus n. sp	11 (2.6%)	0	11 (6.7%)	0

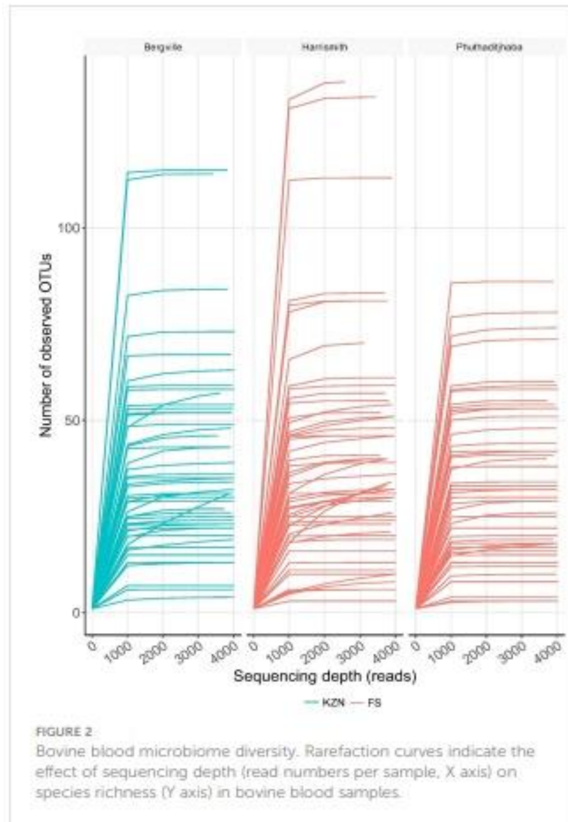

FIGURE 2
Bovine blood microbiome diversity. Rarefaction curves indicate the effect of sequencing depth (read numbers per sample, X axis) on species richness (Y axis) in bovine blood samples.

and not in other locations. Of the 49 *R. appendiculatus* ticks collected, 48 were found in Phuthaditjhaba and only one tick found in Bergville (Table 1). *Rhipicephalus microplus* and *R. decoloratus* were not found in Bergville (Table 1).

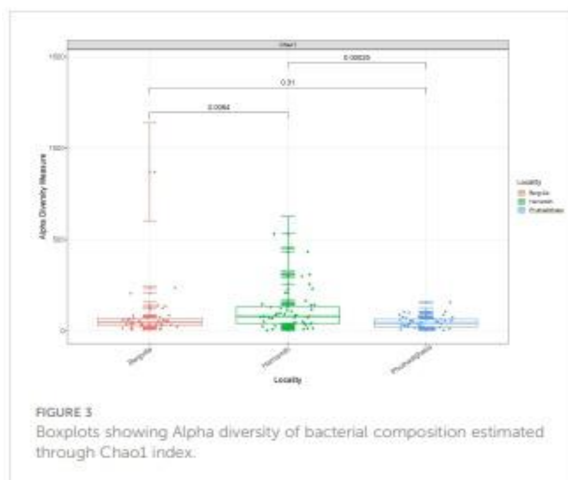

FIGURE 3
Boxplots showing Alpha diversity of bacterial composition estimated through Chao1 index.

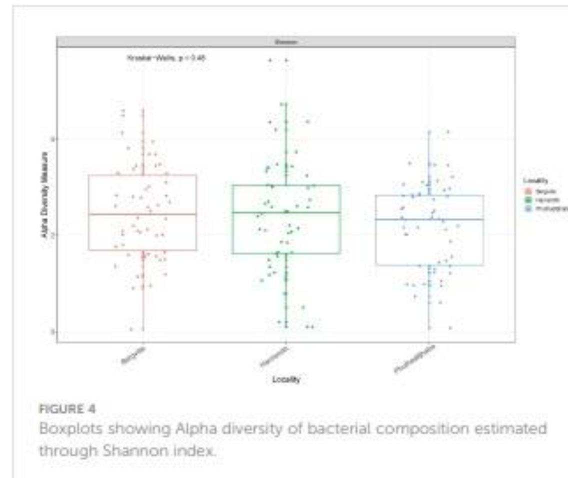

FIGURE 4
Boxplots showing Alpha diversity of bacterial composition estimated through Shannon index.

3.2 Microbial diversity in cattle samples collected from Harrismith, Phuthaditjhaba, and Bergville

The mean species diversity of bacterial populations detected in the bovine samples plotted using the rarefaction curves showed that the sequencing depth was adequate to capture the existing microbial diversity (Figure 2).

The alpha diversity boxplots reflected the minimal, median, degree of dispersion, maxima and outliers of microbial diversity within study sites. The alpha diversities were estimated through Chao1 index, which measures species richness and the Shannon index, that measures both species richness and evenness (30). The microbial diversity of samples from Harrismith indicated significantly higher species richness than Bergville ($p = 0.0064$) and Phuthaditjhaba ($p = 0.00025$) samples as determined using the Chao1 index estimator (Kruskal-Wallis: $p = 0.00065$) (Figure 3). However, the differences in species diversity between the three-study site was insignificant as per the Shannon estimator (Kruskal-Wallis: $p = 0,48$) (Figure 4). The effect size measurements (Table 2) were small to evaluate differences between microbial communities present in the three study sites. Using the pairwise analysis, the α-diversity varied significantly in microbial communities between Bergville and Harrismith and between Harrismith and Phuthadijthaba based on Chao 1 index estimator ($p = 0.0064$ and $p = 0,00025$, respectively). In contrast, there was no significant difference between Bergville and Phuthaditjhaba microbial communities based on Chao 1 index estimator ($p = 0.31$).

