



**Exploring bacterial communities and
genetic diversity harboured by
Amblyomma hebraeum and
Rhipicephalus evertsi evertsi ticks from
livestock in North West Province, South
Africa**

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DECLARATION

I, Kealeboga Mileng, declare that the thesis entitled “Exploring bacterial communities and genetic diversity in *Amblyomma hebraeum* and *Rhipicephalus evertsi evertsi* ticks from livestock in North West Province, South Africa”, submitted for the degree Doctor of Philosophy in Science with Microbiology at the North-West University, has not previously been submitted by me for a degree at this or any other university. I further declare that this is my work in design and execution and that all materials contained herein have been duly acknowledged.

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Abstract

Ticks are important vectors of pathogens that impact both livestock and human health. Among the significant tick species in South Africa, transmitting economically important diseases to livestock are *Amblyomma hebraeum* and *Rhipicephalus evertsi evertsi*. *Amblyomma hebraeum* is a vector for *Ehrlichia ruminantium*, causing heartwater in cattle, sheep, and goats. It also transmits *Rickettsia africae*, responsible for tick-bite fever in human. *R. e. evertsi* transmits *Coxiella* spp. in sheep, and *Anaplasma marginale*, causing anaplasmosis in cattle. This study investigated the bacterial diversity and veterinary important pathogens harboured by *A. hebraeum* and *R. e. evertsi* ticks in sheep and goats from the North West Province of South Africa. Ixodid ticks were collected from different farms in Mahikeng and Matlwang village and were identified using morphological and molecular techniques. Molecular identification targeting the 16S rRNA and 18S rRNA genes sequences was performed on the *A. hebraeum* and *R. e. evertsi* ticks. Bacterial communities associated with these tick species were investigated using next-generation sequencing (NGS) techniques performed using high-throughput sequencing of the V3–V4 hypervariable regions of the 16S rRNA gene on the Illumina MiSeq platform. Key differences in bacterial communities among these tick species were further exploited. The ticks collected from Mahikeng were morphologically identified as *A. hebraeum*. They were characterized by their legs, which had distinctive red and white rings, and a notably ornate scutum with yellow, green, brown, and black markings. Meanwhile, ticks collected from Matlwang were identified as *R. e. evertsi*, characterized by their dark brown bodies, reddish-orange lateral sides and reddish-orange legs. Amplicon gene sequencing revealed significant sequence similarity, with 18S rRNA gene sequences exhibiting 99.36–99.87% identity to known GenBank sequences of the investigated tick species. Phylogenetic analysis based on 16S and 18S rRNA gene sequences demonstrated strong monophyletic clustering, clearly distinguishing between the two tick genera. Next-generation sequencing showed that both tick species were primarily associated with Proteobacteria, with *A. hebraeum* from sheep showing a high abundance of 82.8%, indicating significant reliance on this phylum for survival. *Amblyomma hebraeum* was mainly composed of *Rickettsia* spp., which comprised 61.42% of its bacterial composition. Meanwhile, *R. e. evertsi* exhibited greater bacterial diversity, including various taxa such as *Acinetobacter* and *Pseudomonas* species, particularly from sheep, hosting 8,425 unique taxa (27.8%) compared to *A. hebraeum*'s 4,402 (9%). Both

species shared 1,534 bacterial taxa, representing 63.1% of their core microbiome, pointing to conserved microbial functions. Their predicted functional pathways related to energy metabolism, carbon, and nitrogen cycling highlight their ecological adaptability. The study's findings demonstrate that host species significantly influence the tick microbiome, with ticks from sheep exhibiting more stable and less variable bacterial communities compared to those from goats. The *R. e. evertsi* ticks demonstrated greater bacterial diversity, including more *Actinobacteria* in goat-derived ticks. The molecular identification of ticks provided a genetic library of local or regional tick fauna, especially in underrepresented areas of the local farming system, where tick diversity is high yet under-documented. Data generated in this study will contribute to guiding the development of tick surveillance systems, risk mapping and disease control efforts in South African livestock production systems.

Keywords: *Amblyomma hebraeum*, *Rhipicephalus evertsi evertsi*, 16S rRNA, 18S rRNA, bacterial communities, next-generation sequencing, bacterial diversity

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LIST OF ABBREVIATIONS AND ACRONYMS

<i>A. hebraeum</i> :	<i>Amblyomma hebraeum</i>
AMPure XP:	AMPure XP (magnetic beads for PCR purification)
BLAST:	Basic Local Alignment Search Tool
<i>COI</i> :	Cytochrome c Oxidase subunit I
DADA2:	Divisive Amplicon Denoising Algorithm 2
DNA:	Deoxyribonucleic Acid
vdNTPs:	Deoxynucleotide Triphosphates
FAPROTAX:	Functional Annotation of Prokaryotic Taxa
FNASREC:	Faculty of Natural and Agricultural Sciences Research Ethics Committee
LDA:	Linear Discriminant Analysis
LefSe:	Linear Discriminant Analysis Effect Size
ML:	Maximum Likelihood
MgCl ₂ :	Magnesium Chloride
MiSeq:	Illumina MiSeq (sequencing platform)
NaOH:	Sodium Hydroxide
NCBI:	National Center for Biotechnology Information
NGS:	Next-Generation Sequencing
NMDS:	Non-Metric Multidimensional Scaling
NWU:	North-West University
OTU:	Operational Taxonomic Unit
PCR:	Polymerase Chain Reaction

PCoA:	Principal Component Ordinate Analysis
PhiX:	PhiX Control (sequencing control)
PICRUST:	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
QIIME:	Quantitative Insights Into Microbial Ecology
<i>R. e. evertsi</i> :	<i>Rhipicephalus evertsi evertsi</i>
<i>R. microplus</i> :	<i>Rhipicephalus microplus</i>
<i>R. appendiculatus</i> :	<i>Rhipicephalus appendiculatus</i>
<i>R. mimeticus</i> :	<i>Rhipicephalus mimeticus</i>
RNA:	Ribonucleic Acid
rRNA:	Ribosomal Ribonucleic Acid
TBD:	Tick-borne Disease
TBP:	Tick-borne Pathogen
USA:	United States of America
UV:	Ultraviolet
XT:	Nextera XT (index kit)
g:	gravitational force
rpm:	revolutions per minute
dATP:	Deoxyadenosine triphosphate
mM:	millimolar
dCTP:	deoxycytidine triphosphate
dGTP:	deoxyguanosine triphosphate
dTTP:	deoxythymidine triphosphate
MgCl ₂ :	magnesium chloride

EtBr:	Ethidium Bromide
ML:	maximum likelihood

1. CHAPTER 1

General introduction

1.1. Background

Ticks of the Ixodidae family pose significant threat to livestock health, resulting in economic losses and health challenges. Their infestation can result in reduced milk production in dairy cows, weight loss in young animals, and infertility in breeding stock, all which contributes to significant economic losses for farmers and livestock producers (Rajput *et al.* 2006; Desta, 2016). Apart from being agricultural pests, ticks are vectors of zoonotic pathogens transmissible to humans via bites or direct contact with infected animals, causing diseases (Akutse *et al.*, 2020).

In South Africa, common tick species infesting livestock are *Amblyomma hebraeum*, *Rhipicephalus (Boophilus) microplus*, *Rhipicephalus appendiculatus*, and *Rhipicephalus evertsi evertsi*, being the most common in cattle, goats and sheep (Horak, 2009). The common tick-borne diseases (TBDs) pathogens transmitted by these ticks include *Anaplasma marginale*, *Coxiella burnetii*, which causes Q fever, *Ehrlichia ruminantium*, which causes heartwater disease (also known as cowdriosis) in domestic and wild ruminants such as cattle, sheep and goats, and some wildlife species and *Rickettsia africae*, which causes *Rickettsia africae* which causes African tick bite fever in humans (Khoza, 2024; Mtshali *et al.*, 2015; Thekisoe *et al.*, 2023). These diseases can lead to severe health implications if not detected and treated early. Tick-borne pathogen (TBP) surveillance is imperative for improving diagnostic methods, particularly the detection of emerging pathogens, as molecular techniques have enhanced the ability and capacity to detect novel disease-causing agents (Tijssse-Klasen *et al.*, 2014). A key driver of the emergence and recognition of TBDs has been the rapid expansion of geographic range and abundance of medically important, human-biting tick species over the past several decades (Eisen *et al.* 2016, Sonenshine 2018; Molaei *et al.* 2019).

The taxonomy of ixodid ticks has traditionally relied on morphological analysis, however, it is challenging to identify species using morphology alone, especially when collected in the larval and/or nymphal life stages (McCann *et al.*, 2019). The genetic methods, which include amplification and sequencing of the mitochondrial 16S rRNA, CO1, 12S rRNA 18S rRNA and ITS2 genes, have improved the efficiency of tick identification, the depth of the phylogenetic

research and further aided in elucidating the diversity of different tick species (Lv et al., 2014; Ghosh et al., 2020; Kneubehl et al., 2022). Molecular surveillance of TBDs in livestock has been extensively documented in KwaZulu-Natal and Limpopo provinces, with a moderate body of research emerging from the Eastern Cape, Mpumalanga, and Gauteng (Choopa, 2015; 2021; Lungile, 2024; Khoza, 2024; Mtshali, 2012; Iweriebor *et al.*, 2022). However, data from the North West, Free State, Northern Cape and Western Cape provinces remain limited. Furthermore, molecular surveillance and the characterization of veterinary important bacterial pathogens circulating in livestock in Potchefstroom and Mahikeng cities of North West province remain limited.

Next-generation sequencing (NGS) technology has revolutionised the field of genomics, enabling researchers to better understand infectious diseases (Van Borm *et al.*, 2015; Malikovic *et al.*, 2020). Next generation sequencing' technology can assist track the spread of TBDs through sequencing the DNA or RNA of the disease-causing pathogens harbored by ticks. This approach can provide important information about the pathogens present in ticks and their geographic distribution (Eisen and Paddock, 2021), as well as improved understanding of the complex interactions between ticks, pathogens and hosts (Bonnet *et al.*, 2021; Cabezas-Cruz *et al.*, 2018).

1.2. Problem statement

In the North West province of South Africa, where livestock farming is a crucial part of the ecosystem, tick species such as *A. hebraeum* and *R. e. evertsi* are prevalent (Spickett *et al.*, 2011). These ticks play a significant role in livestock health, and understanding their genetic diversity and microbiome composition is essential. The microbiome, which consists of various microorganisms within ticks, affects their ability to spread diseases and cope with environmental changes (Bonnet and Pollet, 2021). Additionally, the genetic diversity within tick populations indicates their adaptability and potential resistance to control methods (Araya-Anchetta *et al.*, 2015). Given the diverse livestock and environmental conditions in North West province, it is an ideal location to study tick microbiomes.

The *16S rRNA* metabarcoding, also referred to as a meta-taxonomic approach, is a powerful tool that allows for the study of the collective genetic material of microorganisms in each sample. The discovery and characterization of novel and emerging bacterial pathogens in ticks is critical for global health initiatives aimed at improving animal and human welfare. Making it possible to examine the genetic variety of novel and emerging bacterial pathogen of economic

importance in ticks. This study explores these aspects, aiming to improve livestock health management and reduce disease transmission risks in the North West Province.

1.3 Study aim

To investigate the bacterial diversity and veterinary important bacterial pathogens harboured by *Amblyomma hebraeum* and *Rhipicephalus evertsi evertsi* ticks from sheep and goats in the villages of Mahikeng and Potchefstroom cities of North West province of South Africa.

1.4 Objectives of the study

1. Assessing the diversity of ticks infesting sheep and goats in the villages of Mahikeng and Potchefstroom, North West province using morphological and genetic characterization techniques.
2. To determine the bacterial communities associated with *Amblyomma hebraeum* ticks using high-throughput *16S rRNA* gene amplicon sequencing approach.
3. To determine the bacterial communities associated with *Rhipicephalus evertsi evertsi* ticks using high-throughput *16S rRNA* gene amplicon sequencing approach.
4. To conduct a comparative analysis of the bacterial communities associated with *A. hebraeum* and *R. evertsi evertsi* ticks collected from the North West Province, South Africa.

1.5 Ethical Consideration

The study was approved by the North-West University scientific committee of Ecological Interactions and Ecosystem Resilience (EIER) subprogram and the Faculty of Natural and Agricultural Sciences Research Ethics Committee (FNASREC) (NWU-01396-23-A9).

1.6 Thesis chapter outline

- Chapter 1 Introduction, problem statement and justification, aim and objectives.
- Chapter 2: Literature review.
- Chapter 3: Morphological and genetic characterization of small ruminant ticks collected from villages of Mahikeng and Potchefstroom.

- Chapter 4: The bacterial communities harboured by *Amblyomma hebraeum* ticks from villages of Mahikeng city.
- Chapter 5: The bacterial communities harboured by *Rhipicephalus evertsi evertsi* ticks from Matlwang village in Potchefstroom.
- Chapter 6: Comparison of bacterial communities harboured by *A. hebraeum* and *R. e. evertsi* ticks from villages of Mahikeng and Potchefstroom.
- Chapter 7: Conclusion and recommendations

References

1. Abdullah, H.H., El-Molla, A., Salib, F.A., Allam, N.A., Ghazy, A.A. & Abdel-Shafy, S., 2016. Morphological and molecular identification of the brown dog tick *Rhipicephalus sanguineus* and the camel tick *Hyalomma dromedarii* (Acari: Ixodidae) vectors of Rickettsioses in Egypt. *Veterinary World*, 9(10), p.1087.
2. Akutse, K.S., Subramanian, S., Maniania, N., Dubois, T. & Ekesi, S., 2020. Biopesticide research and product development in Africa for sustainable agriculture and food security—Experiences from the International Centre of Insect Physiology and Ecology (icipe). *Frontiers in Sustainable Food Systems*, 4, p.563016.
3. Araya-Anchetta, A., Busch, J.D., Scoles, G.A. & Wagner, D.M., 2015. Thirty years of tick population genetics: a comprehensive review. *Infection, Genetics and Evolution*, 29, pp.164–179.
4. Ashelford, K.E., Chuzhanova, N.A., Fry, J.C., Jones, A.J. & Weightman, A.J., 2006. New screening software shows that most recent large 16S rRNA gene clone libraries contain chimeras. *Applied and Environmental Microbiology*, 72(9), pp.5734–5741.
5. Bonnet, S., Michelet, L., Moutailler, S., Cheval, J., Hebert, C., Vayssier-Taussat, M. & Eloit, M., 2014. Identification of parasitic communities within European ticks using next-generation sequencing. *PLoS Neglected Tropical Diseases*, 8(3), p.e2753.
6. Bonnet, S.I., Binetruy, F., Hernández-Jarguín, A.M. & Duron, O., 2017. The tick microbiome: why non-pathogenic microorganisms matter in tick biology and pathogen transmission. *Frontiers in Cellular and Infection Microbiology*, 7, p.236.
7. Bonnet, S.I. and Pollet, T., 2021. Update on the intricate tango between tick microbiomes and tick-borne pathogens. *Parasite Immunology*, 43(5), p.e12813.
8. Brault, A.C., Savage, H.M., Duggal, N.K., Eisen, R.J. and Staples, J.E., 2018. Heartland virus epidemiology, vector association, and disease potential. *Viruses*, 10(9), p.498.
9. Bryson, N.R., Tice, G.A., Horak, I.G., Stewart, C.G. and Du Plessis, B.J.A., 2002. Ixodid ticks on indigenous goats owned by small-scale farmers in four communal

- grazing areas in South Africa. *Journal of the South African Veterinary Association*, 73(1), pp.26–30.
10. Bukin, Y.S., Galachyants, Y.P., Morozov, I.V., Bukin, S.V., Zakharenko, A.S. and Zemskaya, T.I., 2019. The effect of 16S rRNA region choice on bacterial community metabarcoding results. *Scientific Data*, 6(1), pp.1–14.
 11. Cabezas-Cruz, A., Vayssier-Taussat, M. and Greub, G., 2018. Tick-borne pathogen detection: what's new? *Microbes and Infection*, 20(7–8), pp.441–444.
 12. Choopa, C.N., 2015. *Diagnosis of Tick-borne Diseases in Cattle in Bushbuckridge, Mpumalanga, South Africa and Identification of Theileria Parva Carriers*. University of Pretoria (South Africa).
 13. Desta, B.G.E.A.H., 2016. Review on the impact of ticks on livestock health and productivity. *Journal of Biology, Agriculture and Healthcare*, 6, pp.1–7.
 14. Eisen, L. and Dolan, M.C., 2016. Evidence for personal protective measures to reduce human contact with blacklegged ticks and for environmentally based control methods to suppress host-seeking blacklegged ticks and reduce infection with Lyme disease spirochetes in tick vectors and rodent reservoirs. *Journal of Medical Entomology*, 53, pp.1063–1092.
 15. Eisen, R.J. and Paddock, C.D., 2021. Tick and tickborne pathogen surveillance as a public health tool in the United States. *Journal of Medical Entomology*, 58(4), pp.1490–1502.
 16. Franzosa, E.A., Hsu, T., Sirota-Madi, A., Shafquat, A., Abu-Ali, G., Morgan, X.C. and Huttenhower, C., 2015. Sequencing and beyond: integrating molecular 'omics' for microbial community profiling. *Nature Reviews Microbiology*, 13(6), pp.360–372.
 17. Ghosh, P., Tikar, S.N., Gupta, M.K. and Sukumaran, D., 2020. Molecular characterization using 16S rRNA and COI gene sequence in hard ticks of Gwalior, India. *Defence Life Science Journal*, 5(3), pp.173–179.
 18. Gugliotta, J.L., Goethert, H.K., Berardi, V.P. and Telford, S.R. III., 2013. Meningoencephalitis from *Borrelia miyamotoi* in an immunocompromised patient. *New England Journal of Medicine*, 368(3), pp.240–245.
 19. Hurtado, O.J.B. and Giraldo-Ríos, C., 2018. Economic and health impact of the ticks in production animals. *Ticks and Tick-borne Pathogens*, 9, pp.1–9.

20. Iweriebor, B.C., Afolabi, K.O., Nqoro, A. and Obi, L.C., 2022. Emergence of *Theileria* species in ticks from free-ranging domestic animals in Raymond Mhlaba local municipality, South Africa. *Heliyon*, 8(3).
21. Kalendar, R., Lee, D. and Schulman, A.H., 2014. FastPCR software for PCR, in silico PCR, and oligonucleotide assembly and analysis. In: *DNA Cloning and Assembly Methods*. Totowa, NJ: Humana Press, pp.271–302.
22. Khoza, B.L., 2024. Identification of tick species and their bacterial pathogens from cattle in two provinces of South Africa (Doctoral dissertation, University of Pretoria).
23. Kisten, D., Brinkerhoff, J., Tshilwane, S.I. and Mukaratirwa, S., 2021. A pilot study on the microbiome of *Amblyomma hebraeum* tick stages infected and non-infected with *Rickettsia africae*. *Pathogens*, 10(8), p.941.
24. Kneubehl, A.R., Muñoz-Leal, S., Filatov, S., De Klerk, D.G., Pienaar, R., Lohmeyer, K.H., Bermúdez, S.E., Suriyamongkol, T., Mali, I., Kanduma, E. and Latif, A.A., 2022. Amplification and sequencing of entire tick mitochondrial genomes for a phylogenomic analysis. *Scientific Reports*, 12(1), p.19310.
25. Lungile, K.B., 2024. *Identification of Tick Species and Their Bacterial Pathogens from Cattle in Two Provinces of South Africa* (Master's thesis, University of Pretoria (South Africa)).
26. Lv, J., Wu, S., Zhang, Y., Chen, Y., Feng, C., Yuan, X., Jia, G., Deng, J., Wang, C., Wang, Q. and Mei, L., 2014. Assessment of four DNA fragments (COI, 16S rDNA, ITS2, 12S rDNA) for species identification of the Ixodida (Acari: Ixodida). *Parasites & vectors*, 7(1), p.93.
27. Marufu, M.C., Qokweni, L., Chimonyo, M. and Dzama, K., 2011. Relationships between tick counts and coat characteristics in Nguni and Bonsmara cattle reared on semiarid rangelands in South Africa. *Ticks and Tick-borne Diseases*, 2(3), pp.172–177.
28. McCann, K.M., Grant, W.N., Spratt, D.M. and Hedtke, S.M., 2019. Cryptic species diversity in ticks that transmit disease in Australia. *International Journal for Parasitology: Parasites and Wildlife*, 10, pp.125–131.