Furthermore, microbial composition dissimilarities among the three study sites were analyzed through PCoA plots. The plots showed one cluster of microbial communities using the weighted UniFrac distance metric on PCoA, which considers abundance and the phylogenetic distance between ASVs. Three clusters indicative of the study sites could be observed (PCoA persanova). The Bergville sample composition was divergent from the Harrismith and Phuthaditjhaba groups: however, the Phuthaditjhaba and Harrismith groups clustered quite closely (Figure 5).

TABLE 2 Effect sizes of the differences between the three study sites measured using the Cohen's D with a confidence interval of 95%.

Comparison	Estimate	Standard deviation	Confidence level	Upper confidence interval	Lower confidence interval	Variance
Harrismith vs Phuthaditjhaba	0.04	1	0.95	0.44	-0.36	0.04000786
Harrismith vs Bergville	-0.18	1.04	0.95	0.21	-0.58	0.0401711
Phuthaditjhaba vs Bergville	-0.25	0.94	0.95	0.15	-0.65	0.04030784

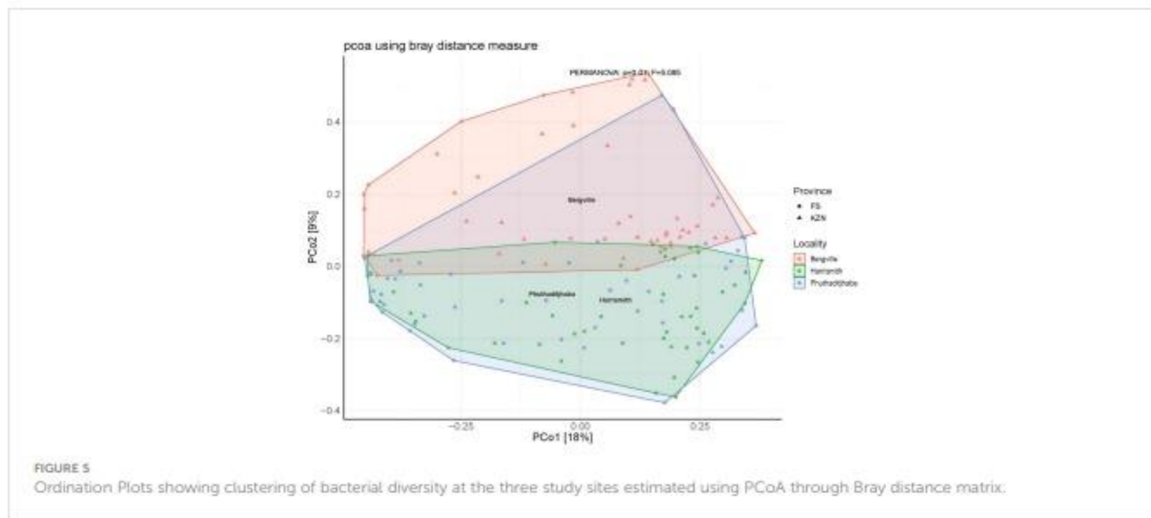

FIGURE 5
Ordination Plots showing clustering of bacterial diversity at the three study sites estimated using PCoA through Bray distance matrix.

Overall, 16 phyla, 30 classes, 71 order, 114 families, 159 genera and 71 species were identified from the three sites (Supplementary Table S1). At the phylum level, the composition of bovine blood microbiomes was dominated by Proteobacteria with a relative abundance of 67.2% (Bergville), 73.8% (Harrismith) and 84.8% (Phuthaditjhaba), followed by Firmicutes at 9.6% (Phuthaditjhaba), 18.9% (Bergville) and 19.6% (Harrismith). Bacteroidota was the third most abundant at 4% (Phuthaditjhaba), 5.3% (Harrismith) and 10.2% (Bergville) (Figure 6; Supplementary Table S2). The relative abundance of the Proteobacteria, Firmicutes and Bacteroidota remained higher in Phuthaditjhaba, Harrismith and Bergville, respectively (Supplementary Table S2). The phyla Fusobacteriota and Spirochaetota were only found in Bergville, while Deinococcota was only found in Phuthaditjhaba (Supplementary Table S2). About half of the detected phyla had a relative abundance of ≥0.1% in the various microbiomes (Bergville=7/13, Harrismith=5/11, Phuthaditjhaba=8/13).

The most dominant classes in the Bergville, Harrismith and Phuthaditjhaba microbiomes were Alphaproteobacteria (64.8, 72.4%, 84.4%), Bacilli (14.3%, 17.5%, 8.2%) and Bacteroidia (10.2%, 5.3%, 4%) (Supplementary Table S2). Class Spirochaetia was unique to the Bergville microbiome, while classes Blastocatellia, Cynobacteria, Deinococei and Phycisphaerae were unique to the Phuthaditjhaba microbiome (Supplementary Table S2). About half of the detected classes had relative abundance of ≥0.1% in Bergville (11/20), Harrismith (8/20) and Phuthaditjhaba (12/23) (Supplementary Table S2).