29. Meissner, H.H., Scholtz, M.M. and Palmer, A.R., 2013. Sustainability of the South African livestock sector towards 2050 Part 1: Worth and impact of the sector. *South African Journal of Animal Science*, 43(3), pp.282–297.
30. Miller, S.A., Dykes, D.D. and Polesky, H.F., 1988. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Research*, 16(3), p.1215.
31. Mnisi, S.S., Mphuthi, M.B., Ramatla, T., Mofokeng, L.S., Thekiso, O. and Syakalima, M., 2022. Molecular detection and genetic characterization of *Ehrlichia ruminantium* harbored by *Amblyomma hebraeum* ticks of domestic ruminants in North West Province, South Africa. *Animals*, 12(19), p.2511.
32. Molaei, G., Little, E.A., Williams, S.C. and Stafford, K.C., 2019. Bracing for the worst—range expansion of the lone star tick in the northeastern United States. *New England Journal of Medicine*, 381(23), pp.2189–2192.
33. Mtshali, K., 2012. Molecular detection of zoonotic tick-borne pathogens in livestock in different provinces of South Africa. Master's thesis, University of Pretoria, South Africa.
34. Mtshali, K., Khumalo, Z.T., Nakao, R., Grab, D.J., Sugimoto, C. and Thekiso, O.M., 2015. Molecular detection of zoonotic tick-borne pathogens from ticks collected from ruminants in four South African provinces. *Journal of Veterinary Medical Science*, 77(12), pp.1573–1579.
35. Musemwe, L., Mushunje, A., Chimonyo, M., Fraser, G., Mapiye, C. and Muchenje, V., 2008. Nguni cattle marketing constraints and opportunities in the communal areas of South Africa: Review. *African Journal of Agricultural Research*, 3(4), pp.239–245.
36. Onyiche, T.E. and MacLeod, E.T., 2023. Hard ticks (Acari: Ixodidae) and tick-borne diseases of sheep and goats in Africa: A review. *Ticks and Tick-borne Diseases*, 14(6), p.102232.
37. Paddock, C.D. and Goddard, J., 2015. The evolving medical and veterinary importance of the Gulf Coast tick (Acari: Ixodidae). *Journal of Medical Entomology*, 52(2), pp.230–252.
38. Paddock, C.D. and Yabsley, M.J., 2007. Ecological havoc, the rise of white-tailed deer, and the emergence of *Amblyomma americanum*-associated zoonoses in

- the United States. In: J.E. Childs, J.S. Mackenzie and J.A. Richt, eds. *Wildlife and Emerging Zoonotic Diseases: The Biology, Circumstances and Consequences of Cross-Species Transmission*. Berlin: Springer, pp.289–324.
39. Padgett, K.A., Bonilla, D., Ereemeeva, M.E., Glaser, C., Lane, R.S., Porse, C.C., Castro, M.B., Messenger, S., Espinosa, A., Hacker, J. and Kjemtrup, A., 2016. The eco-epidemiology of Pacific Coast tick fever in California. *PLoS Neglected Tropical Diseases*, 10(10), p.e0005020.
 40. Pritt, B.S., Respicio-Kingry, L.B., Sloan, L.M., Schrieffer, M.E., Replogle, A.J., Bjork, J., Liu, G., Kingry, L.C., Mead, P.S., Neitzel, D.F. and Schiffman, E., 2016. *Borrelia mayonii* sp. nov., a member of the *Borrelia burgdorferi* sensu lato complex, detected in patients and ticks in the upper midwestern United States. *International journal of systematic and evolutionary microbiology*, 66(11), pp.4878-4880..
 41. Rajput, Z.I., Hu, S.H., Chen, W.J., Arijo, A.G. and Xiao, C.W., 2006. Importance of ticks and their chemical and immunological control in livestock. *Journal of Zhejiang University Science B*, 7(11), pp.912–921.
 42. Rego, R.O., Trentelman, J.J., Anguita, J., Nijhof, A.M., Sprong, H., Klempa, B., Hajdusek, O., Tomás-Cortázar, J., Azagi, T., Strnad, M. and Knorr, S., 2019. Counterattacking the tick bite: towards a rational design of anti-tick vaccines targeting pathogen transmission. *Parasites & Vectors*, 12(1), pp.1–20.
 43. Ringo, A.E., Moumouni, P.F.A., Lee, S.H., Liu, M., Khamis, Y.H., Gao, Y., Guo, H., Zheng, W., Efstratiou, A., Galon, E.M. and Li, J., 2018. Molecular detection and characterization of tick-borne protozoan and rickettsial pathogens isolated from cattle on Pemba Island, Tanzania. *Ticks and Tick-borne Diseases*, 9(6), pp.1437–1445.
 44. Schloss, P.D., Gevers, D. and Westcott, S.L., 2011. Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies. *PLOS One*, 6(12), p.e27310.
 45. Sonenshine, D.E., 2018. Range expansion of tick disease vectors in North America: Implications for spread of tick-borne disease. *International Journal of Environmental Research and Public Health*, 15, p.e478.

46. Spickett, A.M., Heyne, I.H. and Williams, R., 2011. Survey of the livestock ticks of the North West province, South Africa. *Onderstepoort Journal of Veterinary Research*, 78(1), pp.1–12.
47. Takhampunya, R., Korkusol, A., Pongpichit, C., Yodin, K., Rungrojn, A., Chanarat, N., Promsathaporn, S., Monkanna, T., Thaloengsok, S., Tippayachai, B. and Kumfao, N., 2019. Metagenomic approach to characterizing disease epidemiology in a disease-endemic environment in Northern Thailand. *Frontiers in Microbiology*, 10, p.319.
48. Teel, P.D., Ketchum, H.R., Mock, D.E., Wright, R.E. and Strey, O.F., 2010. The Gulf Coast tick: a review of the life history, ecology, distribution, and emergence as an arthropod of medical and veterinary importance. *Journal of Medical Entomology*, 47(5), pp.707–722.
49. Thekiso, O., Ramatla, T., Ringo, A., Mnisi, S., Mphuthi, N., Mofokeng, L., Lekota, K. and Xuan, X., 2023. Molecular detection of *Rickettsia africae* from *Amblyomma hebraeum* ticks in Mafikeng city of North West Province, South Africa. *Research in Veterinary Science*, 164, p.105027.
50. Tjisse-Klasen, E., Koopmans, M.P. and Sprong, H., 2014. Tick-borne pathogen–reversed and conventional discovery of disease. *Frontiers in Public Health*, 2, p.96029.
51. Van Borm, S., Belák, S., Freimanis, G., Fusaro, A., Granberg, F., Höper, D., King, D.P., Monne, I., Orton, R. and Rosseel, T., 2015. Next-generation sequencing in veterinary medicine: how can the massive amount of information from high-throughput technologies improve diagnosis, control, and management of infectious diseases?. In: *Veterinary Infection Biology: Molecular Diagnostics and High-throughput Strategies*, pp.415–436.
52. Walker, J.G., Klein, E.Y. and Levin, S.A., 2014. Disease at the wildlife-livestock interface: acaricide use on domestic cattle does not prevent transmission of a tick-borne pathogen with multiple hosts. *Veterinary Parasitology*, 199(3–4), pp.206–214.

2. CHAPTER 2

Literature review

2.1 Introduction

2.1.1 Background on ticks and their importance

Ticks are significant ectoparasites and vectors of numerous pathogens, impacting both human and animal health globally in the medical and veterinary fields. They are the second most important vectors of human disease-causing agents after mosquitoes and transmit pathogens, resulting in cases of tick-borne diseases (TBDs) worldwide. In South Africa, most common TBD pathogens transmitted by ticks include *Anaplasma marginale*, which causes bovine anaplasmosis, Bovine babesiosis, primarily caused by *Babesia bovis* and *B. bigemina*, *Ehrlichia ruminantium*, which causes Heartwater in livestock and as well as bovine theileriosis, mainly caused by *Theileria parva*, which currently controlled in South Africa (Mtshali *et al.*, 2007; Mtshali *et al.*, 2014; Mnisi *et al.*, 2022; Yusufmia *et al.*, 2010). The transmission dynamics of these disease-causing pathogens are characterised by tick life stages, host species, and environmental conditions, with adult female ticks playing a significant role in pathogen dissemination for specific pathogens (Sasaki *et al.*, 2021). Adult female ticks play a significant role in pathogen dissemination due to their feeding behavior and physiological characteristics. It has been reported large *Babesia* species, adult females are the primary vectors for infecting ruminants, with transovarial (from mother to egg) and transstadial (from one developmental stage to the next) transmission enabling pathogen persistence across tick generations (Karbowski *et al.*, 2018). Climate change is altering the range of tick populations and their hosts, potentially leading to increased emergence rates of specific diseases in areas where they were previously not endemic (Fan *et al.*, 2022; Gasmi *et al.*, 2018). It is, therefore, important to develop control strategies, including vaccines targeting tick antigens and understanding the complex interactions among tick-pathogen-host, since those can have significant implications on disease transmission (de la Fuente *et al.*, 2016). Overall, this review illustrates the profound impact of ticks on public health and confirms the need for continued research and preventative efforts required to reduce their impact on both human and animal health.

2.1.2 Importance of studying bacterial communities and genetic diversity in ticks

Investigating bacterial communities and genetic diversity in ticks is essential for understanding their roles as pathogen vectors and the ecological dynamics of tick populations. Ticks harbour diverse microbiomes that can influence their vector competence and interactions with pathogens (Adegoke *et al.*, 2020). Research has shown that these microbiomes consist of symbiotic and pathogenic bacteria, which can affect pathogen density and transmission dynamics (Carpi *et al.*, 2011; Kurilshikov *et al.*, 2015). For instance, symbiotic and pathogenic bacterial species can modulate the tick's immune response and overall fitness, thereby impacting the transmission of pathogens (Narasimhan *et al.*, 2014; Gall *et al.*, 2016). The genetic diversity of tick-associated pathogens is also crucial for assessing their virulence and transmission potential. It has already been reported that high co-infection rates in ticks can complicate disease management strategies (Kwan *et al.*, 2017; Sánchez-Vicente *et al.*, 2019). Furthermore, the interactions between the tick microbiota and the pathogens they carry can significantly influence the tick's susceptibility to infections, highlighting the importance of understanding these microbial communities (Narasimhan and Fikrig, 2015; De la Fuente *et al.*, 2017).

2.2 General overview of hard ticks

2.2.1 Biology and life cycle of hard ticks

Hard ticks from the family Ixodidae have been the subject of extensive study in South Africa, with recent studies focusing on aspects of their taxonomy, morphology, and ecological roles. These ticks are obligate hematophagous ectoparasites with a complex life cycle that includes egg, larva, nymph, and adult life stages, where each stage is associated with distinct morphological features and host associations (Russell *et al.*, 2013; Inyagwa *et al.*, 2021). The morphological adaptations observed across these stages, particularly in genera such as *Ixodes*, *Amblyomma*, *Dermacentor*, *Hyalomma*, *Haemaphysalis*, and *Rhipicephalus*, have been critical in understanding both their life history strategies and their vector competence for numerous pathogens (Hornok *et al.*, 2023; Makwarela *et al.*, 2023).

The morphological structure of hard ticks comprises a scutum (dorsal shield), a capitulum (mouth part), and Haller's organ, which assists in host identification (Russell *et al.*, 2013; Josek *et al.*, 2017). Exploration of the morphological characteristics of *Ixodes* species from Southern Africa has revealed some distinctive structural traits which may support the theory suggesting

Africa as the origin for bird-associated exophilic ticks (Hornok *et al.*, 2023). These comprise differences in the size and shape of the scutum's hard shield and mouthparts, crucial for successful blood feeding and avoiding the host's immune response (Hornok *et al.*, 2023). Furthermore, morphometric investigations provided insight into the sexual dimorphism observed in hard ticks, where adult females are usually characterised by sexually-distinct features associated with reproductive functions and pathogen transmission dynamics (Makwarela *et al.*, 2024). Moreover, modern studies have incorporated molecular phylogenetic techniques and morphological scrutiny to resolve perplexing issues associated with tick identification and lineage diversification within the region (Hornok *et al.*, 2023; Makwarela *et al.*, 2024).

The tick life cycle begins when an engorged female lays eggs in the environment (Figure 2.1). The eggs hatch into six-legged larvae that attach to a host, feed, and later drop off to molt into nymphs. During the larval stage, ticks are particularly vulnerable and dependent on environmental humidity and temperature, which are critical for survival and subsequent molting to the nymphal stage (Leal *et al.*, 2020; Brunner *et al.*, 2023). The nymph and adult stages are characterised by an increased host range, which not only facilitates the acquisition and transmission of pathogens but also underscores the ecological plasticity of these parasites (Gervasi *et al.*, 2015). Research on cattle ticks in South Africa indicates that aspects of their life cycle such as seasonal activity and timing of development are intricately influenced by biotic and abiotic factors in the region (Lungile, 2024; Oberholster, 2014; Makwarela *et al.*, 2024). Such research is crucial in reducing the economic impact of tick infestations on livestock, which is reported to exceed USD 33 million per year (Makwarela *et al.*, 2023). Tick infestations on livestock have significant economic impacts through both direct and indirect effects. Direct losses result from blood loss, reduced live weight, anemia, and decreased hide quality (Hurtado and Giraldo-Ríos, 2018; Desta, 2016). Indirect losses stem from tick-borne diseases, treatment costs, and control measures (Hurtado and Giraldo-Ríos, 2018; Singh *et al.*, 2022).

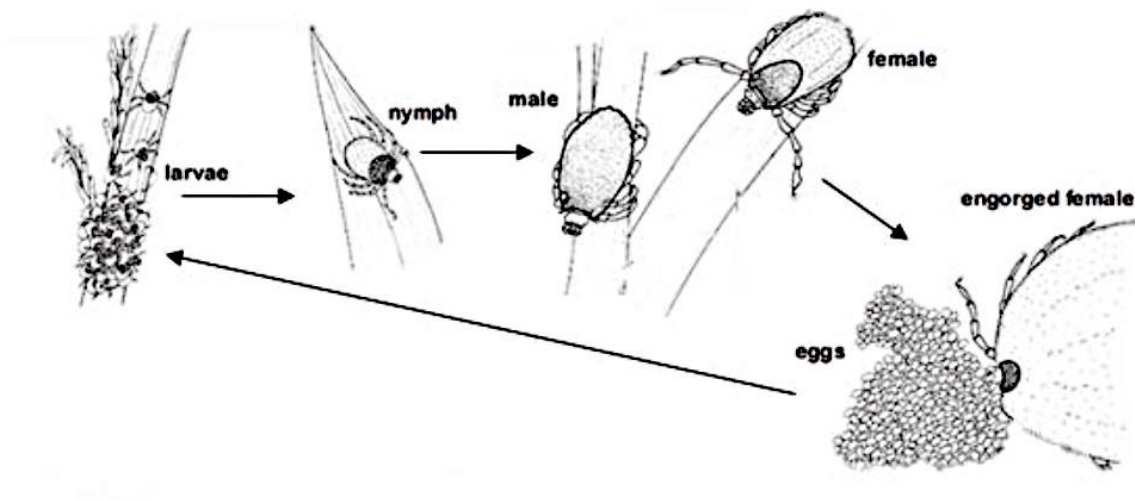


Figure 2.1: Basic life cycle of hard ticks (Walker *et al.*, 2014).

Hard ticks (Acari: Ixodidae) exhibit various life cycle types, primarily classified into one-host, two-host, and three-host systems. The one-host life cycle, such as in blue tick *Rhipicephalus (Boophilus) microplus*, allows the tick to feed throughout its life cycle on a single host, which facilitates both sustained feeding and disease transmission, particularly in livestock (Cleveland *et al.*, 2024). While, in two-host life cycles, the tick feeds on one host during the larval and nymphal stages and a different host for the adult stage. *Hyalomma dromedarii* is a pertinent example, feeding on camels as adults and using small mammals as hosts during its immature stages (Damian *et al.*, 2021). The three-host life cycle is the most variable among ticks and can be demonstrated through *Amblyomma variegatum*, which parasitises a range of hosts throughout its life cycle. This species varies its host choice according to the life stage, where larvae are likely to feed on reptiles and adults on large mammals like cattle (Choudhury *et al.*, 2013). Other example includes *Hyalomma aegyptium*, which primarily uses tortoises of Testudo as adult hosts but employs a variety of other vertebrates, including birds and small mammals, for its larval and nymph stages, thus demonstrating significant ecological flexibility (Paştiu *et al.*, 2012; Barradas *et al.*, 2020). The different life cycle strategies exemplify how hard ticks have radiated into certain ecological zones, underscoring their role as important vectors of zoonotic diseases in Africa and other regions (Gou *et al.*, 2013; Onyiche *et al.*, 2022).

2.2.2 Classification and distribution of *Amblyomma* and *Rhipicephalus* species

Amblyomma is a genus within the family Ixodidae and phylum Arthropoda, containing approximately 130 species worldwide, with 28 of which occurring in Africa and Madagascar (Kushimo *et al.*, 2015). Notably found in Africa, *Amblyomma hebraeum*, *Amblyomma variegatum*, *Amblyomma pomposum*, and *Amblyomma tholloni* are important examples as they are documented for their role as vectors of various pathogens affecting livestock (Mantos *et al.*, 2008; Mandara, 2018; Smit *et al.*, 2024). The distribution of *Amblyomma* species is restricted to warm, humid areas which allow for their life cycle and reproduction (Nava *et al.*, 2008).

On the other hand, the genus *Rhipicephalus* within the family ixodidae and the phylum Arthropoda includes 82 species recognized, species such as *Rhipicephalus (Boophilus) microplus*, *Rhipicephalus appendiculatus*, *Rhipicephalus sanguineus*, and *Rhipicephalus everts* (De Matos *et al.*, 2009; Desalegn *et al.*, 2015; Guglielmone *et al.*, 2010). *Rhipicephalus* is a well-known tick genus infesting cattle and several domestic animals in different parts of Africa. *Rhipicephalus microplus* has recently invaded several West African countries and poses great danger to livestock (Adedayo and Olukunle 2018). In South Africa, *Rhipicephalus microplus* is also expanding its range and invade on regions traditionally inhabited by *Rhipicephalus decoloratus*, leading to a shift in the distribution of these ticks, with potential implications for livestock production and tick-borne diseases (Nyangiwe *et al.*, 2013; Nyangiwe *et al.*, 2017). There is evidence of a changing abundance of the species of *Rhipicephalus* in response to environmental factors. For example, *Rhipicephalus sanguineus* survives in many climatic conditions, while *R. appendiculatus* is very tolerant to high temperatures (Yousseu *et al.*, 2022; Makwarela *et al.*, 2024). Ongoing molecular studies have shown that the scope of diversity and the structure of components within the *Rhipicephalus* have undergone the most change due to control measures (Dantas-Torres *et al.*, 2013). Acaricide use has led to resistance development and genetic bottlenecks in *R. microplus* populations (Cutullé *et al.*, 2009; Robbertse *et al.*, 2016).

2.2.3 Role of ticks as vectors of diseases

Ticks are important vectors for pathogens which affect humans and animals globally (Parola and Raoult, 2001; Estrada-Pena *et al.*, 2008). Ticks transmits various and multiple pathogen types, including viruses, bacteria, and protozoa (Estrada-Peña *et al.*, 2008; Boulanger *et al.*,

2019). Tick-borne diseases (TBDs) present significant veterinary and public health challenges in South Africa. Several zoonotic pathogens, including *Rickettsia africae*, *Rickettsia massiliae*, and *Coxiella burnetii*, have been identified as causative agents of diseases such as African tick bite fever and Q fever in humans (Chitanga *et al.*, 2014; Halajian *et al.*, 2016; Freaan and Blumberg, 2002). In addition to these zoonotic threats, common tick-borne pathogens affecting livestock include *Babesia* spp., *Theileria* spp., and *Anaplasma marginale* (Guo *et al.*, 2019). These pathogens are responsible for a range of diseases that impact animal health and agricultural productivity.

Tick-borne viruses are major public health threats associated with encephalitis and hemorrhagic fever (Charrel *et al.*, 2004; Diaz, 2020). These viruses are classified mainly into three families of Bunyaviridae, Flaviviridae, and Reoviridae (Lani *et al.*, 2014). Important pathogens include Crimean-Congo hemorrhagic fever caused by *Crimean-Congo hemorrhagic fever virus* (CCHFV), tick-borne encephalitis caused by *Tick-borne encephalitis virus* (TBEV), and the newer Bourbon and Heartland viruses (Diaz, 2020). In India, *Hyalomma anatolicum* and *Haemaphysalis spinigera* transmit CCHFV and Kyasanur forest disease-causing pathogens, respectively (Patel *et al.*, 2021). In South Africa, there is limited literature on tick-borne viruses.

Babesiosis and theileriosis exemplify tick-borne protozoan diseases that significantly impact livestock productivity globally (Bakheit *et al.*, 2007). The widespread distribution of tick vectors contributes to public health concerns alongside other bacterial and viral infections (Rochlin and Toledo, 2020). Recent studies have documented an extensive array of tick-borne pathogens in Europe, including bacterial and viral pathogens that impact wildlife, domestic animals, and humans (Moraga-Fernández *et al.*, 2023; de la Fuente *et al.*, 2023; EFSA AHAW, 2010). Additionally, recent studies in Southern Africa have revealed a diverse range of ticks and tick-borne pathogens affecting both livestock and wildlife. A systematic review identified 26 tick species from seven genera in cattle reared by smallholder farmers, with *Rhipicephalus* (*Boophilus*) *decoloratus* and *Rhipicephalus evertsi evertsi* being the most common (Monakale *et al.*, 2024). In wild animals, 49 tick species from nine genera were reported, with *Amblyomma marmoreum* and *Amblyomma hebraeum* among the most frequently observed (Ledwaba *et al.*, 2022). Pathogens such as *Babesia*, *Anaplasma*, *Theileria*, and *Rickettsia* were detected in various hosts (Monakale *et al.*, 2024; Ledwaba *et al.*, 2022; Ledger *et al.*, 2021). Although anaplasmosis, ehrlichiosis, and babesiosis are mainly veterinary important diseases, they are

developing as essential problems of human infection, presenting atypical clinical syndromes that are often hard to recognize (Andonova *et al.*, 2024). The growing burden of these infections adds to existing concerns and highlights the need for improved understanding in their clinical, epidemiological, and laboratory aspects for effective control, rapid diagnosis, and treatment (Rochlin and Toledo, 2020; Andonova *et al.*, 2024).

2.3 Ecology of *Amblyomma hebraeum* and *Rhipicephalus evertsi evertsi* in South Africa

Tick distribution and seasonal activities of *A. hebraeum* and *R. e. evertsi* differ in various localities of South Africa. *Amblyomma hebraeum* is found in regions of the Eastern Cape, Mpumalanga, North West provinces, as well as in Limpopo Province, where recent studies have reported its presence (Schroder and Reilly, 2013; Nel, 2018; Monakale *et al.*, 2024; Makwarela *et al.*, 2023), because it flourishes in grass-covered, shaded, and well-drained areas (Norval, 1977). The *R. e. evertsi* tick is widespread across all nine provinces (Booyesen *et al.*, 2017). Both these species reach peak adult activity during the summer months (Norval, 1977; Rechav, 1982), also when the rest of the species are more active (Rechav, 1982; Yawa *et al.*, 2018). These ectoparasites infest various domestic animals including cattle, donkeys, goats, sheep and even horses (Bryson *et al.*, 2002; Horak *et al.*, 2009; Booyesen *et al.*, 2017).

Amblyomma hebraeum is primarily known as a vector for numerous pathogens such as *Ehrlichia ruminantium*, which causes heartwater in ruminants (Mnisi *et al.*, 2022). This tick species is found in humid areas with thick vegetation that supports its survival and feeding (Ramotadima *et al.*, 2020). It has been reported that *A. hebraeum* also parasitises various wild animals, indicating a broad host range (Sungirai *et al.*, 2016). Specifically in cattle, these ticks feeds on the non-hairy parts of the body, including the perineum and the genital region, resulting in terrible irritation and even superinfection (McCrindle *et al.*, 2019). The *A. hebraeum* found on domestic and wild animals exemplifies its broad host versatility and increased circumstantial adaptation to animals that maintain proper feeding and breeding conditions (Ledger and Béati, 2021). *Rhipicephalus evertsi evertsi* is an economically important tick species that transmits several pathogens to livestock. Studies have shown that *R. e. evertsi* can transmit *Anaplasma ovis* and harbor DNA of *Rickettsia* spp. and *Rickettsia africae* (Teshale *et al.*, 2016). Additionally, this tick species has been found to carry *Anaplasma phagocytophilum*, *Coxiella burnetii*, *Ehrlichia ruminantium*, and various *Rickettsia* species in South African provinces (Mtshali *et al.*, 2015). In contrast to *A. hebraeum*

ticks, *R. e. evertsi* exhibits a unique feeding behaviour. This species is identified as a two-host tick, primarily feeding on cattle and wild ungulates. It's notable that tropic behaviour involves roaming in search of hosts (Mechouk *et al.*, 2022).

Both tick species exhibit unique feeding routines and patterns that could impact their relationships with various hosts. For example, males of both species have been described to practice interrupted feeding, in which they detach from and attach to their hosts several times during one feeding session (Tuppurainen *et al.*, 2010). This behaviour might increase the likelihood of pathogen transmission between different hosts, especially in regions with high host population density (Lubinga *et al.*, 2013). In addition, some *R. e. evertsi* and *A. hebraeum* ticks have been reported co-infesting a single host without any adverse effect on each other's abundance, suggesting a degree of niche segregation based on attachment site preferences (Anderson *et al.*, 2013).

2.3.1 Epidemiological relevance to livestock

The epidemiological importance of *A. hebraeum* and *R. e. evertsi* concerning livestock is important in sub-Saharan Africa, where their high prevalence contributes to the burden of TBDs. *Amblyomma hebraeum* is a vector of *Ehrlichia ruminantium*, which causes heartwater disease of wild and domestic ruminants, that remains a serious obstacle to animal husbandry health and productivity despite the region's abundant resources (Ramotadima *et al.*, 2020; Horak *et al.*, 2021; Dlamkile *et al.*, 2023). Clinical manifestations of heartwater disease in cattle, sheep and goats include high fever, nervous signs such as ataxia and convulsions, respiratory distress, and in severe cases, sudden death without preceding clinical signs (Allsopp, 2010; Yunker, 1996). The disease poses a serious obstacle to animal husbandry in endemic areas of sub-Saharan Africa due to high mortality rates in susceptible livestock, particularly in animals introduced from non-endemic regions. Losses are further compounded by reduced productivity in recovered animals, costs of intensive tick control measures, and the economic burden of treatment and prevention programmes (Allsopp, 2010). There is great concern regarding the distribution of *A. hebraeum* as it is reportedly widespread in different ecological zones of Southern Africa, such as Botswana and South Africa, where it has been linked to increasing clinical cases of heartwater in livestock (Ramotadima *et al.*, 2020; Dlamkile *et al.*, 2023).

The widespread distribution of *R. e. evertsi* has been reported in sub-Saharan Africa, where it is often found together with *A. hebraeum* in many livestock herds (Sungirai *et al.*, 2016; Mapholi *et al.*, 2022). This tick species is also known to be involved in the transmission of several pathogenic organisms, such as *Anaplasma marginale*, causing bovine anaplasmosis in cattle (Masika *et al.*, 1997), and *Babesia bigemina*, causing bovine babesiosis in cattle (De Waal, 2000). It is also an important vector of *Babesia caballi* and *Theileria equi*, which cause equine piroplasmiasis in horses (Onyiche *et al.*, 2019). In addition, *R. e. evertsi* is implicated in the transmission of *Theileria mutans* in cattle (Neitz, 1956) and *Anaplasma centrale*, a mild form of anaplasmosis in cattle (Masika *et al.*, 1997). Although *R. e. evertsi* has been recorded feeding on African buffalo and cattle in *Theileria parva*-endemic areas, current evidence does not strongly support it as a major vector of *T. parva*, with *Rhipicephalus appendiculatus* being the primary vector in East and southern Africa (Maboko *et al.*, 2021). The overlap in the distribution of these two tick species increases the risk of pathogen co-infection in livestock, complicating disease management and control efforts. Detection and surveillance of TBDs rely on a combination of conventional and molecular diagnostic methods. Traditional diagnostic methods like microscopy and serological assays (ELISA, IFA) are widely used but have limitations in sensitivity and specificity (Bakheit *et al.*, 2007; Bluth and Bluth, 2018). Molecular techniques, particularly PCR-based methods, offer improved detection and differentiation of TBPs (Bluth and Bluth, 2018). A notable advancement is the development of high-throughput microfluidic PCR, capable of detecting multiple pathogens in numerous samples simultaneously (Moutailler and Galon, 2024).

Both *A. hebraeum* and *R. e. evertsi* are known to survive and thrive in different environmental conditions, which increases the likelihood of them parasitising livestock and transmitting various TBPs in various regions (Sungirai *et al.*, 2015; Horak *et al.*, 2015). For example, *R. e. evertsi* is reportedly the most prevalent tick in several studies undertaken in Ethiopia and Sudan, and is recognised as a crucial ectoparasite in these regions (ELGhali and Hassan, 2012; Magzoub *et al.*, 2020). In addition, the occurrence of these ticks in some wildlife, such as African buffalo, raises the possibility of wildlife-livestock-human interactions, which may facilitate the transmission of tick-borne pathogens (Anderson *et al.*, 2013; Pillay *et al.*, 2022). *Amblyomma hebraeum* and *R. e. evertsi* are prevalent tick species in South Africa, particularly on livestock across multiple provinces (Booyesen *et al.*, 2017; Makwarela *et al.*, 2024). Numerous studies have documented their ability to thrive under diverse ecological conditions, reflecting their importance as vectors of veterinary pathogens and their ongoing significance

in South African tick-borne disease research (Walker *et al.*, 2003; Horak *et al.*, 2010; Khoza *et al.*, 2024).

2.4. Bacterial communities associated with hard ticks

2.4.1 Microbiome composition in hard ticks

The microbiota profile of hard ticks in Africa has become a target for studying vector competence and pathogen transmission on the continent. Several recent studies have focused on the bacterial microorganisms in hard ticks from different hosts, regions, and ecosystems (Carpi *et al.*, 2011; Van Treuren *et al.*, 2015; Bonnet *et al.*, 2017). Research suggests that tick microbiomes in Africa are highly complex and profoundly affect the health of both animals and humans. Research done by Olivieri *et al.* (2021) and Mulavu *et al.* (2024) has proven the existence of pathogenic and symbiotic endosymbionts in hard ticks and other bacterial pathogens. Olivieri *et al.* (2021) studied ticks collected from various domestic animals, wildlife and environmental sources regionally and discovered a wide range of bacterial species, including rickettsiae, *Anaplasma* spp., *Ehrlichia ruminantium*, *Borrelia garinii*, *Babesia* spp., *Theileria* spp. and *Coxiella burnetii* and other microorganisms that potentially influence vector efficiency. Similarly, Mulavu *et al.* (2024) studied the tick microbiome in Zambia and highlighted that ecological contexts and tick-host interactions shape the composition of bacterial communities.

Research targeting ticks collected from livestock further emphasises the complex nature of these microbiomes. Chigwada *et al.* (2022) used high-throughput 16S rRNA amplicon sequencing to characterise the microbiota of *Amblyomma* and *Hyalomma* ticks infesting Nguni cattle, revealing bacterial communities that included both commensal bacteria and potential pathogenic taxa *Corynebacterium*, *Porphyromonas*, *Anaerococcus*, *Trueperella*, *Helcococcus*, *Rickettsia* and *Coxiellaceae*. Similarly, Chitanga *et al.* (2021) reported on the molecular detection and characterisation of *Rickettsia* species in ixodid ticks from southern Zambia, highlighting that pathogens such as *R. africae* are standard components of the tick microbiota, contributing to the epidemiology of tick-borne diseases in sub-Saharan Africa.

Additionally, studies on wildlife-associated ticks add depth to our understanding of the microbiota present in African tick species. In 2025, Mitchell *et al.* (2025) investigated ticks

infesting white rhinoceros in the Kruger National Park, South Africa, which revealed that *Proteobacteria*, particularly *Coxiella* and *Rickettsia* species, are dominant components of the microbiota of *A. hebraeum* and *Dermacentor rhinoceros*, emphasising the impact of host species and environment on microbiota composition. Moreover, geographical research demonstrates that there are differences in the tick-associated microbiota. In Tunisia, Benyedem *et al.* (2022) provided insights into the microbiota of *Hyalomma* ticks using next-generation sequencing, identifying known pathogens and an array of commensal bacteria whose functions remain fully elucidated. The endo-symbionts *Francisella*, *Mitochondria mitochondrii*, and *Rickettsia* were shown to be the most prevalent in *Hyalomma* spp. *Rickettsia*, *Francisella*, *Ehrlichia*, and *Erwinia* are the most common zoonotic bacteria found in *Hyalomma* ticks. Similarly, Kratou *et al.* (2025) studied the microbiota of *H. dromedarii* ticks cammels from Tunisia and Saudi Arabia, highlighting the influence of geographic origin on the structure of the bacterial community. The study findings indicated a dominant presence of *Francisella* endosymbionts in Tunisian ticks, suggesting adaptive roles of *H. dromedarii* ticks in arid ecosystems. Keystone taxa, particularly *Staphylococcus* and *Corynebacterium*, were identified as central to microbial network structure and resilience. Moreover, network robustness analyses demonstrated enhanced ecological stability in the Tunisian tick microbiota under perturbation, indicated higher resilience to environmental fluctuations compared to Saudi Arabian ticks. Additionally, functional pathway predictions further revealed geographically distinct metabolic profiles between both groups (Tunisia vs. Saudi Arabia and males vs. females), underscoring environmental and biological influences on *H. dromedarii* microbiota assembly.

Furthermore, emerging literature investigates the impact of tick species and developmental stages on the structure and composition of associated microbiota. Kisten *et al.* (2021) conducted a pilot study aimed to determine baseline microbial community within *A. hebraeum* nymphs infected- and non-infected with *Rickettsia africae* from the environment, and within adult ticks infected- and non-infected with *R. africae* collected from cattle sampled from two locations in the Eastern Cape province of South Africa. Additionally, Ackermann *et al.* (2024) described the bacterial microbiome of *Rhipicephalus sanguineus* ticks, which were collected from dogs in rural South Africa, while Khogali *et al.* (2025) explored camel ticks and their relationships with symbionts and pathogens.

These studies emphasised that multiple factors, such as tick species, host animals, geographical location, and environmental conditions, influence the microbiota of hard ticks in Africa. The

presence of both symbiotic and pathogenic bacteria, characterised via advanced high-throughput sequencing and bioinformatics techniques, highlights the complexity of ticks' microbiota and their likely impact on pathogen transmission. This composite knowledge enhances our understanding of tick physiology and vector competence while serving as an indicator for the presence and distribution of TBPs, as well as a basis for possible measures aimed at preventing tick-borne infections in Africa (Chitanga *et al.*, 2021; Olivieri *et al.*, 2021; Kisten *et al.*, 2021; Chigwada *et al.*, 2022; Benyedem *et al.*, 2022; Mulavu *et al.*, 2024; Ackermann *et al.*, 2024; Mitchell *et al.*, 2025; Kratou *et al.*, 2025; Khogali *et al.*, 2025).

2.4.2 Methods for studying bacterial communities in ticks

Over the past ten years, research on tick-associated bacterial communities from Africa have utilised PCR-targeted and high-throughput amplicon sequencing techniques to characterise microbial diversity (Cabezas-Cruz *et al.*, 2018). North African region studies have utilised 16S rRNA-based PCR assays with partial sequencing to screen *Ehrlichia* spp. and other medically important bacteria rapidly. For instance, Sarih *et al.* (2005) designed a broad-range 16S rRNA gene primer set using PCR approach to analyse ticks from Tunisia and Morocco, establishing a robust framework for pathogen detection. Simultaneously, next-generation sequencing (NGS) has successfully been applied to vast bacterial profiling, enabling the study of core microbiota and minor clinically relevant taxa (Portillo *et al.*, 2007; Benyedem *et al.*, 2022). This combination of targeted PCR enhancement and high-throughput amplicon sequencing is a powerful strategy to characterise the microbiome diversity in African tick specimens.

In addition to PCR and amplicon sequencing methods, metabarcoding has become a cornerstone of profiling tick microbiomes, aiding in understanding tick microbial community structure and functional potential, as depicted in Figure 2.2. Benyedem *et al.* (2022) applied Metabarcoding to analyse the microbiota of *Hyalomma* ticks from Tunisia, revealing a wide range of symbiotic and pathogenic microbes, as well as the impact of extrinsic factors like tick gender, developmental stage, and host interactions. Complementary to these efforts, Portillo *et al.* (2007) utilised conventional PCR in conjunction with molecular techniques to detect *R. africae* in ticks from South Africa and Botswana, emphasising the regional epidemiological significance and the importance of using molecular diagnostic tools to track tick-borne diseases. These studies illustrate the need to merge metabarcoding information alongside

classical molecular techniques to fully comprehend the relationships between ticks and infecting bacteria.

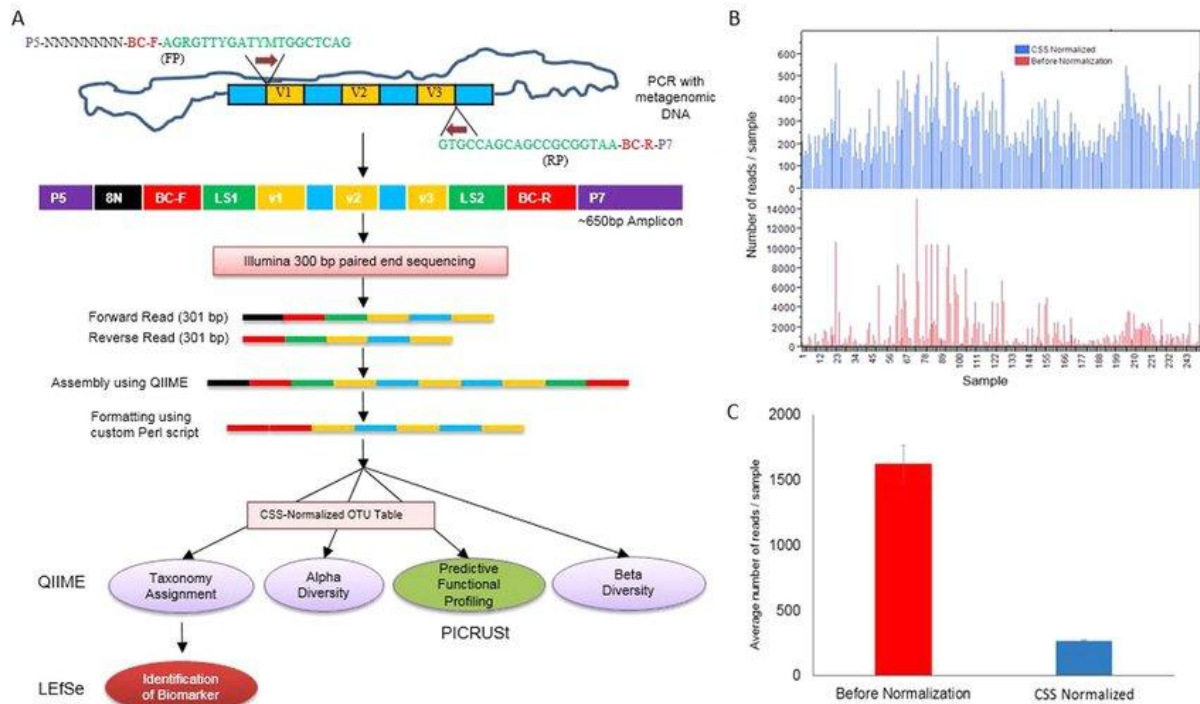


Figure 2.2: Schematic strategy for 16S rRNA amplicon sequencing using next generation sequencing (Mandal *et al.*, 2016). (a) A flow diagram representing library preparation and formatting reads acceptable to QIIME analysis was further analysed using LefSe and PICRUST. The normalised OTU table was used for PICRUST analysis. (FP = forward primer, RP = reverse primer, V = variable region of 16S rRNA gene, P5 and P7 = Illumina sequencing primers, N = random nucleotide, BC-F = barcode of forward primer, BC-R = barcode of reverse primer, and LS = linker sequence). (b) Bar graph showing the read distribution of 252 samples before (red) and after normalisation (blue) of the OTU table. (c) Bargraph showing average number of reads per sample with standard error before (red) and after normalisation (blue).

Moreover, comparative studies using metabarcoding have facilitated understanding the diversity and dynamics of microbes within ticks. Studies conducted outside of Africa indicated that bacterial community composition changes are associated with geographical and host factors (Kurilshikov *et al.*, 2015; Adegoke *et al.*, 2020). Although not exclusively focused on Africa, these approaches offer a systematic approach to studying these populations in African ticks. For instance, alpha and beta diversity analyses, as demonstrated by Takhampunya *et al.* (2021), enhance the understanding of bacterial community differentiation that is expected to

occur in diverse African ecosystems. Such adaptable and flexible methodological approaches are essential to describe complex microbial relationships and for surveillance strategies aimed at predicting and mitigating outbreaks of tick-borne diseases.

2.4.3 Advancements in tick microbiome methods

The exploration of microbial communities within ticks has significantly evolved, largely due to advancements in high-throughput sequencing technologies and the incorporation of various ‘omics’ approaches (Lobanov *et al.*, 2022). Contemporary research emphasizes the use of multi-omics strategies, which include metagenomics, transcriptomics, and proteomics, to develop a comprehensive, systems-level understanding of the tick microbiome and its intricate relationship with the host organism (Arıkan and Muth, 2023). This holistic approach enables detailed cataloguing of microbial taxa as well as the elucidation of gene expression dynamics and protein functions within the microbiome, providing a more nuanced perspective on microbial activities and interactions (Arıkan and Muth, 2023).

Functional metagenomics has also emerged as a powerful tool specifically aimed at identifying genes and metabolic pathways that are crucial for microbial interactions, including symbiotic relationships, pathogen transmission mechanisms, and the prevalence of antimicrobial resistance within tick microbiomes (Simon and Daniel, 2009). This approach is particularly valuable for characterizing previously unknown functions of uncultivable microbes residing within ticks (Cabezas-Cruz *et al.*, 2018), thus broadening our understanding of microbial capabilities and their ecological roles.

2.5. Factors influencing tick microbiota composition

Factors such as the environment and ecology are critical in defining the composition of tick microbiota. Studies indicate that ticks acquire microbes from their surroundings, which are impacted by local bacterial populations and ecological relationships (Kwan *et al.*, 2017; Gurfield *et al.*, 2017). For example, certain bacteria in the environment could modify the microbial community structure in ticks because of the differences in ticks feeding on infected and uninfected hosts (Abraham *et al.*, 2017). Moreover, temperature, humidity, and types of

landscape can also influence the diversity and composition of microbes in different populations of ticks (Aivelo *et al.*, 2021; Krawczyk *et al.*, 2022).

The diversity of tick microbiota is significantly influenced by the host species and their diet. Research has shown that the microbiome of ticks varies depending on the host species they feed on (Rynkiewicz *et al.*, 2015; Zhang *et al.*, 2019). For instance, ticks that feed on various rodent hosts show changes in the microbiome composition, which can affect their ability to harbour and transmit pathogens (Rynkiewicz *et al.*, 2015). Additionally, the tick feeding patterns, specifically during blood meals, are associated with higher microbial diversity due to acquiring the host microorganisms (Andreotti *et al.*, 2011; Benyedem *et al.*, 2022). This dynamic interaction highlights the importance of host-related factors in shaping tick microbiomes.

Tick microbiome composition differs by region due to climate and geographical location. There is evidence supporting significant geographical differences in the microbial composition of ticks, which implies that local environmental factors and isolation by distance play a role (Krawczyk *et al.*, 2022). Different regions can possess different climates, and as a result, the tick microbiomes can differ because climatic factors dictate the microbial reservoirs available in the environment (Kumar *et al.*, 2022; Tóth, 2023). In addition, climatic characteristics, including temperature and rainfall, can influence the activity and habitat selection of ticks, thus affecting their microbiome (Aivelo *et al.*, 2021; Tóth, 2023).

The different tick life stages are important factors influencing their microbial diversity. Studies indicate that microbial diversity is usually highest during the larval stage and declines from the nymphal to the adult stages, where the microbiota is frequently dominated by some specific endosymbionts (Chicana *et al.*, 2019; Adegoke *et al.*, 2021). This pattern suggests that as ticks mature, their interactions with hosts and environmental factors may lead to a more stable but less diverse microbiome (Aivelo *et al.*, 2021; Adegoke *et al.*, 2022). Moreover, potential causes for sex-specific variation in microbiome composition have also been observed, potentially due to variations in physiology and behaviour between male and female ticks (Obregón *et al.*, 2019; Estrada-Peña *et al.*, 2020).