The composition of bovine blood microbiomes at the order level in Bergville, Harrismith and Phuthaditjhaba was numerically dominated by Rickettsiales (64.7%, 72%, 84.3%), respectively, followed by Mycoplasmatales (11%, 17.3%, 8.1%) and Bacteroidales (10.2%, 5%, 4%) (Supplementary Table S2). There was a significant difference in the relative abundance among the three study sites (Kruskal Wallis test, p=0.0281). At least 46 orders of bacteria were identified in each of the three microbiomes, of which 13.0% (6/46), 16.0% (8/50) and 19.6% (10/51) were exclusively unique in the Bergville, Harrismith and Phuthaditjhaba microbiome, respectively (Supplementary Table S2). There were more orders with relative percent abundance ≥0.5% in the Bergville microbiome (12/46) than in the Harrismith (6/50) and Phuthaditjhaba (5/51) (Supplementary Table S2).

The unique and shared distribution of bacterial families, genera and species found in the Phuthaditjhaba, Harrismith and Bergville samples are shown by comprehensive Venn diagrams (Figures 7A–C). We detected 114 bacterial families, of which 69, 77 and 81 were found in Phuthaditjhaba, Bergville and Harrismith microbiome, respectively (Figures 7A–C; Supplementary Table S2). Moreover, 41

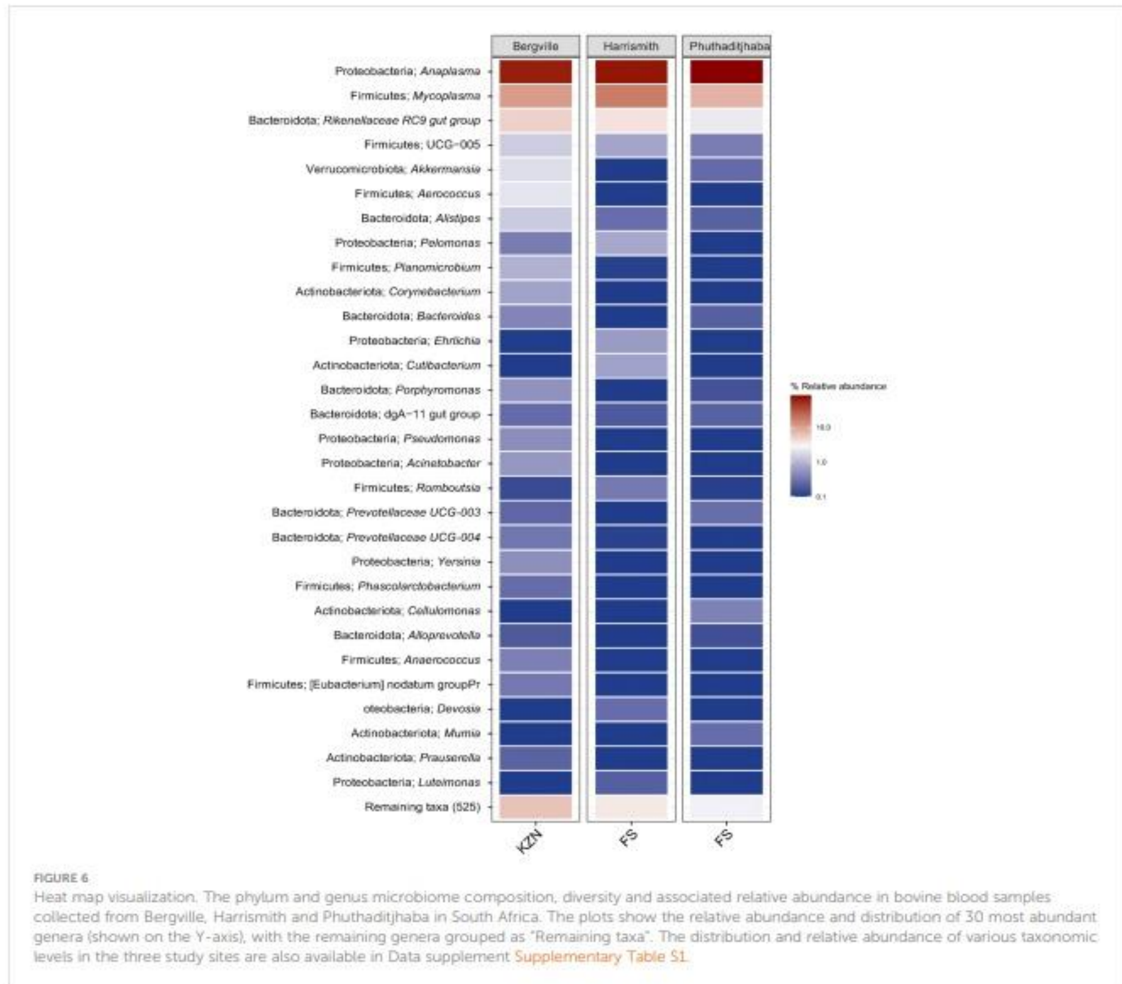

FIGURE 6
Heat map visualization. The phylum and genus microbiome composition, diversity and associated relative abundance in bovine blood samples collected from Bergville, Harrismith and Phuthaditjhaba in South Africa. The plots show the relative abundance and distribution of 30 most abundant genera (shown on the Y-axis), with the remaining genera grouped as "Remaining taxa". The distribution and relative abundance of various taxonomic levels in the three study sites are also available in Data supplement Supplementary Table S1.

bacterial families were shared across the three study sites (Figure 7A). The Phuthaditjhaba microbiome had an exclusively unique association with 13 bacterial families, while 12 and 17 bacterial families were uniquely present in Bergville and Harrismith bovine blood samples (Figure 7; Supplementary Table S2). There was significant variation in the relative percent abundance (Kruskal-Walli's test, p =0.0045) of the bacteria at the family level among the three study sites. More than 80% of the bacterial microbiome in Bergville, Harrismith and Phuthaditjhaba was largely dominated by members of three families: *Anaplasmataceae* (65.0%, 72.1%, 84.7%, respectively), *Mycoplasmataceae* (11.1%, 17.4%, 8.1%) and *Rikenellaceae* (6.6%, 4.3%, 2.7%). There were more families with relative percent abundance ≥0.5% in the Bergville microbiome (14/77) than in the Harrismith (7/81) and Phuthaditjhaba (4/69).

Overall, we detected 159 microbiome genera, of which 94, 108 and 82 were found in Bergville, Harrismith and Phuthaditjhaba, respectively (Figure 7B). Forty-two genera were shared across the three study sites (Figure 7B), while 23, 31 and 22 exclusively associated microbiome genera were found in the Bergville, Harrismith and Phuthaditjhaba, respectively (Figure 7B; Supplementary Table S2). There were significant differences in the relative percent abundance (Kruskal-Wallis test, P=0.00042) of the bacteria at the genus level in the locality microbiome. *Anaplasma* was the most abundant bacterial genus in the Phuthaditjhaba, Harrismith and Bergville samples, with a relative abundance of 85.4%, 72.4% and 67.1%, respectively. This was followed by *Mycoplasma* (8.2%, 17.6% and 11.4%, respectively) (Supplementary Table S2). The Phuthaditjhaba microbiome was also predominated with *Rikenellaceae RC9 gut group* (2.3%). Other abundant genera in the Bergville blood microbiome were *Rikenellaceae RC9 gut group* (5.2%), *Aerococcus* (2.1%), *Akkermansia* (1.9%), *Alistipes* (1.4%), *UCG-005* (1.4%), *Planomicrobium* (0.9%), *Corynebacterium* (0.7%), *Acinetobacter* (0.6%), *Porphyromonas* (0.5%) and *Pseudomonas* (0.5%). Other top abundant genera in the Harrismith microbiome were *Pelomonas* (0.7%), *Cutibacterium* (0.7%), *UCG-005* (0.6%) and *Ehrlichia* (0.6%) (Supplementary Table S2). The rest