2.6. Tick microbiome and genetic diversity interactions

Recent studies have highlighted the significance of microbial communities' in shaping the tick genetic diversity and their capacity to transmit pathogens. Studies show that the tick gut microbiota varies significantly based on tick species, sex, and environmental factors. Obregón *et al.* (2019) showed that the taxonomic and functional composition of the tick gut microbiota differs remarkably between male ticks and females, and this may reflect some level of specialisation that corresponds to reproductive and life cycle processes. Additionally, Kwan *et al.* (2017) provided evidence that ticks, like other animals, have and acquire microbial diversity from the environment, which significantly influences the composition and diversity of their microbiota. Such acquisition of microorganisms from the environment may result in changes in the genetics of the tick populations, as different microbial communities may provide other benefits and disadvantages relating to pathogen transmission.

Research shows that diversity in the tick genetics and microbiome affects the transmission of TBDs. Rynkiewicz *et al.* (2015) reported that the bacterial population identified within the tick collected from XX correlated with the blood microbiomes of the rodents, suggesting potential interactions that could be influencing the presence or dynamics of the pathogens. Moreover, Zhang *et al.* (2019) reported that fed ticks had higher microbe diversity than unfed ticks, which suggests that feeding has the potential to change the microbial community structure and, therefore, change the ability of the tick to act as a vector for pathogens.

Gall *et al.* (2017) highlighted that lab colonisation has the effect of homogenising the microbiome associated with field-collected ticks, implying that some genetic components interact with microbial communities in affecting tick populations. Significant differences were observed in the microbial community structure among various tick species, which may indicate genetic diversity and adaptations to ecological niches (Namina *et al.*, 2023). These study findings highlight the importance of considering microbial and genetic factors when studying tick-borne disease dynamics.

2.7. Regional context: Livestock and tick-borne diseases in North West Province, South Africa

Livestock farming in the North West Province of South Africa is crucial to the region's economy and food security. There are approximately 7500 commercial farms within the

province, which significantly add value to the agricultural output of South Africa (Seoraj-Pillai and Pillay, 2016). This livestock farming sub-sector comprises smallholder farmers and large-scale commercial farmers who predominantly produce cattle, sheep and goats (Mokoena, 2023). The area's climate is semi-arid, with an average annual rainfall of about 481 mm, which makes livestock farming particularly difficult because of limited water resources and poor-quality pasture (Botai *et al.*, 2016). Furthermore, the economic feasibility of livestock farming is often threatened by various factors, including disease outbreaks and environmental factors such as drought (Botai *et al.*, 2016).

Tick-borne diseases (TBDs) are a significant concern for livestock health in the North West Province. The prevalence of ticks like *A. hebraeum*, *R. microplus*, *R. e. evertsi*, and *R. mimeticus* has been documented, demonstrating their colonisation in the area (Horak *et al.*, 2015; Nyangiwe *et al.*, 2017). These ticks serve as vectors for multiple disease-causing pathogens such as babesiosis and anaplasmosis, which can result in serious health problems in livestock, resulting in financial losses for farmers (Spickett *et al.*, 2011). Additionally, North West Province hosts key wildlife-rich areas such as Pilanesberg National Park and the Bloemhof Dam Nature Reserve, which support substantial biodiversity within mixed agricultural landscapes (Carruthers, 2011). While direct data on wildlife livestock contact in North West is limited, evidence from broader regional studies identifies watering points and shared grazing areas as potential hotspots for disease transmission among livestock—and possibly at the wildlife–livestock interface (Hlokwe *et al.*, 2016). These combined patterns suggest multiple pathways through which ticks and their pathogens could disseminate across the province

The economic impact of ticks and TBDs on livestock production is significant, affecting farmers' livelihoods through treatment costs, reduced productivity, and animal mortality (Desta, 2016; Nath *et al.*, 2018). Acaricide resistance poses a challenge, increasing control costs and limiting efficacy (Nath *et al.*, 2018). Ticks also reduce the quality of hides and skin, further impacting economic returns (Desta, 2016). While tick control through acaricides remains common, concerns about resistance and environmental contamination necessitate integrated approaches combining chemical and non-chemical methods (Nath *et al.*, 2018). Economic evaluations of tick control programs are complex, considering factors such as production impacts, market access, and non-production benefits (Tisdell *et al.*, 1999). In South Africa, government-led tick control programs may not be economically justified based on cost-

benefit analysis. The overall impact of these elements highlights the necessity of tackling tick-borne diseases to ensure the viability of livestock farming in the North West Province.

References

1. Aboagye-Antwi, F., Heylen, D.J.A. and Kumsa, B., 2023. Tick communities of cattle in smallholder rural livestock production systems in sub-Saharan Africa.
2. Abraham, N.M., Liu, L., Jutras, B.L., Yadav, A.K., Narasimhan, S., Gopalakrishnan, V., Ansari, J.M., Jefferson, K.K., Cava, F., Jacobs-Wagner, C. and Fikrig, E., 2017. Pathogen-mediated manipulation of arthropod microbiota to promote infection. *Proceedings of the National Academy of Sciences*, 114(5), pp.E781-E790.
3. Ackermann, R.E., Gall, C.A., Brayton, K.A., Collins, N.E., Wyk, I., Wentzel, J., Kolo, A.O. and Oosthuizen, M.C., 2024. The bacterial microbiome and pathogen reservoir potential of *Rhipicephalus sanguineus* ticks in South Africa. *Ticks and Tick-borne Diseases*, 15(1–2), pp.37–45.
4. Adedayo, A.F. and Olukunle, F.B., 2018. First evidence of an established *Rhipicephalus (Boophilus) microplus* population in Nigeria. *Alexandria Journal of Veterinary Sciences*, 56(1), pp.182–186.
5. Adegoke, A., Kumar, D., Bobo, C., Rashid, M.I., Durrani, A.Z., Sajid, M.S. and Karim, S., 2020. Tick-borne pathogens shape the native microbiome within tick vectors. *Microorganisms*, 8(9), p.1299.
6. Adegoke, A., Kumar, D., Budachetri, K. and Karim, S., 2022. Hematophagy and tick-borne Rickettsial pathogen shape the microbial community structure and predicted functions within the tick vector, *Amblyomma maculatum*. *Frontiers in Cellular and Infection Microbiology*, 12, p.1037387.
7. Aivelo, T., Lemoine, M. and Tschirren, B., 2022. Elevational changes in bacterial microbiota structure and diversity in an arthropod-disease vector. *Microbial Ecology*, 84(3), pp.868-878.
8. Ali, A., Khan, M.A., Zahid, H., Yaseen, P.M., Qayash Khan, M., Nawab, J., Ur Rehman, Z., Ateeq, M., Khan, S. and Ibrahim, M., 2019. Seasonal dynamics, record of ticks infesting humans, wild and domestic animals, and molecular phylogeny of *Rhipicephalus microplus* in Khyber Pakhtunkhwa, Pakistan. *Frontiers in Physiology*, 10, p.793.
9. Allsopp, B.A., 2010. Natural history of *Ehrlichia ruminantium*. *Veterinary parasitology*, 167(2-4), pp.123-135.

10. Anderson, K., Ezenwa, V.O. and Jolles, A.E., 2013. Tick infestation patterns in free-ranging African buffalo (*Syncerus caffer*): effects of host innate immunity and niche segregation among tick species. *International Journal for Parasitology: Parasites and Wildlife*, 2, pp.1-9.
11. Andonova, R., Bashchobanov, D., Gadzhovska, V. and Popov, G., 2024. Tick-Borne Diseases—Still a Challenge: A Review. *Biologics*, 4(2), pp.130-142.
12. Andreotti, R., Pérez de León, A.A., Dowd, S.E., Guerrero, F.D., Bendele, K.G. and Scoles, G.A., 2011. Assessment of bacterial diversity in the cattle tick *Rhipicephalus (Boophilus) microplus* through tag-encoded pyrosequencing. *BMC Microbiology*, 11, pp.1-11.
13. Arıkan, M. and Muth, T., 2023. Integrated multi-omics analyses of microbial communities: a review of the current state and future directions. *Molecular Omics*, 19(8), pp.607-623.
14. Awa, D.N., Adakal, H., Luogbou, N.D.D., Wachong, K.H., Leinyuy, I. and Achukwi, M.D., 2015. Cattle ticks in Cameroon: is *Rhipicephalus (Boophilus) microplus* absent in Cameroon and the Central African region?. *Ticks and Tick-borne Diseases*, 6(2), pp.117-122.
15. Bakheit, M.A., Seitzer, U., Mbatı, P.A. and Ahmed, J.S., 2007. Serological diagnostic tools for the major tick-borne protozoan diseases of livestock. *Parassitologia*, 49, p.53.
16. Barker, S.C. and Murrell, A., 2004. Systematics and evolution of ticks with a list of valid genus and species names. *Parasitology*, 129(S1), pp.S15-S36.
17. Barradas, P.F., Lima, C., Cardoso, L., Amorim, I., Gärtner, F. and Mesquita, J.R., 2020. Molecular evidence of *Hemolivia mauritanica*, *Ehrlichia* spp. and the endosymbiont *Candidatus* Midichloria mitochondrii in *Hyalomma aegyptium* infesting *Testudo graeca* tortoises from Doha, Qatar. *Animals*, 11(1), p.30.
18. Benyedem, H., Lekired, A., Mhadhbi, M., Dhibi, M., Romdhane, R., Chaari, S., Rekik, M., Ouzari, H.I., Hajji, T. and Darghouth, M.A., 2022. First insights into the microbiome of Tunisian *Hyalomma* ticks gained through next-generation sequencing with a special focus on *H. scupense*. *Plos One*, 17(5), p.e0268172.
19. Bluth, M.J. and Bluth, M.H., 2018. Molecular pathology techniques: advances in 2018. *Clinics in Laboratory Medicine*, 38(2), pp.215-236.

20. Bonnet, S.I., Binetrui, F., Hernández-Jarguín, A.M. and Duron, O., 2017. The tick microbiome: why non-pathogenic microorganisms matter in tick biology and pathogen transmission. *Frontiers in cellular and infection microbiology*, 7, p.236.
21. Booysen, S., Horak, I.G., Heyne, H., Smit, W.J. and Halajian, A., 2017. Parasites of domestic and wild animals in South Africa. L. Ixodid ticks infesting horses and donkeys. *Onderstepoort Journal of Veterinary Research*, 84(1), pp.1-6.
22. Botai, C.M., Botai, J.O., Dlamini, L.C., Zwane, N.S. and Phaduli, E., 2016. Characteristics of droughts in South Africa: A case study of Free State and North West provinces. *Water*, 8(10), p.439.
23. Boulanger, N., Boyer, P., Talagrand-Reboul, E. and Hansmann, Y., 2019. Ticks and tick-borne diseases. *Médecine et Maladies Infectieuses*, 49(2), pp.87-97.
24. Brunner, J.L., LaDeau, S.L., Killilea, M., Valentine, E., Schierer, M. and Ostfeld, R.S., 2023. Off-host survival of blacklegged ticks in eastern North America: A multistage, multiyear, multisite study. *Ecological Monographs*, 93(3), p.e1572.
25. Bryson, N.R., Tice, G.A., Horak, I.G., Stewart, C.G. and Du Plessis, B.J.A., 2002. Ixodid ticks on indigenous goats owned by small-scale farmers in four communal grazing areas in South Africa. *Journal of the South African Veterinary Association*, 73(1), pp.26-30.
26. Cabezas-Cruz, A., Pollet, T., Estrada-Peña, A., Allain, E., Bonnet, S.I. and Moutailler, S., 2018. Handling the microbial complexity associated to ticks. *Ticks and Tick-borne Pathogens*, 1.
27. Carpi, G., Cagnacci, F., Wittekindt, N.E., Zhao, F., Qi, J., Tomsho, L.P., Drautz, D.I., Rizzoli, A. and Schuster, S.C., 2011. Metagenomic profile of the bacterial communities associated with *Ixodes ricinus* ticks. *Public Library of Science ONE*, 6(10), e25604.
28. Carpi, G., Cagnacci, F., Wittekindt, N.E., Zhao, F., Qi, J., Tomsho, L.P., Drautz, D.I., Rizzoli, A. and Schuster, S.C., 2011. Metagenomic profile of the bacterial communities associated with *Ixodes ricinus* ticks. *Public Library of Science ONE*, 6(10), p.e25604.
29. Carruthers, J., 2011. Pilanesberg National Park, North West Province, South Africa: Uniting economic development with ecological design – a history, 1960s to 1984. *Koedoe: African Protected Area Conservation and Science*, 53(1), a1028.

30. Charrel, R.N., Attoui, H., Butenko, A.M., Clegg, J.C., Deubel, V., Frolova, T.V., Gould, E.A., Gritsun, T.S., Heinz, F.X., Labuda, M. and Lashkevich, V.A., 2004. Tick-borne virus diseases of human interest in Europe. *Clinical Microbiology and Infection*, 10(12), pp.1040-1055.
31. Chicana, B., Couper, L.I., Kwan, J.Y., Tahiraj, E. and Sweil, A., 2019. Comparative microbiome profiles of sympatric tick species from the far-western United States. *Insects*, 10(10), p.353.
32. Chigwada, A.D., Mapholi, N.O., Ogola, H.J.O., Mbizeni, S. and Masebe, T.M., 2022. Pathogenic and endosymbiotic bacteria and their associated antibiotic resistance biomarkers in *Amblyomma* and *Hyalomma* ticks Infesting Nguni Cattle (*Bos* spp.). *Pathogens*, 11(4), p.432.
33. Chitanga, S., Chibesa, K., Sichibalo, K., Mubemba, B., Nalubamba, K.S., Muleya, W., Changula, K. and Simulundu, E., 2021. Molecular detection and characterization of *Rickettsia* species in Ixodid ticks collected from cattle in Southern Zambia. *Frontiers in Veterinary Science*, 8, p.684487.
34. Chitanga, S., Gaff, H. and Mukaratirwa, S., 2014. Tick-borne pathogens of potential zoonotic importance in the southern African Region. *Journal of the South African Veterinary Association*, 85(1), pp.01-03.
35. Choudhury, M.K., Shiferaw, Y. and Hussien, A., 2015. Toxicity of *Millettia ferruginea* darasana (family: Fabaceae) against the larvae and adult ticks of *Amblyomma variegatum* Fabricius a three-host tick in cattle. *Journal of Parasitic Diseases*, 39, pp.298-302.
36. Clavijo, J.J., Vásquez, C.L. and Valera, N.C., 2009. Garrapatas duras de los géneros *Amblyomma* Koch y *Rhipicephalus* Koch (Acari: Ixodidae) presentes en la colección de zoología agrícola, decanato de agronomía, Universidad Centro Occidental Lisandro Alvarado, Venezuela. *Entomotropica*, 24(1), pp.41-50.
37. Clayton, K.A., Gall, C.A., Mason, K.L., Scoles, G.A. and Brayton, K.A., 2015. The characterization and manipulation of the bacterial microbiome of the Rocky Mountain wood tick, *Dermacentor andersoni*. *Parasites & Vectors*, 8, pp.1-5
38. Cleveland, C.A., Friedman, M., Thompson, A.T., Haynes, E., Coker, S.M., Bryan II, J.A., Sidouin, M., Ouakou, P.T., Ngandolo, B.N.R. and Yabsley, M.J., 2025. Multi-

- season survey of ixodid tick species collected from domestic dogs in Chad, Africa. *Veterinary Parasitology: Regional Studies and Reports*, 57, p.101165.
39. Cutullé, C., Jonsson, N.N. and Seddon, J., 2009. Population structure of Australian isolates of the cattle tick *Rhipicephalus* (*Boophilus*) *microplus*. *Veterinary Parasitology*, 161(3-4), pp.283-291.
 40. Damian, D., Damas, M., Wensman, J.J. and Berg, M., 2021. Molecular diversity of hard tick species from selected areas of a wildlife-livestock interface ecosystem at Mikumi National Park, Morogoro Region, Tanzania. *Veterinary Sciences*, 8(3), p.36.
 41. Dantas-Torres, F., Latrofa, M.S., Annoscia, G., Giannelli, A., Parisi, A. and Otranto, D., 2013. Morphological and genetic diversity of *Rhipicephalus sanguineus* sensu lato from the New and Old Worlds. *Parasites & Vectors*, 6, pp.1-17.
 42. de la Fuente, J., Antunes, S., Bonnet, S., Cabezas-Cruz, A., Domingos, A.G., Estrada-Peña, A., Johnson, N., Kocan, K.M., Mansfield, K.L., Nijhof, A.M. and Papa, A., 2017. Tick-pathogen interactions and vector competence: Identification of molecular drivers for tick-borne diseases. *Frontiers in Cellular and Infection Microbiology*, 7, p.114.
 43. de la Fuente, J., Estrada-Peña, A., Rafael, M., Almazán, C., Bermúdez, S., Abdelbaset, A.E., Kasaija, P.D., Kabi, F., Akande, F.A., Ajagbe, D.O. and Bamgbose, T., 2023. Perception of ticks and tick-borne diseases worldwide. *Pathogens*, 12(10), p.1258.
 44. de la Fuente, J., Estrada-Peña, A., Venzal, J.M., Kocan, K.M. and Sonenshine, D.E., 2008. Overview: ticks as vectors of pathogens that cause disease in humans and animals. *Frontiers in Bioscience*, 13, pp.6938–6946.
 45. de la Fuente, J., Kopáček, P., Lew-Tabor, A. and Maritz-Olivier, C., 2016. Strategies for new and improved vaccines against ticks and tick-borne diseases. *Parasite Immunology*, 38(12), pp.754-769.
 46. De Matos, C., Siteo, C., Neves, L., Bryson, N.R. and Horak, I.G., 2008. Ixodid ticks on dogs belonging to people in rural communities and villages in Maputo Province, Mozambique. *Onderstepoort Journal of Veterinary Research*, 75(2), pp.103-108.
 47. De Matos, C., Siteo, C., Neves, L., Nöthling, J.O. and Horak, I.G., 2009. The comparative prevalence of five ixodid tick species infesting cattle and goats in

- Maputo Province, Mozambique. *Onderstepoort Journal of Veterinary Research*, 76(2), pp.201-208.
48. De Waal, D.T., 2000. Anaplasmosis control and diagnosis in South Africa. *Annals of the New York Academy of Sciences*, 916(1), pp.474-483.
 49. Desalegn, T., Fikru, A. and Kasaye, S., 2015. Survey of tick infestation in domestic ruminants of Haramaya District, Eastern Hararghe, Ethiopia. *Journal of Bacteriology & Parasitology*, 6(5), p.246.
 50. Desta, B.G.E.A.H., 2016. Review on the impact of ticks on livestock health and productivity. *Journal of Biology, Agriculture and Healthcare*, 6, pp.1–7.
 51. Diaz, J.H., 2020. Emerging tickborne viral infections: what wilderness medicine providers need to know. *Wilderness & Environmental Medicine*, 31(4), pp.489-497.
 52. Djiman, T.A., Biguezoton, A.S. and Saegerman, C., 2024. Tick-borne diseases in Sub-Saharan Africa: a systematic review of pathogens, research focus, and implications for public health. *Pathogens*, 13(8), p.697.
 53. Dlamkile, Z., Neves, L., Morar-Leather, D., Brandt, C., Pretorius, A., Steyn, H. and Liebenberg, J., 2023. Characterisation of South African field *Ehrlichia ruminantium* using multilocus sequence typing. *Onderstepoort Journal of Veterinary Research*, 90(1), pp.1-8.
 54. Due, C., Fox, W., Medlock, J.M., Pietzsch, M. and Logan, J.G., 2013. Tick bite prevention and tick removal. *BMJ (British Medical Journal)*, 347, f7123.
 55. EFSA Panel on Animal Health and Welfare (AHAW), 2010. Scientific Opinion on Geographic Distribution of Tick-borne Infections and their Vectors in Europe and the other Regions of the Mediterranean Basin. *EFSA Journal*, 8(9), p.1723.
 56. ElGhali, A. and Hassan, S.M., 2012. Ticks infesting animals in the Sudan and southern Sudan: past and current status: research communication. *Onderstepoort Journal of Veterinary Research*, 79(1), pp.1-6.
 57. Estrada-Peña, A., Cabezas-Cruz, A. and Obregón, D., 2020. Behind taxonomic variability: the functional redundancy in the tick microbiome. *Microorganisms*, 8(11), p.1829.
 58. Fan, X., Ma, R., Yue, C., Liu, J., Yue, B., Yang, W., Li, Y., Gu, J., Yan, X. and Qi, D., 2023. A snapshot of climate drivers and temporal variation of *Ixodes ovatus*

- abundance from a giant panda living in the wild. *International Journal for Parasitology: Parasites and Wildlife*, 20, pp.162–169.
59. Frean, J. and Blumberg, L., 2002. Tick bite fever and Q fever-a South African perspective. *South African medical journal*, 92(3).
 60. Gall, C.A., Reif, K.E., Scoles, G.A., Mason, K.L., Mousel, M., Noh, S.M. and Brayton, K.A., 2016. The bacterial microbiome of *Dermacentor andersoni* ticks influences pathogen susceptibility. *International Society for Microbial Ecology Journal*, 10(8), pp.1846–1855.
 61. Gall, C.A., Scoles, G.A., Magori, K., Mason, K.L. and Brayton, K.A., 2017. Laboratory colonization stabilizes the naturally dynamic microbiome composition of field collected *Dermacentor andersoni* ticks. *Microbiome*, 5, pp.1-10.
 62. Gasmi, S., Bouchard, C., Ogden, N.H., Adam-Poupart, A., Pelcat, Y., Rees, E.E., Milord, F., Leighton, P.A., Lindsay, L.R., Koffi, J.K. and Thivierge, K., 2018. Evidence for increasing densities and geographic ranges of tick species of public health significance other than *Ixodes scapularis* in Québec, Canada. *Public Library of Science ONE*, 13(8), e0201924.
 63. Gervasi, S.S., Civitello, D.J., Kilvitis, H.J. and Martin, L.B., 2015. The context of host competence: a role for plasticity in host–parasite dynamics. *Trends in Parasitology*, 31(9), pp.419-425.
 64. Getahun Asebe, Y.H., Basu, A. and Shibru, D., 2016. Overview of the biology, epidemiology and control methods against hard ticks: a review. *Global Journal of Science Frontier Research*, 16(C2), pp.33–45.
 65. Guglielmone, A.A. and Nava, S., 2006. The Argentine ticks of the genus *Amblyomma* (Acari: Ixodidae): distribution and hosts. *RIA. Revista de Investigaciones Agropecuarias*, 35(3), pp.133-153.
 66. Guglielmone, A.A., Robbins, R.G., Apanaskevich, D.A., Petney, T.N., Estrada-Peña, A., Horak, I.G., Shao, R. and Barker, S.C., 2010. The Argasidae, Ixodidae and Nuttalliellidae (Acari: Ixodida) of the world: a list of valid species names. *Zootaxa*, 2528(1), pp.1–28.
 67. Guglielmone, A.A., Robbins, R.G., Apanaskevich, D.A., Petney, T.N., Estrada-Peña, A., Horak, I.G., Shao, R. and Barker, S.C., 2010. The Argasidae, Ixodidae and