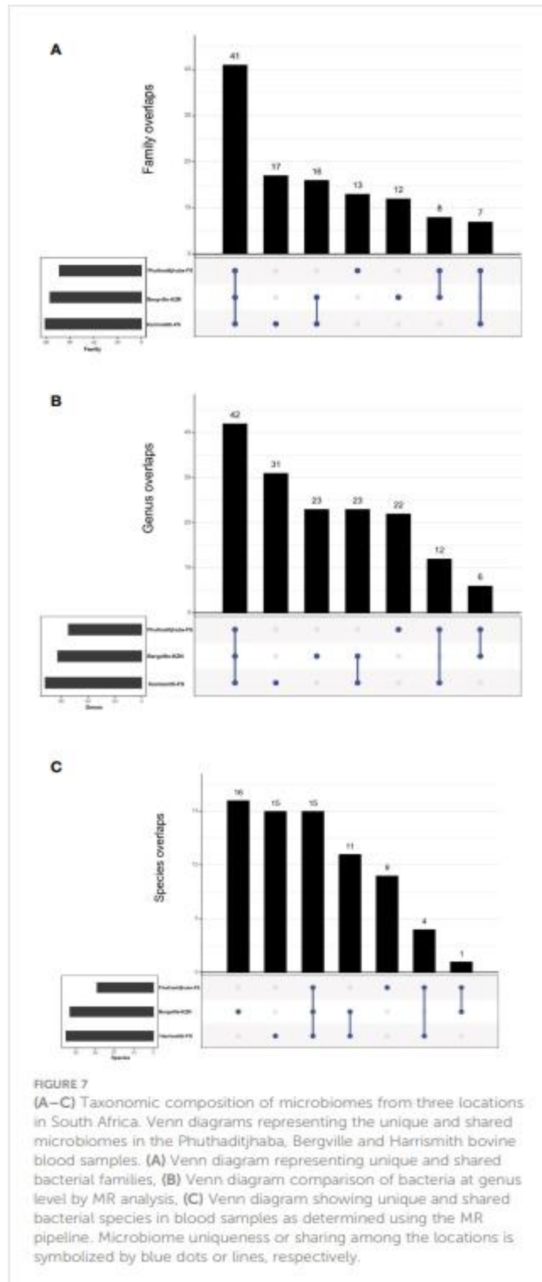

FIGURE 7
(A–C) Taxonomic composition of microbiomes from three locations in South Africa. Venn diagrams representing the unique and shared microbiomes in the Phuthaditjhaba, Bergville and Harrismith bovine blood samples. (A) Venn diagram representing unique and shared bacterial families, (B) Venn diagram comparison of bacteria at genus level by MR analysis, (C) Venn diagram showing unique and shared bacterial species in blood samples as determined using the MR pipeline. Microbiome uniqueness or sharing among the locations is symbolized by blue dots or lines, respectively.

of the genera (82, 102, 79) had <0.5% relative abundance in the three study sites (Bergville, Harrismith, Phuthaditjhaba, respectively) (Supplementary Table S2).

We investigated the species-level differences of microbial communities across the three study sites, which showed no significant differences (Kruskal Wallis test, p=0.081) in the microbiome relevant abundance (Supplementary Table S2). Of the 71 detected species (159 genera), 12.67%, 22.54% and 21.13% had a sole association with Phuthaditjhaba, Bergville and Harrismith study sites respectively (Figure 7C). *Anaplasma marginale* (relative abundance 56.2%, 43.5%, 54.2%, respectively) was the most abundant bacterial pathogen in the Bergville, Harrismith and Phuthaditjhaba samples, followed by *A. platys* (22.6%, 31.5%, 32.9%) and *Mycoplasma wenyonii* (14%, 19.6%, 7.8%) (Supplementary Table S2). Other predominant species in Bergville bovine blood microbiome were *Aerococcus vaginalis* (2.6%), *Acinetobacter lwoffii* (0.6%), *Pseudomonas poae* (0.5%) and *Yersinia pestis* (0.5%). In contrast, *Anaplasma bovis* (1.3%), *Anaplasma centrale* (0.9%), *Anaplasma ovis* (0.8%), *Cutibacterium namnetense* (0.8%) and *Ehrlichia canis* (0.6%) were the other relatively predominant species in Harrismith. Moreover, *Anaplasma bovis* (3.0%) and *Mycoplasma haemobos* (1.0%) were the other predominant bacterial species in Phuthaditjhaba (Supplementary Table S2). The rest of the species identified in these samples had relative abundance lower than 0.5% (Supplementary Table S2). The presence of few predominating bacterial genera in the three study sites could have suggested that these differences might also occur in composition at the species level, but instead most of the genera identified in each microbiome were represented on average by two species.

4 Discussion

The most abundant tick species collected in the three study sites was *R. evertsi evertsi* (78.7%). The tick species is the most widespread rhipicephalid in Africa (26), and transmits the bacterium *A. marginale* (31), the main cause of anaplasmosis in cattle, as well as protozoan parasites *Theileria equi* and *Babesia caballi* (32), which cause piroplasmosis in horses, and induces tick paralysis in sheep (33). This was not surprising as previous studies have shown that the tick is widely distributed (26) and is the most prevalent tick species present in different parts of South Africa (6, 14, 24). Cattle sampled in Bergville belonged to different farmers under one systematic dipping regimen. This could mean that *R. evertisi evertsi* is resistant to the dipping chemicals used at these sites. In reference to a pilot study conducted in Bergville in March 2015 at the same dip tanks (25), the most prevalent tick species was also *R. evertsi evertsi* (55%). In the Free State, *R. evertsi evertsi* was identified to be 44.7% and was prevalent in cattle over a period of two years (34). In the present study, Phuthadijthaba had the most diverse tick species, namely *R. evertsi evertsi, H. rufipes, R. decoloratus, R. appendiculatus, R. simus* and *R. africanus*, followed by Harrismith which had five tick species, namely *H. rufipes, H. truncatum, R. decolaratus* and *R. microplus*, in addition to *R. eversti evesti*, while Bergville had the least tick species diversity, with *H. truncatum* and *R. appendiculatus* as well as *R. evertsi evertsi*. Other relatively abundant tick species were *R. appendiculatus, R. simus* and *R. afranicus*. *Rhipicephalus appendiculatus* was the second most collected tick and was present in Phuthaditjhaba and Bergville. *Rhipicephalus appendiculatus* transmits *Theileria parva*, the main causative agent of Corridor disease in cattle, *T. taurotragi* that

causes benign bovine theileriosis and *A. bovis* and *R. conorii.* The latter causes tick typhus in humans. The presence of this tick was not recorded in the eastern Free State during a 3-year long tick survey study (23) and during the following years (9, 35). Whereas in Bergville it was identified in 2017 (25). *Rhipicephalus simus* transmits *A. centrale*, the cause of subacute bovine anaplasmosis.