- Nuttalliellidae (Acari: Ixodida) of the world: a list of valid species names. *Zootaxa*, 2528(1), pp.1–28.
68. Guo, H., Moumouni, P.F.A., Thekiso, O., Gao, Y., Liu, M., Li, J., Galon, E.M., Efstratiou, A., Wang, G., Jirapattharasate, C. and Ringo, A.E., 2019. Genetic characterization of tick-borne pathogens in ticks infesting cattle and sheep from three South African provinces. *Ticks and tick-borne diseases*, 10(4), pp.875-882.
 69. Gurfield, N., Grewal, S., Cua, L.S., Torres, P.J. and Kelley, S.T., 2017. Endosymbiont interference and microbial diversity of the Pacific coast tick, *Dermacentor occidentalis*, in San Diego County, California. *PeerJ*, 5, p.e3202.
 70. Halajian, A., Palomar, A.M., Portillo, A., Heyne, H., Luus-Powell, W.J. and Oteo, J.A., 2016. Investigation of Rickettsia, Coxiella burnetii and Bartonella in ticks from animals in South Africa. *Ticks and Tick-borne Diseases*, 7(2), pp.361-366.
 71. Hlokwé, T.M., De Klerk-Lorist, L.M. and Michel, A.L., 2016. Wildlife on the move: a hidden tuberculosis threat to conservation areas and game farms through introduction of untested animals. *Journal of Wildlife Diseases*, 52(4), pp.837-843.
 72. Hofmeester, T.R., Year. The wild life of tick-borne pathogens. Doctoral dissertation, Wageningen University and Research, Wageningen, The Netherlands.
 73. Horak, I.G., Heyne, H. and Donkin, E.F., 2010. Parasites of domestic and wild animals in South Africa XLVIII. Ticks (Acari: Ixodidae) infesting domestic cats and wild felids in southern Africa. *Onderstepoort Journal of Veterinary Research*, 77(1), pp.1-7.
 74. Horak, I.G., Heyne, H., Williams, R., Gallivan, G.J., Spickett, A.M., Bezuidenhout, J.D. and Estrada-Peña, A., 2018. The genus *Rhipicephalus* Koch, 1844. In: I.G. Horak, H. Heyne, R. Williams and G.J. Gallivan, eds. *The Ixodid Ticks (Acari: Ixodidae) of Southern Africa*. Springer, Cham, pp.287–510.
 75. Horak, I.G., Jordaan, A.J., Van Dalen, E.M. and Heyne, H., 2015. Distribution of endemic and introduced tick species in Free State Province, South Africa. *Journal of the South African Veterinary Association*, 86(1), pp.1-9.
 76. Horak, I.G., Nyangwiwe, N. and De Matos, C., 2009. Species composition and geographic distribution of ticks infesting cattle, goats, and dogs in a temperate and

- a subtropical region of south-east Africa. *Onderstepoort Journal of Veterinary Research*, 76(3), pp.263-276.
77. Hornok, S., Kontschán, J., Takács, N., Heyne, H., Kovács, Á.B., Plantard, O., Keve, G., Fedorov, D., Gyuranecz, M. and Halajian, A., 2023. Molecular-phylogenetic analyses of Ixodes species from South Africa suggest an African origin of bird-associated exophilic ticks (subgenus *Trichotoixodes*). *Parasites & Vectors*, 16(1), p.392.
 78. Hurtado, O.J.B. and Giraldo-Ríos, C., 2018. Economic and health impact of the ticks in production animals. In *Ticks and tick-borne pathogens*. IntechOpen.
 79. Inyagwa, C.M., Obonyo, F.O., Mukolwe, D.L. and Mungube, E.O., 2021. An overview of hard and soft ticks and their control methods. *Combating and Controlling Nagana and Tick-Borne Diseases in Livestock*, pp.166-194.
 80. Jongejan, F. and Uilenberg, G., 2004. The global importance of ticks. *Parasitology*, 129(S1), pp.S3-S14.
 81. Jongejan, F., Berger, L., Busser, S., Deetman, I., Jochems, M., Leenders, T., de Sitter, B., van Der Steen, F., Wentzel, J. and Stoltsz, H., 2020. *Amblyomma hebraeum* is the predominant tick species on goats in the Mnisi Community area of Mpumalanga Province, South Africa, and is co-infected with *Ehrlichia ruminantium* and *Rickettsia africae*. *Parasites & Vectors*, 13, pp.1-12.
 82. Josek, T., Allan, B.F. and Alleyne, M., 2018. Morphometric analysis of chemoreception organ in male and female ticks (Acari: Ixodidae). *Journal of Medical Entomology*, 55(3), pp.547-552.
 83. Karbowiak, G. and Biernat, B., 2016. The role of particular tick developmental stages in the circulation of tick-borne pathogens affecting humans in Central Europe. 2. Tick-borne encephalitis virus. *Annals of Parasitology*, 62(1).
 84. Karbowiak, G., Biernat, B., Stańczak, J., Werszko, J., Szewczyk, T. and Sytykiewicz, H., 2018. The role of particular ticks developmental stages in the circulation of tick-borne pathogens in Central Europe. 6. Babesia. *Annals of Parasitology*, 64(4), pp.265–284.
 85. Karim, S., Budachetri, K., Mukherjee, N., Williams, J., Kausar, A., Hassan, M.J., Adamson, S., Dowd, S.E., Apanskevich, D., Arijo, A. and Sindhu, Z.U., 2017. A

- study of ticks and tick-borne livestock pathogens in Pakistan. *PloS Neglected Tropical Diseases*, 11(6), p.e0005681.
86. Khogali, R., Bastos, A., Getange, D., Bargul, J.L., Kalayou, S., Ongeso, N., Verhoeven, J.T.P., Kabii, J., Ngiela, J., Masiga, D. and Villinger, J., 2025. Exploring the microbiomes of camel ticks to infer vector competence: insights from tissue-level symbiont-pathogen relationships. *Scientific Reports*, 15(1), p.5574.
 87. Khoza, B.L., 2024. *Identification of tick species and their bacterial pathogens from cattle in two provinces of South Africa* (Doctoral dissertation, University of Pretoria).
 88. Kim, M., Kim, J.Y., Yi, M.H., Lee, I.Y., Yong, D., Jeon, B.Y. and Yong, T.S., 2021. Microbiome of *Haemaphysalis longicornis* tick in Korea. *The Korean Journal of Parasitology*, 59(5), p.489.
 89. Kisten, D., Brinkerhoff, J., Tshilwane, S.I. and Mukaratirwa, S., 2021. A pilot study on the microbiome of *Amblyomma hebraeum* tick stages infected and non-infected with *Rickettsia africae*. *Pathogens*, 10(8), p.941.
 90. Kratou, M., Maitre, A., Abuin-Denis, L., Selmi, R., Belkahia, H., Alanazi, A.D., Gattan, H., Al-Ahmadi, B.M., Shater, A.F., Mateos-Hernández, L. and Obregón, D., 2025. Microbial community variations in adult *Hyalomma dromedarii* ticks from single locations in Saudi Arabia and Tunisia. *Frontiers in Microbiology*, 16, p.1543560.
 91. Krawczyk, A.I., Röttjers, L., Fonville, M., Takumi, K., Takken, W., Faust, K. and Sprong, H., 2022. Quantitative microbial population study reveals geographical differences in bacterial symbionts of *Ixodes ricinus*. *Microbiome*, 10(1), p.120.
 92. Kumar, D., Downs, L.P., Adegoke, A., Machtinger, E., Oggenfuss, K., Ostfeld, R.S., Embers, M. and Karim, S., 2022. An exploratory study on the microbiome of northern and southern populations of *Ixodes scapularis* ticks predicts changes and unique bacterial interactions. *Pathogens*, 11(2), p.130.
 93. Kurilshikov, A., Livanova, N.N., Fomenko, N.V., Tupikin, A.E., Rar, V.A., Kabilov, M.R., Livanov, S.G. and Tikunova, N.V., 2015. Comparative metagenomic profiling of symbiotic bacterial communities associated with *Ixodes persulcatus*, *Ixodes pavlovskyi* and *Dermacentor reticulatus* ticks. *PloS one*, 10(7), p.e0131413.

94. Kushimo, O., 2013. The tick genus *Amblyomma* in Africa: phylogeny and multilocus DNA barcoding. Master of Science (M.S.) thesis, Georgia Southern University.
95. Kwan, J.Y., Griggs, R., Chicana, B., Miller, C. and Sweit, A., 2017. Vertical vs. horizontal transmission of the microbiome in a key disease vector, *Ixodes pacificus*. *Molecular Ecology*, 26(23), pp.6578-6589.
96. Leal, B., Zamora, E., Fuentes, A., Thomas, D.B. and Dearth, R.K., 2020. Questing by tick larvae (Acari: Ixodidae): a review of the influences that affect off-host survival. *Annals of the Entomological Society of America*, 113(6), pp.425-438.
97. Ledger, K.J., Beati, L. and Wisely, S.M., 2021. Survey of ticks and tick-borne rickettsial and protozoan pathogens in Eswatini. *Pathogens*, 10(8), p.1043.
98. Ledwaba, M.B., Nozipho, K., Tembe, D., Onyiche, T.E. and Chaisi, M.E., 2022. Distribution and prevalence of ticks and tick-borne pathogens of wild animals in South Africa: A systematic review. *Current Research in Parasitology & Vector-Borne Diseases*, 2, p.100088.
99. Leenders, M.J.T., 2015. Impact of *Amblyomma* ticks on the health of goats in the Mnisi area, near Kruger Park, South Africa, with the aim to develop an intervention strategy to prevent lameness and exposure to heartwater. Master's thesis, University of Pretoria, Pretoria, South Africa.
100. Lobanov, V., Gobet, A. and Joyce, A., 2022. Ecosystem-specific microbiota and microbiome databases in the era of big data. *Environmental Microbiome*, 17(1), p.37.
101. Lubinga, J.C., Tuppurainen, E.S.M., Mahlare, R., Coetzer, J.A.W., Stoltz, W.H. and Venter, E.H., 2015. Evidence of transstadial and mechanical transmission of lumpy skin disease virus by *Amblyomma hebraeum* ticks. *Transboundary and Emerging Diseases*, 62(2), pp.174-182.
102. Lungile, K.B., 2024. Identification of tick species and their bacterial pathogens from cattle in two provinces of South Africa. Master's thesis, University of Pretoria, Pretoria, South Africa.
103. Maboko, B.B., Sibeko-Matjila, K.P., Pierneef, R., Chan, W.Y., Josemans, A., Marumo, R.D., Mbizeni, S., Latif, A.A. and Mans, B.J., 2021. South African buffalo-

- derived *Theileria parva* is distinct from other buffalo and cattle-derived *T. parva*. *Frontiers in Genetics*, 12, p.666096.
104. Magzoub, A., El Ghali, A., Hussien, M.O., Juma, Y. and Mohammed, S.B., 2021. Prevalence of ticks (Acari: Ixodidae) and *Theileria lestoquardi* in sheep at El Huda and El Nuhud animals production research stations, Sudan. *Journal of Parasitic Diseases*, 45(1), pp.146-152.
 105. Mahlobo-Shwabede, S.I., Zishiri, O.T., Thekiso, O.M. and Makalo, M.J., 2021. Molecular detection of *Coxiella burnetii*, *Rickettsia africae* and *Anaplasma* species in ticks from domestic animals in Lesotho. *Pathogens*, 10(9), p.1186.
 106. Makwarela, T.G., Djikeng, A., Masebe, T.M., Nkululeko, N., Nesengani, L.T. and Mapholi, N.O., 2024. Vector abundance and associated abiotic factors that influence the distribution of ticks in six provinces of South Africa. *Veterinary World*, 17(8), p.1765.
 107. Makwarela, T.G., Nyangiwe, N., Masebe, T., Mbizeni, S., Nesengani, L.T., Djikeng, A. and Mapholi, N.O., 2023. Tick diversity and distribution of hard (Ixodidae) cattle ticks in South Africa. *Microbiology Research*, 14(1), pp.42-59.
 108. Makwarela, T.G., Nyangiwe, N., Masebe, T.M., Djikeng, A., Nesengani, L.T., Smith, R.M. and Mapholi, N.O., 2024. Morphological and molecular characterization of tick species infesting cattle in South Africa. *Veterinary Sciences*, 11(12), p.638.
 109. Mandal, R.K., Jiang, T., Al-Rubaye, A.A., Rhoads, D.D., Wideman, R.F., Zhao, J., Pevzner, I. and Kwon, Y.M., 2016. An investigation into blood microbiota and its potential association with bacterial chondronecrosis with osteomyelitis (BCO) in broilers. *Scientific Reports*, 6(1), p.25882.
 110. Mandara, S., 2018. The distribution of *Amblyomma variegatum* and *A. hebraeum* in Zimbabwe and their infection with *Ehrlichia ruminantium* and *Rickettsia africae*. Master's thesis, University of Pretoria, Pretoria, South Africa.
 111. Mapholi, N.O., Banga, C., Dzama, K., Matika, O., Riggio, V., Nyangiwe, N. and Maiwashe, A., 2022. Prevalence and tick loads in Nguni cattle reared in different environmental conditions across four provinces of South Africa. *Veterinary World*, 15(8), p.1943.

112. Masika, P.J., Sonandi, A. and Van Averbek, W., 1997. Perceived causes, diagnosis and treatment of babesiosis and anaplasmosis in cattle by livestock farmers in communal areas of the central Eastern Cape Province, South Africa. *Journal of the South African Veterinary Association*, 68(2), pp.40-44.
113. McCrindle, C.M., Maime, M.J., Botha, E.A., Webb, E.C. and Smuts, M.P., 2019. Scrotal tick damage as a cause of infertility in communal bulls in Moretele, South Africa. *Journal of the South African Veterinary Association*, 90(1), pp.1-7.
114. Mechouk, N., Mihalca, A.D., Deak, G. and Bouslama, Z., 2022. Synopsis of the ticks of Algeria with new hosts and localities records. *Parasites & Vectors*, 15(1), p.302.
115. Mitchell, J.K., Matthee, S., Ndhlovu, A., Miller, M., Buss, P. and Matthee, C.A., 2025. The microbiome and Coxiella diversity in *Amblyomma hebraeum* and *Dermacentor rhinoceros* ticks found on white rhinoceros. *Microbial Ecology*, 88(1), pp.48.
116. Mnisi, S.S., Mphuthi, M.B., Ramatla, T., Mofokeng, L.S., Thekiso, O. and Syakalima, M., 2022. Molecular detection and genetic characterization of *Ehrlichia ruminantium* harbored by *Amblyomma hebraeum* ticks of domestic ruminants in North West Province, South Africa. *Animals*, 12(19), p.2511.
117. Mokoena, O.P., Ntuli, T.S., Ramarumo, T. and Seeletse, S.M., 2023. Exploring the challenges faced by the small-scale dairy farmers in Bojanala Platinum District Municipality in North-West Province, South Africa. *South African Journal of Agricultural Extension*, 51(3), pp.186-209.
118. Monakale, K.S., Ledwaba, M.B., Smith, R.M., Gaorekwe, R.M. and Malatji, D.P., 2024. A systematic review of ticks and tick-borne pathogens of cattle reared by smallholder farmers in South Africa. *Current Research in Parasitology & Vector-borne Diseases*, 6, p.100205.
119. Moraga-Fernández, A., Muñoz-Hernández, C., Sánchez-Sánchez, M., Fernández de Mera, I.G. and de la Fuente, J., 2023. Exploring the diversity of tick-borne pathogens: The case of bacteria (*Anaplasma*, *Rickettsia*, *Coxiella* and *Borrelia*), protozoa (*Babesia* and *Theileria*) and viruses (*Orthonairovirus*, tick-borne encephalitis virus and louping ill virus) in the European continent. *Veterinary Microbiology*, 286, p.109892.

120. Moutailler, S. and Galon, C., 2024. Real-time microfluidic PCRs: a high-throughput method to detect 48 or 96 tick-borne pathogens in 48 or 96 samples. In: L. Gilbert, ed. *Borrelia burgdorferi: Methods and Protocols*. New York, NY: Springer US, pp. 1–17.
121. Mtshali, K., Khumalo, Z.T., Nakao, R., Grab, D.J., Sugimoto, C. and Thekiso, O.M., 2015. Molecular detection of zoonotic tick-borne pathogens from ticks collected from ruminants in four South African provinces. *Journal of Veterinary Medical Science*, 77(12), pp.1573-1579.
122. Mtshali, M.S., De la Fuente, J., Ruybal, P., Kocan, K.M., Vicente, J., Mbatia, P.A., Shkap, V., Blouin, E.F., Mohale, N.E., Moloi, T.P. and Spickett, A.M., 2007. Prevalence and genetic diversity of *Anaplasma marginale* strains in cattle in South Africa. *Zoonoses and public health*, 54(1), pp.23-30.
123. Mtshali, P.S., Tsotetsi, A.M., Thekiso, M.M.O. and Mtshali, M.S., 2014. Nested PCR detection and phylogenetic analysis of *Babesia bovis* and *Babesia bigemina* in cattle from peri-urban localities in Gauteng province, South Africa. *Journal of Veterinary Medical Science*, 76(1), pp.145-150.
124. Mulavu, M., Khumalo, C.S., Moonga, L., Hayashida, K., Mubemba, B., Changula, K., Simulundu, E., Muleya, W. and Chitanga, S., 2024. Defining the bacterial microbiome of ticks in Chongwe and Chisamba Districts of Zambia. *Infectious Medicine*, 3(4), p.100131.
125. Namina, A., Kazarina, A., Lazovska, M., Akopjana, S., Ulanova, V., Kivrane, A., Freimane, L., Sadovska, D., Kimsis, J., Bormane, A. and Capligina, V., 2023. Comparative microbiome analysis of three epidemiologically important tick species in Latvia. *Microorganisms*, 11(8), p.1970.
126. Narasimhan, S. and Fikrig, E., 2015. Tick microbiome: the force within. *Trends in Parasitology*, 31(7), pp.315-323.
127. Narasimhan, S., Rajeevan, N., Liu, L., Zhao, Y.O., Heisig, J., Pan, J., Eppler-Epstein, R., DePonte, K., Fish, D. and Fikrig, E., 2014. Gut microbiota of the tick vector *Ixodes scapularis* modulate colonization of the Lyme disease spirochete. *Cell Host & Microbe*, 15(1), pp.58-71.
128. Narasimhan, S., Schuijt, T.J., Abraham, N.M., Rajeevan, N., Coumou, J., Graham, M., Robson, A., Wu, M.J., Daffre, S., Hovius, J.W. and Fikrig, E., 2017.

Modulation of the tick gut milieu by a secreted tick protein favors *Borrelia burgdorferi* colonization. *Nature Communications*, 8(1), p.184.