Rhipicephalus afranicus n sp., is a recently described species in South Africa (36) and was subsequently identified in Uganda (37). The genetic divergence between the Ugandan and Southern African sequences is 2.4%, demonstrating that two different populations of this species may be occurring between Southern and East Africa (37). A study conducted in Israel speculate that this tick species could be the source of transmission for *A. bovis* causing bovine anaplasmosis in cattle (36). In our knowledge, this study is the first to report the presence of *Rhipicephalus afranicus* n sp. in Phuthaditjhaba.

Only three *R. microplus* ticks were collected, and this was in Harrismith. The tick is an invasive species of Asian origin and considered one of the most widespread ectoparasites of livestock. This tick has been recovered in the Free State Province from very few sites including wild animals (9, 19, 35). This is an interesting finding from this study, and it has been found in one locality (Harrismith) because there are very few reports of this tick species in the Free State province. Therefore, considering its high dispersal rate as reported in South-East Africa (19, 38), the occurrence of *R. microplus* as determined by this study warrants further investigation about its distribution in the Free State.

The tick species identified in this study are associated with transmission of bacterial pathogens: *Anaplasma, Rickettsia* and *Erhlirchia.* The bacterial microbiome in Bergville, Harrismith and Phuthaditjhaba was largely dominated by members of three families: *Anaplasmataceae* (65.0%, 72.1%, 84.7%, respectively), *Mycoplasmataceae* (11.1%, 17.4%, 8.1%) and *Rikenellaceae* (6.6%, 4.3%, 2.7%). The high proportion of *Anaplasmataceae* is in correlation with the abundance of biological vectors present at the three study sites. The latter two families are associated with respiratory and gastrointestinal tract microbiomes in various animals.

Anaplasma species are widespread in South Africa, with a seemingly wide host range. *Anaplasma marginale* was the most abundant species in the three study sites, confirming that the ticks present are vectors of this pathogen. The second most abundant pathogen was *A. platys*, which has previously been detected in ruminants' blood (39, 40). A recent 16S microbiome analysis revealed the presence of both species and nine novel species in nine different free-roaming wildlife species (African buffalo, impala, kudu, zebra, warthog, hyena, leopard, lion, and elephant) in the Kruger National Park and surroundings game reserves (18). *Anaplasma platys* is an emergent zoonotic pathogen (41, 42) that causes canine cyclic thrombocytopenia (43) and the presence of this species in cattle may pose a threat to human health. The pathogen has a worldwide distribution, and the suspected tick vector is *R. sanguineus sensu lato* ticks (44). The pathogen was detected in 12 African tick species in seven African countries in all regions (45), which may imply that various tick species are involved in its epidemiology. Detection of *A. platys* in cattle can be attributed to the close association of dogs with cattle in the study areas. Given the widespread occurrence of *R. sanguineus s.l*, this is likely to increase the risk of infections in humans. Further studies on the occurrence of *platys* in other mammalian and tick and evaluation of the vectorial capacity of tick species can assist in elucidating the epidemiology of *A. platys.* The other species found in the present study was *M. wenyonii*, whose mode of transmission is unknown, but presumably through mechanical transmission by blood-sucking arthropods (46). Many cattle infected with *M. wenyonii* do not exhibit clinical illness, unless is rare when concurrent illness results in immunosuppression (46, 47).

High abundance of *A. marginale*, the main cause of bovine anaplamsosis, can be attributed to the various tick species involved in transmission (*Rhipicephalus, Dermacentor, Ixodes, Hyalomma*) and other means of transmission, namely mechanical transmission by contaminated fomites and biting flies and the transplacental route (48, 49). A recent study involving African ticks showed that the pathogen was detected in 17 species of the genera *Amblyomma, Rhipicephalus* and *Hyalomma* (45). High *A. marginale* abundance in the study area may increase selection for genetically distinct strains and thus further complicating the efforts towards the development of a safe and effective vaccine. *Rikenellaceae* RC9 gut group, belonging to the *Rikenellaceae* family, plays an important role in the digestion of crude fiber (50). Therefore, the presence of the *Rikenellaceae* RC9 gut group indicates the roughage diet consumed by cattle in the three study sites. The presence of this bacteria could suggest that while drawing blood from the caudal vein there were remnants of feaces that got into the vacutainer.

5 Conclusions

We determined tick species distribution and abundance in three selected study sites in two provinces of South Africa, and established diversity of bacterial tick-borne pathogens from blood of cattle using the 16S rRNA PacBio sequencing method. *Rhipicephalus evertsi evertsi*, followed by *R. simus* and *R. appendiculatus* were the most abundant tick species. High abundance of *R. evertsi evertsi* has important implications for the transmission and occurrence of bovine anaplasmosis and equine piroplasmosis. Effective control of the ticks will minimize severe disease cases, taking into consideration the development and spread of acaricide resistance and maintenance of endemic stability. There was a high diversity of tick-borne bacterial pathogens in the blood of cattle, that can be attributed to emergence and change in distribution of pathogens as well close interactions among domestic and peri-domestic animals and humans. *Anaplasma marginale* and *A. platys* were the most abundance tick-borne bacterial pathogens, highlighting the risk of anaplasmosis in cattle, dogs, and humans. Control of anaplasmosis requires consideration of various factors such as climate change, acaricide resistance and animal movements.