129. Nath, S., Mandal, S., Pal, S., Jadhao, S., Ottalwar, N. and Sanyal, P., 2018. Impact and management of acaricide resistance-pertaining to sustainable control of ticks. *International Journal of Livestock Research*, 8(10), pp.46-60.
130. Nava, S., Mangold, A.J. and Guglielmone, A.A., 2008. Aspects of the life cycle of *Amblyomma parvum* (Acari: Ixodidae) under natural conditions. *Veterinary Parasitology*, 156(3-4), pp.270-276.
131. Neitz, W.O., 1956. Classification, transmission, and biology of piroplasms of domestic animals. *Annals of the New York Academy of Sciences*, 64(2), pp.56–111.
132. Norval, R.A.I., 1977. Ecology of the tick *Amblyomma hebraeum* Koch in the Eastern Cape Province of South Africa. I. Distribution and seasonal activity. *The Journal of Parasitology*, pp.734-739.
133. Nyangiwe, N., Harrison, A. and Horak, I.G., 2013. Displacement of *Rhipicephalus decoloratus* by *Rhipicephalus microplus* (Acari: ixodidae) in the eastern cape province, South Africa. *Experimental and Applied Acarology*, 61(3), pp.371-382.
134. Nyangiwe, N., Horak, I.G., Van der Mescht, L. and Matthee, S., 2017. Range expansion of the economically important Asiatic blue tick, *Rhipicephalus microplus*, in South Africa. *Journal of the South African Veterinary Association*, 88(1), pp.1-7.
135. Oberholster, T., 2014. Characterisation of the genetic diversity of the southern cattle tick, *Rhipicephalus microplus*, populations from South Africa. Doctoral dissertation, University of Pretoria, Pretoria, South Africa.
136. Obregón, D., Bard, E., Abrial, D., Estrada-Peña, A. and Cabezas-Cruz, A., 2019. Sex-specific linkages between taxonomic and functional profiles of tick gut microbiomes. *Frontiers in Cellular and Infection Microbiology*, 9, p.298.
137. Olivieri, E., Kariuki, E., Floriano, A.M., Castelli, M., Tafesse, Y.M., Magoga, G., Kumsa, B., Montagna, M. and Sasser, D., 2021. Multi-country investigation of the diversity and associated microorganisms isolated from tick species from

- domestic animals, wildlife and vegetation in selected African countries. *Experimental and Applied Acarology*, 83, pp.427-448.
138. Onyiche, T.E., Suganuma, K., Igarashi, I., Yokoyama, N., Xuan, X. and Thekisoe, O., 2019. A review on equine piroplasmosis: epidemiology, vector ecology, risk factors, host immunity, diagnosis and control. *International journal of environmental research and public health*, 16(10), p.1736.
 139. Parola, P. and Raoult, D., 2001. Ticks and tickborne bacterial diseases in humans: an emerging infectious threat. *Clinical Infectious Diseases*, 32(6), pp.897-928.
 140. Paștiu, A.I., Matei, I.A., Mihalca, A.D., D'Amico, G., Dumitrache, M.O., Kalmár, Z., Sándor, A.D., Lefkaditis, M., Gherman, C.M. and Cozma, V., 2012. Zoonotic pathogens associated with *Hyalomma aegyptium* in endangered tortoises: evidence for host-switching behaviour in ticks?. *Parasites & Vectors*, 5, pp.1-6.
 141. Patel, R., Singh, R., Gupta, B., Rai, A., Dubey, S., Dhakad, B.S. and Soni, D., 2020. Tick borne viral zoonotic diseases: a review. *Journal of Entomology and Zoology Studies*, 8(6), pp.2034–2041.
 142. Pillay, A., Nyangiwe, N. and Mukaratirwa, S., 2023. Low genetic diversity and population structuring of *Amblyomma hebraeum* and *Rickettsia africae* from coastal and inland regions in the Eastern Cape Province of South Africa. *Medical and Veterinary Entomology*, 37(2), pp.275-285.
 143. Portillo, A., Pérez-Martínez, L., Santibáñez, S., Blanco, J.R., Ibarra, V. and Oteo, J.A., 2007. Detection of *Rickettsia africae* in *Rhipicephalus (Boophilus) decoloratus* ticks from the Republic of Botswana, South Africa. *The American Journal of Tropical Medicine and Hygiene*, 77(2), pp.376-377.
 144. Rafidah Lani, R.L., Moghaddam, E.M., Haghani, A.H., Chang, C.L., AbuBakar, S.A. and Zandi, K.Z., 2014. Tick-borne viruses: a review from the perspective of therapeutic approaches. *Ticks and Tick-borne Diseases*, 5(5), pp.457–465.
 145. Ramotadima, I., Hyera, J. and Lebani, K., 2021. Presence and persistence of *Amblyomma hebraeum* augments the burden of heartwater in the southern district of Botswana. *Research Square*.

146. Rechav, Y., 1982. Dynamics of tick populations (Acari: Ixodidae) in the Eastern Cape Province of South Africa. *Journal of Medical Entomology*, 19(6), pp.679-700.
147. Reid, H., 1987. Controlling tick-borne diseases of sheep in Britain. *In Practice*, 9(5), p.189.
148. Rochlin, I. and Toledo, A., 2020. Emerging tick-borne pathogens of public health importance: a mini-review. *Journal of Medical Microbiology*, 69(6), pp.781-791.
149. Rodríguez-Vivas, R.I., Hodgkinson, J.E. and Trees, A.J., 2012. Resistencia a los acaricidas en *Rhipicephalus (Boophilus) microplus*: situación actual y mecanismos de resistencia. *Revista mexicana de ciencias pecuarias*, 3, pp.9-24.
150. Russell, R.C., Otranto, D. and Wall, R.L., 2013. Ticks (hard) (Acari: Ixodidae). In: R.C. Russell, D. Otranto and R.L. Wall, eds. *The Encyclopaedia of Medical and Veterinary Entomology*. Wallingford, UK: CABI, pp.331–366.
151. Rynkiewicz, E.C., Hemmerich, C., Rusch, D.B., Fuqua, C. and Clay, K., 2015. Concordance of bacterial communities of two tick species and blood of their shared rodent host. *Molecular Ecology*, 24(10), pp.2566-2579.
152. Sado Yousseu, F., Simo Tchegnag, H., Kamgang, B., Djonabaye, D., McCall, P.J., Ndip, R.N. and Wondji, C.S., 2022. Infestation rates, seasonal distribution, and genetic diversity of ixodid ticks from livestock of various origins in two markets of Yaoundé, Cameroon. *Medical and Veterinary Entomology*, 36(3), pp.283-300.
153. Sanchez-Vicente, S., Tagliafierro, T., Coleman, J.L., Benach, J.L. and Tokarz, R., 2019. Polymicrobial nature of tick-borne diseases. *mBio*, 10(5), pp.10-1128.
154. Sarih, M.H., M'Ghirbi, Y., Bouattour, A., Gern, L., Baranton, G. and Postic, D., 2005. Detection and identification of *Ehrlichia* spp. in ticks collected in Tunisia and Morocco. *Journal of Clinical Microbiology*, 43(3), pp.1127-1132.
155. Sasaki, K., Honma, M., Nakao, M., Sasaki, M., Hashimoto, Y., Ishida-Yamamoto, A. and Yoshii, K., 2021. Survey to detect tick-borne encephalitis virus from human-feeding ticks in Hokkaido, Japan. *The Journal of Dermatology*, 48(7), pp.1094-1097.
156. Seoraj-Pillai, N. and Pillay, N., 2016. A meta-analysis of human–wildlife conflict: South African and global perspectives. *Sustainability*, 9(1), p.34.

157. Simon, C. and Daniel, R., 2009. Achievements and new knowledge unravelled by metagenomic approaches. *Applied Microbiology and Biotechnology*, 85, pp.265-276.
158. Singh, K., Kumar, S., Sharma, A.K., Jacob, S.S., RamVerma, M., Singh, N.K., Shakya, M., Sankar, M. and Ghosh, S., 2022. Economic impact of predominant ticks and tick-borne diseases on Indian dairy production systems. *Experimental parasitology*, 243, p.108408.
159. Smit, A., Mulandane, F., Labuschagne, M., Wójcik, S.H., Malabwa, C., Sili, G., Mandara, S., Dlamkile, Z., Ackermann, R., Rose Vineer, H. and Stoltz, W.H., 2024. Intra-and interspecific variation of Amblyomma ticks from southern Africa. *Parasites & Vectors*, 17(1), p.364.
160. Spickett, A.M., Heyne, I.H. and Williams, R., 2011. Survey of the livestock ticks of the North West Province, South Africa. *Onderstepoort Journal of Veterinary Research*, 78(1), pp.1-12.
161. Sungirai, M., Abatih, E.N., Moyo, D.Z., Clercq, P.D. and Madder, M., 2017. Shifts in the distribution of ixodid ticks parasitizing cattle in Zimbabwe. *Medical and Veterinary Entomology*, 31(1), pp.78-87.
162. Sungirai, M., Madder, M., Moyo, D.Z., De Clercq, P. and Abatih, E.N., 2015. An update on the ecological distribution of the Ixodidae ticks in Zimbabwe. *Experimental and Applied Acarology*, 66, pp.269-280.
163. Takhampunya, R., Sakolvaree, J., Chanarat, N., Youngdech, N., Phonjatturas, K., Promsathaporn, S., Tippayachai, B., Tachavarong, W., Srinoppawan, K., Poole-Smith, B.K. and McCardle, P.W., 2021. The bacterial community in questing ticks from Khao Yai National Park in Thailand. *Frontiers in Veterinary Science*, 8, p.764763.
164. Teshale, S., Kumsa, B., Menandro, M.L., Cassini, R. and Martini, M., 2016. Anaplasma, Ehrlichia and rickettsial pathogens in ixodid ticks infesting cattle and sheep in western Oromia, Ethiopia. *Experimental and Applied Acarology*, 70(2), pp.231-237.
165. Thapa, S., Zhang, Y. and Allen, M.S., 2019. Effects of temperature on bacterial microbiome composition in *Ixodes scapularis* ticks. *MicrobiologyOpen*, 8(5), p.e00719.

166. Tisdell, C.A., Harrison, S.R. and Ramsay, G.C., 1999. The economic impacts of endemic diseases and disease control programmes. *Revue Scientifique et Technique-Office International des Epizooties*, 18(2), pp.380-398.
167. Tóth, A.G., Farkas, R., Papp, M., Kilim, O., Yun, H., Makrai, L., Maróti, G., Gyurkovszky, M., Krikó, E. and Solymosi, N., 2023. *Ixodes ricinus* tick bacteriome alterations based on a climatically representative survey in Hungary. *Microbiology Spectrum*, 11(6), pp.e01243-23.
168. Tuppurainen, E.S., Stoltz, W.H., Troskie, M., Wallace, D.B., Oura, C.A.L., Mellor, P.S., Coetzer, J.A.W. and Venter, E.H., 2011. A potential role for ixodid (hard) tick vectors in the transmission of lumpy skin disease virus in cattle. *Transboundary and Emerging Diseases*, 58(2), pp.93-104.
169. Van Treuren, W., Ponnusamy, L., Brinkerhoff, R.J., Gonzalez, A., Parobek, C.M., Juliano, J.J., Andreadis, T.G., Falco, R.C., Ziegler, L.B., Hathaway, N. and Keeler, C., 2015. Variation in the microbiota of *Ixodes* ticks with regard to geography, species, and sex. *Applied and environmental microbiology*, 81(18), pp.6200-6209.
170. Vechtova, P., Fussy, Z., Cegan, R., Sterba, J., Erhart, J., Benes, V. and Grubhoffer, L., 2020. Catalogue of stage-specific transcripts in *Ixodes ricinus* and their potential functions during the tick life-cycle. *Parasites & Vectors*, 13, pp.1-19.
171. Walker, A.R., Bouattour, A., Camicas, J.L., Estrada-Peña, A., Horak, I.G., Latif, A.A., Pegram, R.G. and Preston, P.M., 2014. *Ticks of domestic animals in Africa: a guide to identification of species*. Edinburgh: Bioscience Reports.
172. Walker, J.B., 1991. A review of the ixodid ticks (Acari: Ixodidae) occurring in southern Africa. *Onderstepoort Journal of Veterinary Research*, 58(2), pp.81–105.
173. Wu-Chuang, A., Obregon, D., Estrada-Peña, A. and Cabezas-Cruz, A., 2022. Thermostable keystone bacteria maintain the functional diversity of the *Ixodes scapularis* microbiome under heat stress. *Microbial Ecology*, pp.1-12.
174. Yawa, M., Nyangiwe, N., Muchenje, V., Kadzere, C.T., Mpendulo, T.C. and Marufu, M.C., 2018. Ecological preferences and seasonal dynamics of ticks (Acari: Ixodidae) on and off bovine hosts in the Eastern Cape Province, South Africa. *Experimental and Applied Acarology*, 74, pp.317-328.

175. Yunker, C.E., 1996. Heartwater in sheep and goats: a review. *Onderstepoort Journal of Veterinary Research*, 63(2), pp.159–170.
176. Yusufmia, S.B.A.S., Collins, N.E., Nkuna, R., Troskie, M., Van den Bossche, P. and Penzhorn, B.L., 2010. Occurrence of *Theileria parva* and other haemoprotozoa in cattle at the edge of Hluhluwe-iMfolozi Park, KwaZulu-Natal, South Africa. *Journal of the South African Veterinary Association*, 81(1), pp.45-49.
177. Yuval, B. and Spielman, A., 1990. Duration and regulation of the developmental cycle of *Ixodes dammini* (Acari: Ixodidae). *Journal of Medical Entomology*, 27(2), pp.196-201.
178. Zhang, Y.K., Yu, Z.J., Wang, D., Bronislava, V., Branislav, P. and Liu, J.Z., 2019. The bacterial microbiome of field-collected *Dermacentor marginatus* and *Dermacentor reticulatus* from Slovakia. *Parasites & Vectors*, 12, pp.1-10.
179. Zolnik, C.P., Prill, R.J., Falco, R.C., Daniels, T.J. and Kolokotronis, S.O., 2016. Microbiome changes through ontogeny of a tick pathogen vector. *Molecular Ecology*, 25(19), pp.4963-4977.

3. CHAPTER 3

Morphological and genetic characterization of ticks collected from small ruminants in Mahikeng and Potchefstroom, North West Province

3.1. Introduction

The North West Province of South Africa has a large population of livestock, companion animals, and wildlife, with various economically important tick-borne diseases (TBDs) known to be endemic in the province. Ticks and tick-borne diseases are responsible for significant economic losses through livestock mortality and morbidity, as well as costs associated with controlling ticks and TBDs estimated at more than 33 million USD annually in South Africa (Makwarela *et al.*, 2023). The North West Province of South Africa harbors a diverse range of tick species, with surveys identifying 22 different species infesting livestock (Spickett *et al.*, 2011). Key species include *Amblyomma hebraeum*, *Rhipicephalus appendiculatus*, *Rhipicephalus evertsi evertsi*, and *Boophilus microplus*, each playing a role in transmitting economically important diseases (Spickett *et al.*, 2011; Bryson *et al.*, 2002). Several tick genera, in particular *Rhipicephalus*, *Amblyomma*, and *Haemaphysalis*, have been reported to parasitize domestic animals (Iweriebor *et al.*, 2022; Adelabu *et al.*, 2024). According to Nyangiwe *et al.* (2018), climate change and anthropogenic activities such as the transportation of livestock have facilitated the range expansion of invasive tick species such as *Rhipicephalus microplus* and *Amblyomma variegatum* across Africa

The taxonomy of ixodid ticks has traditionally relied on morphological analysis, however, it is challenging to identify species using morphology alone, especially when collected in the larval and/or nymphal life stages (McCann *et al.*, 2019). Characters that are useful for species identification, in addition to features of mouthparts and scutal patterns, can be instrumental, but face other challenges of phenotypic variation, sexual dimorphism, and cryptic species, which makes precise identification difficult due to the high relatedness between some species (Beati and Keirans, 2001).

The genetic methods, which include amplification and sequencing of the mitochondrial 16S ribosomal RNA (rRNA), cytochrome oxidase subunit I (COI), 12S rRNA, and 18S rRNA genes, are employed to identify and characterize the tick species, have improved the efficiency

of tick identification, the depth of the phylogenetic research and further aided in elucidating the diversity of different tick species (Lv *et al.*, 2014; Ghosh *et al.*, 2020; Kneubehl *et al.*, 2022). These genetic markers reveal evolutionary relationships, genetic diversity, and species boundaries that are not apparent from morphology alone (Norris *et al.*, 1996). Efficient and widely used markers for tick species identification are the nuclear 18S ribosomal RNA (rRNA) gene and the mitochondrial 12S rRNA gene (Cruickshank 2002; Dabert and Dabert, 2006). The 18S rRNA gene is particularly efficient for genus-level recognition (Cruickshank, 2002), while 16S rRNA, *COI* gene, and ITS2 (Internal Transcribed Spacer 2) markers are primarily used to resolve taxonomy at the species level (Dabert, 2006). Moreover, the 12S rRNA gene is suitable for identifying closely related tick lineages (Abdullah *et al.*, 2016). Additionally, morphological and molecular methods have been used to identify *Haemaphysalis longicornis* and *Rhipicephalus microplus* (Qin *et al.*, 2011), indicating that these methods can supplement each other to ensure accurate tick identification. Several South African studies have similarly demonstrated the integration of morphological and molecular techniques for reliable tick classification and pathogen detection (Adelabu *et al.*, 2024; Mhlambo *et al.*, 2023; Mtshali *et al.*, 2015).

Recent studies in South Africa's North West Province have revealed a notable presence of ticks and tick-borne pathogens in domestic ruminants, emphasizing an ongoing concern for the health and productivity of livestock in the area (Spickett *et al.*, 2011; Mnisi *et al.*, 2022; Thekisoe *et al.*, 2023). A thorough morphological and genetic identification of tick species in this area is crucial to understand the diversity of tick species circulating in the area to predict and mitigate emerging TBDs that may affect human health and livestock production through crafting focused control methods and further building on the knowledge of the epidemiology of TBDs in South Africa. Therefore, it is important to conduct morphological and genetic studies of livestock ticks in this region to advance sustainable livestock management and safeguard both animal and public health. This chapter sought to investigate the diversity of tick species currently occurring infesting small stock in Mahikeng and Potchefstroom villages of North West Province of South Africa using a combination of morphological and genetic tools.

3.2 Materials and methods

3.2.1 Study site

The study was conducted in selected villages of the North West province of South Africa, which are, Matlwang village [26° 44' 9.66" S 26° 55' 47.65" E] of Potchefstroom (Figure 3.1) and Masutlhe [25°48'12.0"S 25°24'50.1"E], Ramatlabama [25°42'06.0"S 25°40'40.9"E], Dihatshwane[25°54'25.6"S25°45'12.7"E] and Rooigrond [25°55'18.3"S 25°47'58.7"E] of Mahikeng city (Figure 3.2).

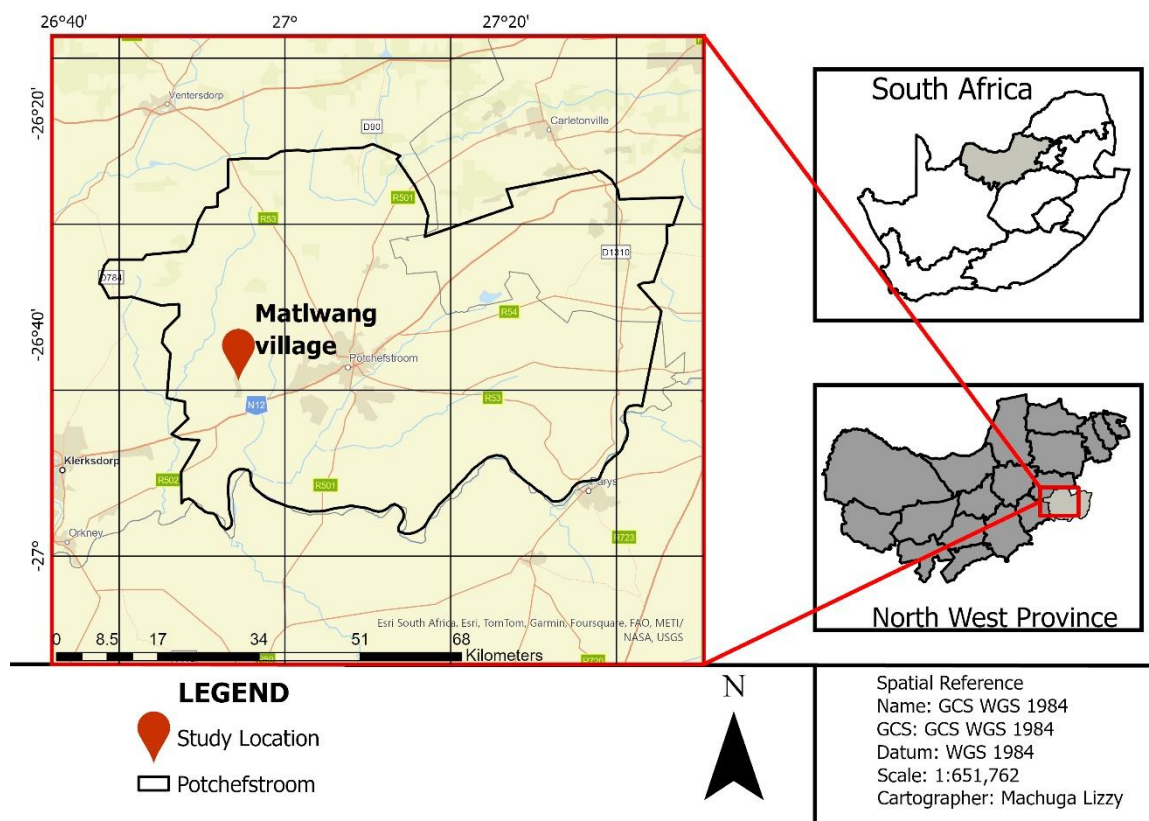


Figure 3.1: A map indicating the location of Matlwang village in Potchefstroom. The study area is indicated with a red point marker.

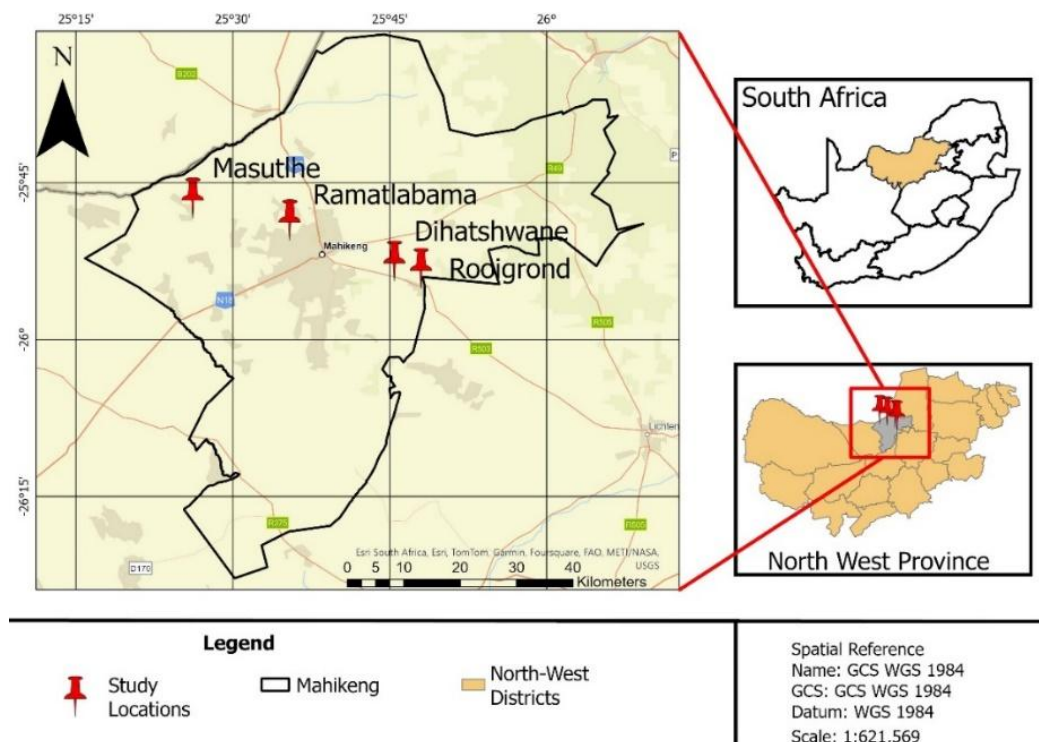


Figure 3.2: A map indicating the location of Masutlhe, Ramatlabama, Dihatshwane, and Rooigrond in Mahikeng. The study areas are indicated with red point markers.

3.2.2 Collection of ticks

In January – February 2024, ixodid ticks were collected randomly from sheep and goats of different farms, depending on the area of abundance and visibility; this included heads, ears, neck, abdominal areas, and perineum using sterile fine-tipped forceps. The collected ixodid ticks were adequately noted and were stored in individual vials containing 70% ethanol. The tick specimens were transferred to the laboratory for identification and further analysis.

3.2.3 Characterization of ticks

The Nikon AZ 100 M multi-zoom microscope (Nikon Inc., Tokyo, Japan) was used to identify and capture sequential photographs of different features of the ticks. The ticks were identified to the species level by discerning unique characters that differentiated between species following published morphological keys by published keys by Walker (2003) and Latif (2013).

3.2.4. Preparation of ticks from Potchefstroom for DNA extraction

Tick species were separated according to the host, location, and gender, where male ticks were arranged into pools (each pool formed from 3-5 ticks). A total of thirty pools are representative of Matlwang. Each tick specimen was cut with sterile scalpels within a 1.5 ml microcentrifuge

tube. DNA samples were stored at -20°C at the laboratory (North-West University Potchefstroom campus) until further use.

3.2.5 Preparation of ticks from Mafikeng for DNA extraction

Tick species were categorized based on host and location, and subsequently organized into pools, each consisting of 3 to 5 ticks. In total, thirty pools were representative of Mahikeng. Each tick specimen was cut with sterile scalpels within a 1.5 ml microcentrifuge tube. DNA samples were stored at -20°C at the laboratory (North West University Potchefstroom campus) until further use.

3.2.6 DNA extraction protocol

Prior to DNA extraction, ticks were washed twice with 70% ethanol and distilled water and allowed to air dry on sterile paper. DNA was extracted from the pool of ticks using the QIAGEN DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions as follows: Each sample was placed in 1.5 ml microcentrifuge tube and homogenized in 180 μL of ATL buffer using metal pellet and TissueLyzer LT instrument (Qiagen, Germany) to ensure thorough tissue disruption. Subsequently, 20 μL of proteinase K was added to each sample, and the tubes were incubated at 56°C for 1–3 hours with occasional vortexing to facilitate complete lysis. After digestion, 200 μL of AL buffer was added to the lysates, which were vortexed for 15 seconds and incubated at 70°C for 10 minutes to enhance DNA binding. Ethanol (200 μL , 96–100%) was added, and the mixtures were briefly vortexed.

The lysates were transferred to DNeasy Mini spin columns placed in 2 mL collection tubes and centrifuged at $6000 \times g$ (8000 rpm) for 1 minute, after which the flow-through was discarded. The spin columns were washed with 500 μL of Buffer AW1 and centrifugation at $6000 \times g$ (8000 rpm) for 1 minute. After discarding the flow-through, 500 μL of Buffer AW2 was added, and the columns were centrifuged at $20,000 \times g$ (14,000 rpm) for 3 minutes to ensure thorough washing. The columns were then transferred to clean 1.5 mL microcentrifuge tubes, and DNA was eluted by adding 200 μL of Buffer AE directly onto the membrane. Following a 1-minute incubation at room temperature, the columns were centrifuged at $6000 \times g$ (8000 rpm) for 1 minute. The eluted DNA was stored at -20°C for subsequent analysis.

3.2.7 Genetic identification of ticks using 16S rRNA and 18S rRNA PCR assays

Polymerase chain reaction (PCR) for genetic identification of ticks was performed targeting the mitochondrial 16S rRNA and nuclear 18S rRNA genes using primers listed in Table 3.1.

Each PCR reaction consisted of a total reaction of 25 µL consisting of 12.5 µL of a 2X DreamTaq Green Mastermix (0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP, 0.4 mM dTTP, 4 mM MgCl₂, and loading buffer) (ThermoFisher Scientific, South Africa), 8.5 µL of nuclease-free water, 2 µL of the template DNA, and 1 µL of 10 µM of the forward primer, 1 µL of 10 µM of the reverse primer. Conditions of the PCR were as follows: one step of denaturation at 94°C for 2 min; 35 cycles of denaturation at 94°C for 30 s, annealing at a specific temperature for each gene assay annealing at for 30 seconds (Table 3.1) for 30 s, and elongation at 68°C for 2 min; and one step of final elongation at 68°C – 5 min using ProFlex PCR System (Applied Biosystems, USA). The amplified PCR mixture was confirmed by electrophoresis on 1.5% agarose gel, stained with Ethidium Bromide (EtBr), and visualized under UV light.

Table 3.1: Primers utilized in amplification and sequencing of genes.

DNA marker	5'- primer sequences-3'	Amplified fragment size	Annealing Temperature (°C)	Reference
16S rRNA	16S-F -5'-CTGCTCAATGATTTTTTAAATTGCTGTGG-3' 16S-R -5'-CCGGTCTGAACTCAGATCAAGT-3'	460bp	72	Hornok <i>et al.</i> , 2023
18S rRNA	18S-F - 5'-CATTAAATCAGTTATGGTTCC-3' 18S-R - 5'-CGCCGCAATACGAATGC-3'	780bp	72	Hornok <i>et al.</i> , 2023

3.2.8 Sequencing

Positive PCR products were sent to Inqaba Biotechnical Industries (Pty) Ltd in Pretoria, South Africa, for Sanger sequencing using the Applied Biosystems 3500 Genetic Analyzer (Thermo Fisher Scientific). The sequencing was performed as a bi-directional reaction to ensure accurate base calling. The acquired gene sequences were aligned on the GenBank database using the nucleotide Basic Local Alignment Search Tool (BLASTn) (www.ncbi.nlm.nih.gov/BLAST) from the National Centre for Biotechnology Information (NCBI) to identify sequences with high similarity. Sequences obtained in this study were then deposited to the NCBI database for accession numbers.

3.2.9 Phylogenetic analysis

FinchTV version 1.4 (Treves, 2010) was used to edit the base calls of the sequenced gene displayed on a chromatogram. Ambiguous bases were trimmed from the 5' and 3' end using the FinchTV version 1.4 (Treves, 2010). Multiple sequence alignment was conducted using BioEdit version 7.7.1 (Hall *et al.*, 2011). The phylogenetic tree analysis was constructed using

a maximum likelihood (ML) tree based on MEGAX (Tamura *et al.*, 2011). The bootstrap values were estimated for 1000 replicates using the Kimura 2-parameter model (Kumar *et al.*, 2018).

3.3 Results

A total of 368 tick samples were collected for this study. Among these, 192 ticks were collected from sheep and goats in Matlwang (n = 192) village across four different kraals. Additionally, one hundred sixty-eight ticks were collected from sheep and goats in the regions of Masutlhe (n = 48), Ramatlabama (n = 45), Dihatshwane (n = 38), and Rooigrond (n = 37) in Mahikeng, North West Province. From these 360 ticks, genomic DNA was extracted from 60 pooled samples. Morphological identification of tick species were subsequently confirmed genetically using PCR and sequencing of the 16S rRNA and 18S rRNA gene markers.

3.3.1 Morphological identification of ticks

All the ixodid ticks collected from Mahikeng were identified morphologically as *Amblyomma hebraeum* (Figure 3.3) based on the presence of red and white rings on the legs, a conspicuously ornate conscutum with yellow, green, brown, and black markings, and long mouthparts. Additionally, the ixodid ticks collected from Matlwang were identified as *Rhipicephalus evertsi evertsi*, as illustrated in Figure 3.4. These ticks are characterized by their dark brown bodies, reddish-orange lateral sides and legs. Adult male specimens were carefully examined based on their physical attributes, allowing for differentiation from other species within the *Rhipicephalus* and *Amblyomma* genera. The identification of the male tick species was facilitated by the dorsal shield (conscutum) that extends over their entire abdomen.

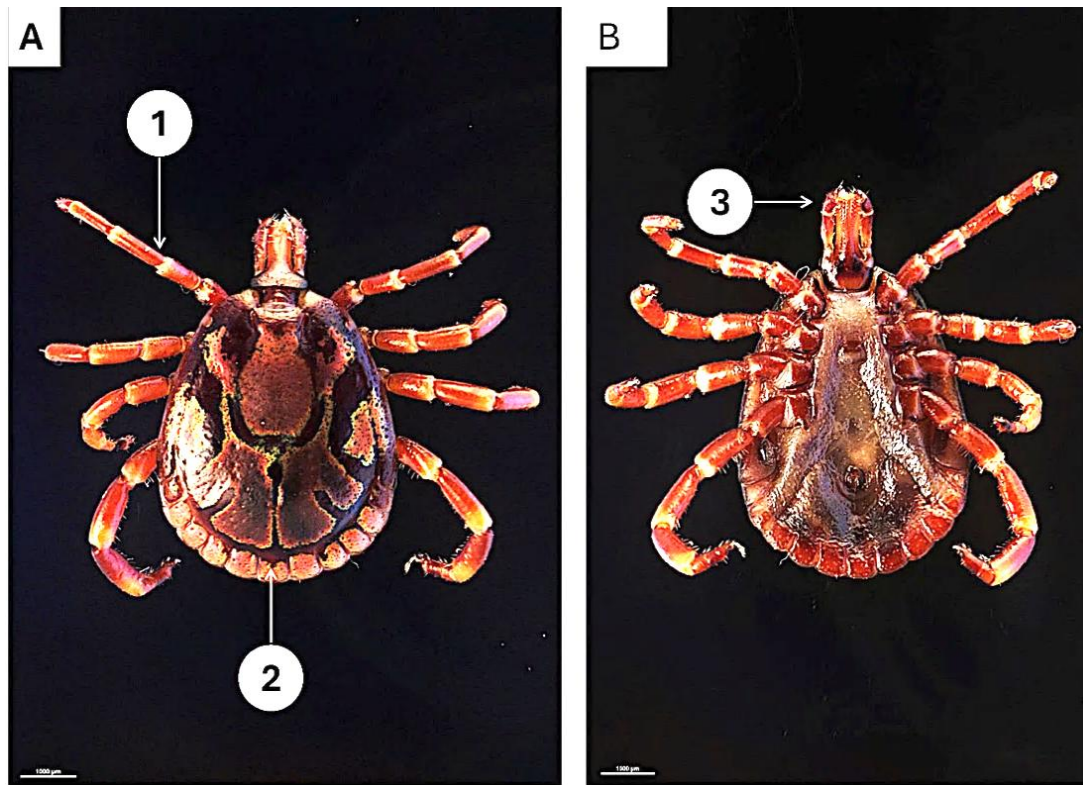


Figure 3.3: The *A. hebreum* male. A Dorsal side: (1) legs with red and white rings, (2) conscutum ornate and conspicuously coloured with yellow, green, brown and black markings. B Ventral (3) long mouthpart.

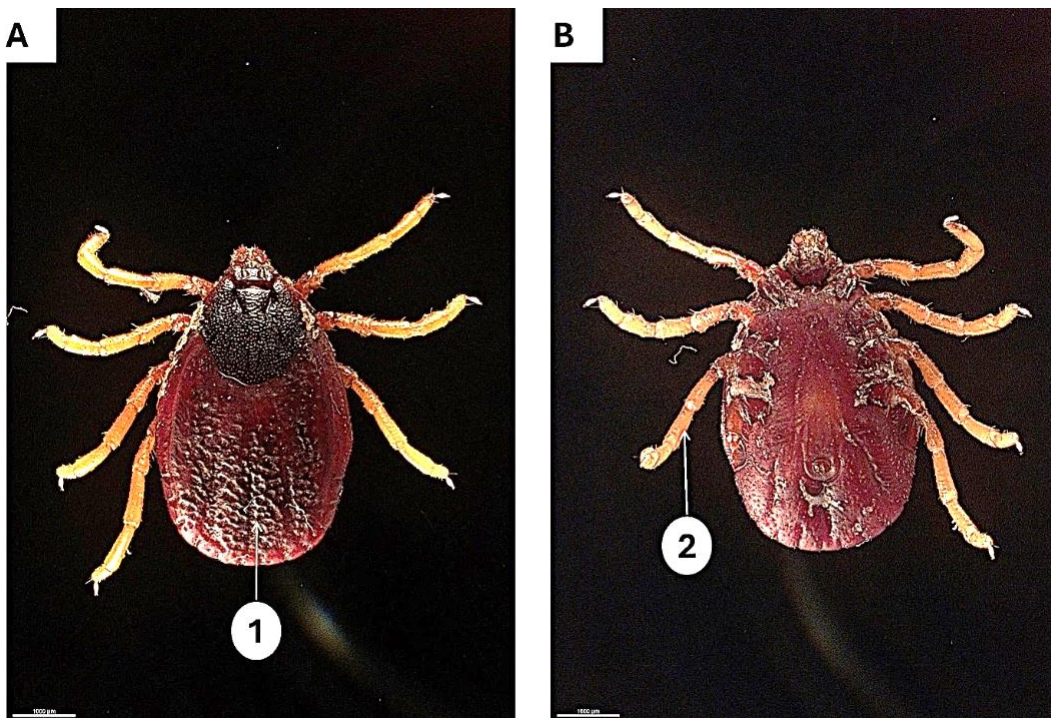


Figure 3.4: The *R. e. evertsi* male. A Dorsal side (1) dark brown, and the rest of the body (lateral sides) reddish-orange. B Ventral (2) legs are reddish-orange.

3.3.2 Genetic identification of ticks

3.3.2.1 Tick identification using 16S rRNA gene sequencing

The amplification of tick DNA targeting the 16S rRNA gene was successfully performed, resulting in a PCR product of 460 bp (Supplementary Figure S1). The tick sequence queries matched the top ranked BLAST hits, confirming morphologic identification as *A. hebreum*. The sequenced tick samples in this study i.e. *A. hebreum* RMC4, *A. hebreum* MO3, *A. hebreum* RC5, *A. hebreum* MC2, *A. hebreum* RMC1, *A. hebreum* RC2, *A. hebreum* MO4, *A. hebreum* RMO4, *A. hebreum* RO3, *A. hebreum* RO5, *A. hebreum* RMO5 and *A. hebreum* MO5) showed nucleotide similarity to *A. hebreum* sequences (GenBank accession numbers: L34316.1 and NC067897.1) with query coverage ranging between 94 and 99%, with sequence identity between 99.27 and 100% (Table 3.2). The *A. hebreum* sequences generated in this study were all submitted to the Genbank database with the following accession numbers: PP80989, PP809810, PP809812, PP809813, PP809811, PP809814, PP809815, PP809816, PP809817, PP809818, PP809824 and PP809819 (Table 3.2).

The *R. e. evertsi* sequences generated in this study (Sample ID: *R. e. evertsi* MG1, *R. e. evertsi* MG2, *R. e. evertsi* MG3, *R. e. evertsi* MG4, *R. e. evertsi* MG6, *R. e. evertsi* MS1, *R. e. evertsi* MS2, *R. e. evertsi* MS3, *R. e. evertsi* MS4 and *R. e. evertsi* MS15) matched with similar reference sequence of *R. e. evertsi* (GenBank accession number: KJ613642.1) on the NCBI database with query covers of 93% and 94%, matching identifications of 99.23% and 100%%, and E-values of 0 (Table 3.2). The *R. e. evertsi* sequences generated in this study were all submitted to GenBank with the following accession numbers: PP809820, PP809822, PP809823, PP809821, PP809825, PP809826, PP809827, PP809830, PP809828 and PP809829 (Table 3.2).

The phylogenetic analysis of *Rhipicephalus* and *Amblyomma* tick species, including *R. e. evertsi* and *A. hebreum* from South African livestock, along with additional sequences from Uganda, Spain, Nigeria, Taiwan, Egypt, and Sweden, revealed two major clades with strong monophyletic structure. The phylogenetic tree displayed well-supported major clades that aligned with genus-level divisions, using *Ixodes ricinus* from Sweden as an out-group (Figure 3.6). Two major clades were determined using the 16S rRNA genes, separating *R. e. evertsi* from *A. hebreum*. The *R. evertsi* were collected from sheep in Matlwang, while *A. hebreum* were collected from sheep in Mahikeng. The *R. e. evertsi* formed a major clade supported by a high

bootstrap value > 79%, indicating genetic cohesion among South African ticks collected from sheep with other *Rhipicephalus* species from cattle in South Africa. Moreover, the South African *A. herbarium* clusters closely with OR699119.1 from Uganda. The *Amblyomma* species cluster, highlighted in green includes *A. hebraeum* from South African livestock. Additionally, *Amblyomma* sp. from Nigeria, Taiwan, and Egypt, formed a genetically distinct group from *Rhipicephalus*. The sequenced South African *A. hebraeum* from sheep clustered together, demonstrating their common origin from Mahikeng.

Table 3.2: BLASTn results of *16S rRNA* gene sequences of tick species sequenced in this study.

Sample ID	Max score	Total score	Query cover (%)	E value	GenBank Accession no	Identification percentage (%)	Accession numbers (This study)
<i>Amblyomma hebraeum</i> RMC4	745	745	96	0.0	L34316.1	100	PP80989
<i>Amblyomma hebraeum</i> MO3	756	756	99	0.0	L34316.1	99.29	PP809810
<i>Amblyomma hebraeum</i> RC5	765	765	99	0.0	L34316.1	99.52	PP809812
<i>Amblyomma hebraeum</i> MC2	763	763	99	0.0	L34316.1	99.29	PP809813
<i>Amblyomma hebraeum</i> RMC1	750	750	99	0.0	L34316.1	99.28	PP809811
<i>Amblyomma hebraeum</i> RC2	741	741	97	0.0	L34316.1	99.27	PP809814
<i>Amblyomma hebraeum</i> MO4	745	745	95	0.0	L34316.1	100	PP809815
<i>Amblyomma hebraeum</i> RMO4	745	745	99	0.0	L34316.1	99.27	PP809816
<i>Amblyomma hebraeum</i> RO3	754	754	99	0.0	L34316.1	99.52	PP809817
<i>Amblyomma hebraeum</i> RO5	743	743	99	0.0	NC067897.1	99.5	PP809818
<i>Amblyomma hebraeum</i> RMO5	756	756	99	0.0	L34316.1	99.29	PP809824
<i>Amblyomma hebraeum</i> MO5	758	758	99	0.0	L34316.1	99.76	PP809819
<i>Rhipicephalus evertsi evertsi</i> MG1	719	719	94	0.0	KJ613642.1	99.49	PP809820
<i>Rhipicephalus evertsi evertsi</i> MG2	713	713	93	0.0	KJ613642.1	99.74	PP809822
<i>Rhipicephalus evertsi evertsi</i> MG3	706	706	94	0.0	KJ613642.1	99.74	PP809823
<i>Rhipicephalus evertsi evertsi</i> MG4	721	721	94	0.0	KJ613642.1	100	PP809821
<i>Rhipicephalus evertsi evertsi</i> MG6	702	702	93	0.0	KJ613642.1	98.97	PP809825
<i>Rhipicephalus evertsi evertsi</i> MS1	712	712	94	0.0	KJ613642.1	98.99	PP809826
<i>Rhipicephalus evertsi evertsi</i> MS2	702	702	93	0.0	KJ613642.1	99.23	PP809827
<i>Rhipicephalus evertsi evertsi</i> MS3	710	710	94	0.0	KJ613642.1	99.24	PP809830
<i>Rhipicephalus evertsi evertsi</i> MS4	715	715	94	0.0	KJ613642.2	99.74	PP809828
<i>Rhipicephalus evertsi evertsi</i> MS15	715	715	94	0.0	KJ613642.3	99.74	PP809829

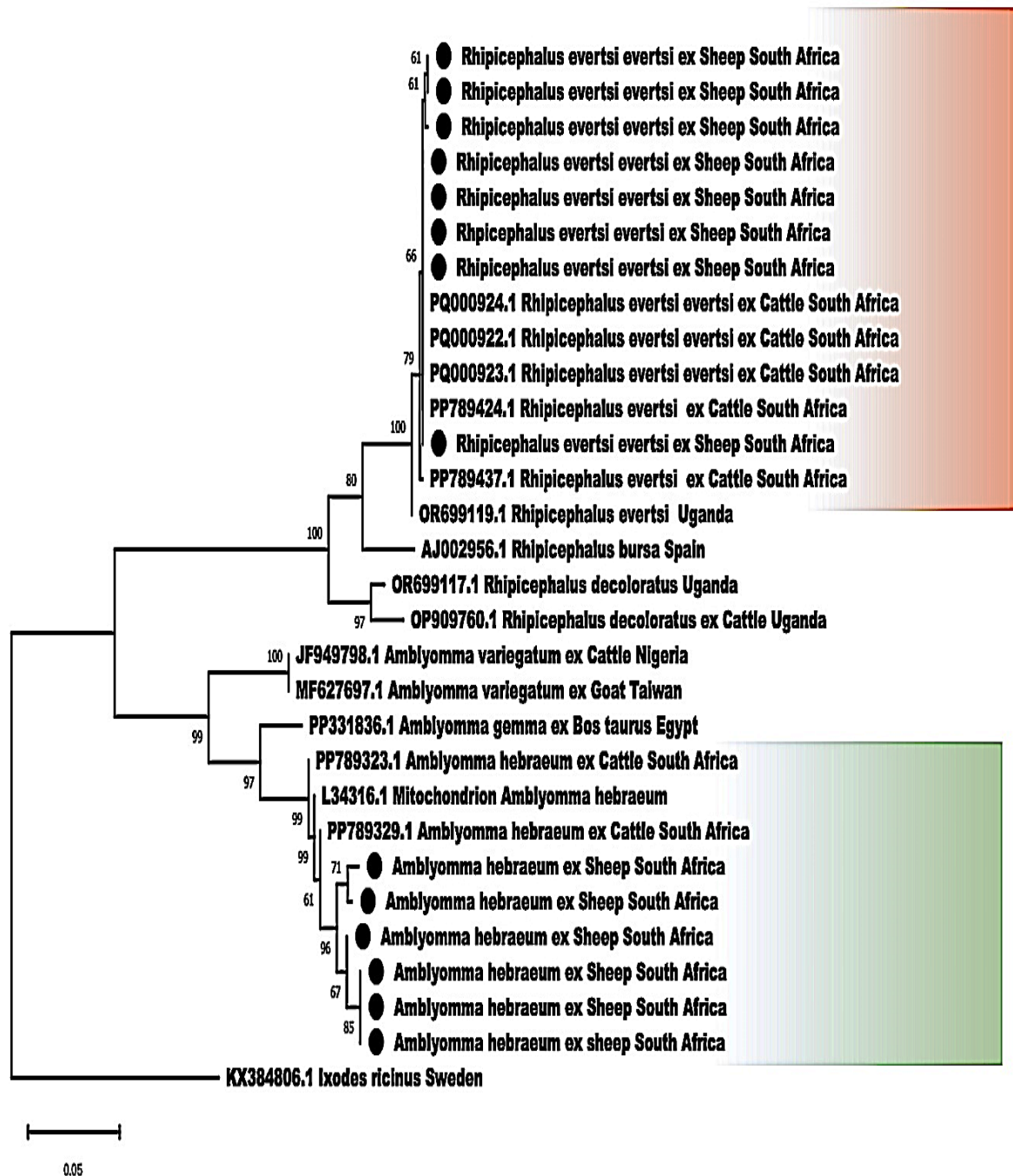


Figure 3.6: Phylogenetic tree of 16S rRNA gene sequences based on maximum likelihood on the *R. evertsi* and *A. hebraeum* tick species. The sequenced tick species in this study are highlighted in circles. The sequence of *Ixodes ricinus* from Sweden (KX384806.1) was used as an outgroup. The levels of bootstrap support for phylogenetic groupings are given at each node. The obtained sequences are represented with black circles.