6 Limitations of the study

Few cattle were sampled from a limited number of locations; however, given the common practice of communal grazing, it is probable that other cattle in and around the sampled locations harbor similar pathogens, as observed in this study.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, PRJNA1031221.

Ethics statement

The animal studies were approved by Animal Ethics Committee of the University of Pretoria, South Africa. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

BK: Formal analysis, Writing – original draft, Investigation. CB: Formal analysis, Writing – original draft, Methodology, Writing – review & editing. SM: Formal analysis, Writing – review & editing. NN: Writing – review & editing, Methodology. TM: Writing – review & editing. SN: Writing – review & editing, Methodology. MO: Writing – review & editing, Conceptualization. ZM: Conceptualization, Writing – review & editing, Formal analysis, Funding acquisition, Methodology, Writing – original draft.

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Acknowledgments

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fitd.2024.1399364/full#supplementary-material

SUPPLEMENTARY TABLE 1
16S combined rank totals.

SUPPLEMENTARY TABLE 2
Locality abundances.

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Appendix 3. Research Ethics Approval Certificate

Faculty of Veterinary Science
Research Ethics Committee 15 July 2024

LETTER OF APPROVAL

Ethics Reference No	**REC029-21**
Protocol Title	**Detection of bacterial tick-borne pathogens in two provinces of South Africa using a microbiome sequencing approach**
Principal Investigator	**Miss BL Khoza**
Supervisors	**Prof MC Oosthuizen** **Miss ZT Khumalo**

Dear Miss BL Khoza,

We are pleased to inform you that your submission conforms to the requirements of the Faculty of Veterinary Sciences Research Ethics committee.

Please note the following about your ethics approval:

1. Please use your reference number (REC029-21) on any documents or correspondence with the Research Ethics Committee regarding your research.
2. Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
3. Please note that ethical approval is granted for the duration of the research as stipulated in the original application (for Post graduate studies e.g. Honours studies: 1 year, Masters studies: two years, and PhD studies: three years) and should be extended when the approval period lapses.
4. The digital archiving of data is a requirement of the University of Pretoria. The data should be accessible in the event of an enquiry or further analysis of the data.

Ethics approval is subject to the following:

1. The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.
2. Note: All FVS animal research applications for ethical clearance will be automatically rerouted to the Animal Ethics committee (AEC) once the applications meet the requirements for FVS ethical clearance. As such, all FVS REC applications for ethical clearance related to human health research will be automatically rerouted to the Health Sciences Research Ethics Committee, and all FVS applications involving a questionnaire will be automatically rerouted to the Humanities Research Ethics Committee. Also take note that, should the study involve questionnaires aimed at UP staff or students, permission must also be obtained from the relevant Dean and the UP Survey Committee. Research may not proceed until all approvals are granted.

We wish you the best with your research.

Yours sincerely

PROF M. OOSTHUIZEN
Chairperson: Research Ethics Committee

Appendix 4. Animal Ethics Approval Certificate 2023

Faculty of Veterinary Science
Animal Ethics Committee

05 December 2023

Approval Certificate
Annual Renewal

AEC Reference No.: REC029-21 Line 2
Title: Detection of bacterial tick-borne pathogens in two provinces of South Africa using a microbiome sequencing approach
Researcher: Miss BL Khoza
Student's Supervisor: Miss ZT Khumalo

Dear Miss BL Khoza,

The **Annual Renewal** as supported by documents received between 2023-10-10 and 2023-11-20 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2023-11-20.

Please note the following about your ethics approval:

1. The use of species is approved:

Species	Approved
Cattle - Nguni breed	150
Samples	Approved
Blood sample - Blood - KwaZulu Na - Live	150
tick species - Ticks - KwaZulu Na - Live	450

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2024-12-05.

3. Please remember to use your protocol number (REC029-21) on any documents or correspondence with the AEC regarding your research.

4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

5. All incidents must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.

6. The committee also requests that you record major procedures undertaken during your study for own-archiving, using any available digital recording system that captures in adequate quality, as it may be required if the committee needs to evaluate a complaint. However, if the committee has monitored the procedure previously or if it is generally can be considered routine, such recording will not be required.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

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Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

Appendix 5. Animal Ethics Approval Certificate 2022

Faculty of Veterinary Science
Animal Ethics Committee

14 October 2022

Approval Certificate
Annual Renewal
(EXT1)

AEC Reference No.:	REC029-21 Line 1
Title:	Detection of bacterial tick-borne pathogens in two provinces of South Africa using a microbiome sequencing approach
Researcher:	Miss BL Khoza
Student's Supervisor:	Miss ZT Khumalo

Dear Miss BL Khoza,

The **Annual Renewal** as supported by documents received between 2022-09-12 and 2022-09-26 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2022-09-26.

Please note the following about your ethics approval:

1. The use of species is approved:

Species	Approved
Cattle - Nguni breed	150
Samples	Approved
Blood sample - KwaZulu Na - Live	150
tick species - KwaZulu Na - Live	450

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2023-10-14.
3. Please remember to use your protocol number (REC029-21) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
5. All incidents must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.
6. The committee also requests that you record major procedures undertaken during your study for own-archiving, using any available digital recording system that captures in adequate quality, as it may be required if the committee needs to evaluate a complaint. However, if the committee has monitored the procedure previously or if it is generally can be considered routine, such recording will not be required.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

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Fakulteit Veeartsenykunde
Lefapha la Disaense tša Bongakadiruiwa

Appendix 6. Animal Ethics Approval Certificate 2021

Faculty of Veterinary Science
Animal Ethics Committee

9 September 2021

Approval Certificate
New Application

AEC Reference No.: REC029-21
Title: Detection of bacterial tick-borne pathogens in two provinces of South Africa using a microbiome sequencing approach
Researcher: Miss BL Khoza
Student's Supervisor: Prof MC Oosthuizen

Dear Miss BL Khoza,

The **New Application** as supported by documents received between 2021-04-21 and 2021-09-03 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2021-09-03.

Please note the following about your ethics approval:

1. The use of species is approved:

Species	**Number**
Cattle - Nguni breed	150
Samples Blood	150 (10 ml each)
Ticks species	450

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2022-09-09.
3. Please remember to use your protocol number (REC029-21) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
5. **All incidents** must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.
6. The committee also requests that you record major procedures undertaken during your study for own-archiving, using any available digital recording system that captures in adequate quality, as it may be required if the committee needs to evaluate a complaint. However, if the committee has monitored the procedure previously or if it is generally can be considered routine, such recording will not be required.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

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Email: marleze.rheeder@up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

We wish you the best with your research.

Yours sincerely

H. Lutermann

Dr Heike Lutermann

DEPUTY CHAIRMAN: UP-Animal Ethics Committee

Appendix 7. Section 20 permit from Department of Agriculture, Land Reform and Rural Development.

Directorate Animal Health, Department of Agriculture, Land Reform & Rural Development
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@dalrrd.gov.za
Reference: 12/11/1//1/6/ 1700/MG

Dr Zamantungwa Khumalo
University of Pretoria
Faculty of Veterinary Science
Department of Veterinary Tropical Diseases
Onderstepoort
0110
E-mail:zamantungwak@nicd.ac.za

Dear Dr Zamantungwa Khumalo,

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)

Your application received per email, requesting permission under Section 20 of the Animal Diseases Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers. I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions:

Conditions:

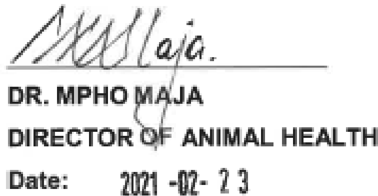
1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. The study is approved as per the application form dated 07 October 2020 and the correspondence thereafter. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this study under this Section 20 permit. Please apply in writing to HerryG@dalrrd.gov.za;
3. All potentially infectious material utilised, collected or generated during the study are to be destroyed at the completion of the study. A registered waste removal company must dispose the material generated from the study Records must be kept for five years for auditing purposes;

4. Ticks and blood samples must be collect from cattle locations in Free State and KwaZulu Natal where letters from an authorised State Veterinarian have been obtained indicating absence of reported cases of Controlled diseases;
5. Collected samples must be transported to the Department of Veterinary Tropical Diseases BSL2+ laboratory in containers that are leak proof for DNA extraction;
6. A dispensation is issued for long term storage of Extracted nucleic acid. Approval for further distribution or usage of the stores samples must be obtained from Director Animal Health;
7. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 approval.

Title of research/study: Detection of bacterial tick-borne pathogens in two provinces of South Africa using a microbe sequencing approach.

Researcher: Miss Bongekile Khoza,
Institution: University of Pretoria
Our ref Number: 12/11/1/1/MG
Your ref:
Expiry date: 31 December 2023

Kind regards,



DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2021-02-23

SUBJECT: Detection of bacterial tick-borne pathogens in two provinces of South Africa using a microbe sequencing approach

Appendix 8. Section 20 permit (Amendment 1) from Department of Agriculture, Land Reform and Rural Development.

agriculture, land reform
& rural development

Department:
Agriculture, Land Reform and Rural Development
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Land Reform and Rural Development
Private Bag X138, Pretoria 0001

Enquiries: Ms Mama Laing • Tel: +27 12 319 7442 • Fax: +27 12 319 7470 • E-mail: MamaL@Dalrrd.gov.za
Reference: 12/11/1/1/MG (2446)

Dr Zamantungwa Khumalo
University of Pretoria
Faculty of Veterinary Science
Department of Veterinary Tropical Diseases
Onderstepoort, 0110
Email: zamantungwak@nicd.ac.za

Dear Dr Zamantungwa Khumalo,

RE: AMENDMENT OF SECTION 20 APPROVAL IN TERMS OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984)

Title of research project / study: "Detection of bacterial tick-borne pathogens in two Provinces" Reference 12/11/1/1/1708/MG:

The laboratory, as indicated in condition 5 of the above mentioned section 20, where the testing of the collected samples shall be done has been changed to DVTD BSL2 laboratory.

All other conditions as specified in the Section 20 approval shall remain in full effect;

Take note that the reference number of the section 20 permit has been changed to: 12/11/1/1/1708/MG.

Kind regards,

DIRECTOR: ANIMAL HEALTH
Date: 2022 -06- 27

– 1 –