3.3.2.2 PCR amplification of 18S rRNA, sequencing, and phylogenetic analysis

The amplification of tick DNA targeting the 18S rRNA gene successfully produced a PCR product of 780 bp size (Supplementary Figure S2), which were successfully sequenced. Sequence entries submitted to BLASTn, showed the top-ranked hits were identified to tick species level with query covers of > 98% and 99%, matching identifications between 99.36% and 99.87%, and E-values of 0 with tick species (Table 2.3). The sequences generated in this study were all submitted to GenBank and assigned as *A. hebreum*; PP826352, PP830056, PP830059, PP830060, PP830061, PP830062, PP830064, PP842196 and PP830071 (Table 2.3). Similarly, the top-ranked *R. evertsi* from the query sequences sequenced in this study were selected for phylogenetic purposes. The sequences of *R. evertsi* generated in this study were all submitted to GenBank under accession numbers: PP830093, PP830094, PP830095, PP830096, PP830097, PP830098, PP830099, PP830101, PP830100, PP830103 and PP830102.

The maximum likelihood phylogenetic tree revealed two main clades with strong monophyletic groupings, each representing the two main genera. The major clade in grey shade consisted of *Rhipicephalus* species, showing strong bootstrap support of above 90%, clustering with *R. evertsi* from South Africa with *Rhipicephalus* sp. from various regions, including *R. sanguineus* from Israel and Uzbekistan, *R. microplus* from India, and *R. pusillus* from Spain. This clustering indicated significant genetic cohesion within the *Rhipicephalus* genus across geographic regions. The second clade in blue shade, comprising *Amblyomma* species, included both South African *A. hebraeum* sequenced in this study (highlighted in black circles) and sequences from Italy, forming a distinct group from *Rhipicephalus* with high bootstrap values supporting their separation. Within this clade, the South African *A. hebraeum* sequences clustered closely together, indicating limited genetic diversity among these samples.

Table 3.3: BLASTn results of the *18S rRNA* gene sequences from tick species sequenced in this study.

Sample ID	Max score	Total score	Query cover (%)	E value	GenBank Accession no	Identification percentage (%)	Accession number (This study)
<i>Amblyomma hebraeum</i> RMC4	1395	1395	99	0.0	KY457490.1	99.74	PP826352
<i>Amblyomma hebraeum</i> MC2	1402	1402	99	0.0	KY457489.1	99.36	PP830056
<i>Amblyomma hebraeum</i> RMC1	1384	1384	99	0.0	KY457490.1	99.87	PP830059
<i>Amblyomma hebraeum</i> MO4	1393	1393	99	0.0	KY457489.1	99.61	PP830060
<i>Amblyomma hebraeum</i> RMO4	1386	1386	98	0.0	KY457490.1	99.74	PP830061
<i>Amblyomma hebraeum</i> RO3	1400	1400	99	0.0	KY457489.1	99.87	PP830062
<i>Amblyomma hebraeum</i> RO5	1400	1400	99	0.0	KY457490.1	99.48	PP830064
<i>Amblyomma hebraeum</i> RMO5	1389	1389	99	0.0	KY457489.1	99.61	PP842196
<i>Amblyomma hebraeum</i> MO5	1386	1386	98	0.0	KY457490.1	99.74	PP830071
<i>Rhipicephalus evertsi</i> MG1	1400	1400	98	0.0	KY457503.1	99.87	PP830093
<i>Rhipicephalus evertsi</i> MG2	1391	1391	99%	0.0	KY457503.1	99.48%	PP830094
<i>Rhipicephalus evertsi</i> MG3	1391	1391	97%	0.0	KY457503.1	99.87%	PP830095
<i>Rhipicephalus evertsi</i> MG4	1400	1400	99%	0.0	KY457503.1	99.61%	PP830096
<i>Rhipicephalus evertsi</i> MG5	1397	1397	99%	0.0	KY457503.1	99.48%	PP830097
<i>Rhipicephalus evertsi</i> MG6	1400	1400	99%	0.0	KY457503.1	99.87%	PP830098
<i>Rhipicephalus evertsi</i> MS1	1404	1404	99%	0.0	KY457503.1	99.74%	PP830099
<i>Rhipicephalus evertsi</i> MS2	1406	1406	99%	0.0	KY457503.1	99.87%	PP830101
<i>Rhipicephalus evertsi</i> MS3	1395	1395	99%	0.0	KY457503.1	99.48%	PP830100
<i>Rhipicephalus evertsi</i> MS4	1389	1389	99%	0.0	KY457503.1	99.61%	PP830103
<i>Rhipicephalus evertsi</i> MS15	1391	1391	98%	0.0	KY457503.1	99.87%	PP830102

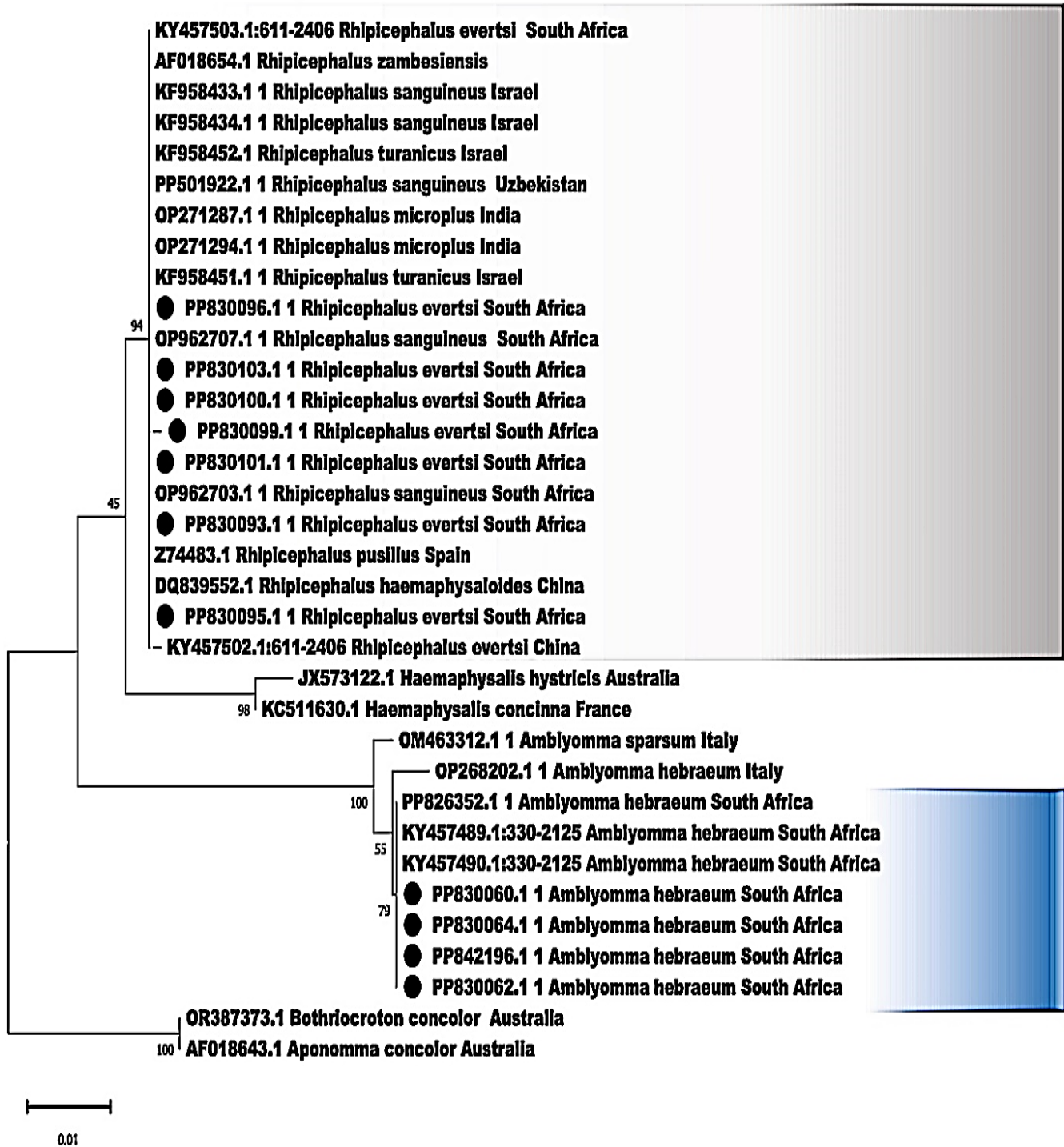


Figure 3.8: Phylogenetic tree of 18S rRNA gene sequences based on maximum likelihood on the *R. evertsi* and *A. hebraeum* tick species. The GenBank sequences IOR387373.1 and AF018643.1 of *Aponomma concolor* from Australia were used as an outgroup. The sequenced ticks in this study are represented with black circles. The levels of bootstrap support for phylogenetic groupings are given at each node. The obtained sequences are represented with black circles.

3.4 Discussion

The North West Province is primarily inhabited by Batswana people who are known for their traditional farming practices and community-oriented agricultural system (Omotayo *et al.*, 2020). This communal farming approach is crucial for food security and socio-economic stability in rural areas of South Africa, particularly in the North West Province, where agriculture is a crucial livelihood strategy (Oduniyi and Tekana, 2019). Ticks are significant ectoparasites, primarily feeding on the blood of various livestock. They are classified into two prominent families hard ticks and soft ticks, and are also important vectors of various pathogens, such as those causing tick-borne diseases in livestock (Boulanger, *et al.*, 2019). The current study used comprehensive morpho-genetic approaches to identify and analyse the diversity of ixodid ticks sampled from livestock in the Mahikeng and Potchefstroom regions of North West Province, South Africa. This combined morphological and molecular identification has provided a reliable species determination. Ticks in this study belong to the genera *Rhipicephalus* and *Amblyomma*. These reported findings support the observations by Horak *et al.* (2018) that showed that ticks belonging to these genera are indigenous to South African ecology and affect wild and domestic animals.

In this study, tick specimens collected from Masutlhe, Ramatlabama, Dihatshwane, and Rooigrond in Mahikeng were identified as *A. herbaeum*, which is generally prevalent in South Africa, particularly in the North West Province (Spickett *et al.*, 2011). According to Walker (2003) and Latif (2013), *A. hebraeum*, commonly known as the tropical bont tick, is distinguished by its vibrant colouration and pronounced features. The conscutum and scutum are heavily punctuated, exhibiting a deep brown colour with a prominent dark marbled pattern, often mixed with metallic blue or greenish hues. *A. hebraeum* displays ornate patterns, particularly in the males, with intricate white or cream-coloured markings on the scutum. The body wall beneath the conscutum mixes reddish and brown tones, particularly noticeable in unfed adults. The morphological findings of *A. herbaeum* specimens collected in the current study are as described above.

In this study, the 16S rRNA gene sequences from ticks collected from small stock in Mahikeng villages matched other *A. herbaeum* sequences on GenBank with percentage identity values ranging from 99.23 - 100%. Additionally, the 18S rRNA gene sequences of same ticks also had identity values ranging from 99.36 - 99.87%, with respective *A. herbaeum* sequences from GenBank. In a phylogenetic tree based on 16S rRNA, the *A. herbaeum* sequences from sheep in the current study and cattle in other South African region formed a distinct monophyletic

group with high bootstrap values, indicating strong genetic relatedness. Likewise, this can be taken as an evident result that the input sequences are indeed *A. hebraeum*, matching well with the other such sequences, enabling them to form a distinct cluster. The tree included some additional reference species, such as *A. variegatum*, which are separately positioned on the tree, indicating genetic differences from the main clusters of interest.

Similarly, in the 18S rRNA phylogenetic tree, the input sequences of *A. hebraeum* cluster together, confirming that they belong to the *A. hebraeum* species. The *A. hebraeum* sequences formed a distinct cluster, with minimal genetic diversity evident among sequences from South Africa, corroborating their identification as *A. hebraeum*. The analysis also included additional *Amblyomma* species, such as *A. sparsum* from Italy, which are positioned separately on the tree, highlighting genetic divergence within the genus. The inclusion of other genera like *Bothriocroton* and *Aponomma* further emphasises the genetic differences from the main clusters of *Rhipicephalus* and *Amblyomma*. The phylogenetic tree indicates that the *Amblyomma* clade is monophyletic and genetic identification findings of this study using *A. hebraeum* in the current study are similar to those of Monakale *et al.*, (2024), where molecular data supported morphological identification and the phylogenetic tree showed no distinct clustering based on village locality, indicating that ticks of the same species were distributed across all study sites. The ticks collected from small livestock in Mahikeng are confirmed to be *A. hebraeum* through morphological and genetic identification methods.

In this current study, tick specimens collected from Matlwang village in Potchefstroom were morphologically identified as *R. e. evertsi*, prevalent in South Africa across the North West Province (Spickett *et al.*, 2011). According to Walker (2003) and Latif (2013), the conscutum and scutum of *R. e. evertsi* are densely punctuated with very dark-brown and a reddish-orange body wall. The eyes are convex and orbited, and the legs are reddish-orange. The adanal plates are triangular and large. The circum-spiracular integument is covered with dense prominent setae. Caudal appendages are absent in fed specimens. The presence of distinct festoons and a well-defined scutum further aids in identifying this tick species. The morphological findings of *R. e. evertsi* in the current study are described above.

In this study, the generated 16S rRNA gene sequences from ticks collected from small stock in Matlwang village in Potchefstroom matched with other *R. e. evertsi* sequences from GenBank, with matching identifications between 99.23% and 100%. Additionally, the generated 18S rRNA gene sequences of ticks from the same area also had matching identity values ranging

between 99.48% and 99.87%, with respective *R. e. evertsi* reference sequences on the NCBI database. In the phylogenetic analysis based on 16S rRNA sequences, the *R. e. evertsi* sequences obtained from the current study of from sheep and cattle in South Africa form a distinct cluster, monophyletic group (indicated by a single common ancestor). This is evident from the strong clustering with high bootstrap support (> 79%). The reference sequences from South Africa also grouped together with the other such sequences originating from other countries such as Uganda, which further confirms that the reference sequences are also very closely related. Similarly, the 18S rRNA phylogenetic tree, the input sequences of *R. evertsi* cluster together, confirming that they indeed belong to the *R. e. evertsi* species. The samples from South Africa form a distinct cluster with high bootstrap support (above 70% in most nodes), highlighting minimal genetic diversity among them. Additionally, some *R. sanguineus* samples from South Africa are seen clustering closely. Other *Rhipicephalus* species, such as *R. sanguineus*, *R. turanicus*, and *R. microplus*, cluster separately, reflecting clear genetic divergence from *R. e. evertsi*. The phylogenetic tree indicates that the *R. e. evertsi* cluster is paraphyletic within the broader *Rhipicephalus* genus, as other species disrupt its exclusivity. However, the overall *Rhipicephalus* genus shows signs of monophyly with respect to the outgroups. This supports the evolutionary distinctiveness of both genera while highlighting possible regional diversification within *Rhipicephalus*. The genetic identification findings of this study using of *R. e. evertsi* in the current study are similar to Monakale *et al.*, (2024) observations where molecular data supported morphological identification, and the phylogenetic tree showed no distinct clustering based on village locality, indicating that ticks of the same species were distributed across all study sites.

The 16S rRNA gene sequences have consistently yielded reliable results for the identification of tick species (Abouelhassan *et al.*, 2019; Monakale *et al.*, 2024). In contrast, the 18S rRNA gene has been employed to investigate the phylogenetic relationships among tick subfamilies, corroborating some findings from 16S rRNA studies while differing in others (Black *et al.*, 1997; Cruickshank, 2002). Morphological identification was consistent with the genetic results. Consequently, it can be confidently asserted that the ticks collected from small livestock in Mahikeng and Matlwang are indeed *A. hebreum* and *R. e. evertsi*. The findings of this study support existing literature, which indicates that species from the genera *Rhipicephalus* and *Amblyomma* predominantly contribute to tick infestations affecting livestock in South Africa (Booyesen *et al.*, 2017; Makwarela *et al.*, 2023; Monakale *et al.*, 2024). This situation poses a significant threat, as many of these species are recognized carriers and transmitters of

economically important disease-causing agents, including heartwater, bovine babesiosis, and anaplasmosis (Booyesen *et al.*, 2017).

Genetic identification utilizing 16S rRNA and 18S rRNA gene markers demonstrated precise classification of both mature and immature specimens. The BLASTn results reaffirmed a high sequence identity ranging from 99.48% to 100% between the sequenced ticks and reference sequences from GenBank, thereby validating the morphological identifications. These findings align with other studies that have emphasized the effectiveness of molecular tools in accurately identifying species and elucidating their phylogenetic relationships (Muruthi *et al.*, 2016; Monakale *et al.*, 2024).

The application of molecular markers not only confirmed species identity but also provided insights into the genetic relationships within and across species. The high sequence homology with reference sequences from other countries, such as Uganda and Italy, observed among local samples supports the existence of genetic affiliation across geographical borders, which may necessitate research on tick dispersion patterns and adaptability. This observation is important because the global distributions of ticks are modified by climatic changes and anthropogenic environmental factors, including processes like livestock transport (Nyangiwe *et al.*, 2018). Therefore, continuous monitoring and genotyping surveillance are necessary in the prevention and control of the spread of tick-borne diseases for livestock health.

References

1. Abdullah, H.H., El-Molla, A., Salib, F.A., Allam, N.A., Ghazy, A.A. and Abdel-Shafy, S., 2016. Morphological and molecular identification of the brown dog tick *Rhipicephalus sanguineus* and the camel tick *Hyalomma dromedarii* (Acari: Ixodidae) vectors of Rickettsioses in Egypt. *Veterinary World*, 9(10), p.1087.
2. Abouelhassan, E.M., El-Gawady, H.M., Abdel-Aal, A.A., El-Gayar, A.K. and Esteve-Gassent, M.D., 2019. Comparison of some molecular markers for tick species identification. *Journal of Arthropod-Borne Diseases*, 13(2), p.153.
3. Adelabu, O.A., Iweriebor, B.C. and Obi, C.L., 2024. Evidence of zoonotic rickettsiae in ixodid ticks of domestic animals in some communal farms in the Eastern Cape Province, South Africa. *Journal of Advanced Veterinary and Animal Research*, 11(2), p.254.
4. Beati, L. and Keirans, J.E., 2001. Analysis of the systematic relationships among ticks of the genera *Rhipicephalus* and *Boophilus* (Acari: Ixodidae) based on mitochondrial 12S ribosomal DNA gene sequences and morphological characters. *Journal of Parasitology*, 87(1), pp.32–48.
5. Black 4th, W.C. and Piesman, J., 1994. Phylogeny of hard- and soft-tick taxa (Acari: Ixodida) based on mitochondrial 16S rDNA sequences. *Proceedings of the National Academy of Sciences*, 91(21), pp.10034–10038.
6. Booysen, S., Horak, I.G., Heyne, H., Smit, W.J. and Halajian, A., 2017. Parasites of domestic and wild animals in South Africa. L. Ixodid ticks infesting horses and donkeys. *Onderstepoort Journal of Veterinary Research*, 84(1), pp.1–6.
7. Boulanger, N., Boyer, P., Talagrand-Reboul, E. and Hansmann, Y., 2019. Ticks and tick-borne diseases. *Médecine et Maladies Infectieuses*, 49(2), pp.87–97.
8. Brahma, R.K., Dixit, V., Sangwan, A.K. and Doley, R., 2014. Identification and characterization of *Rhipicephalus (Boophilus) microplus* and *Haemaphysalis bispinosa* ticks (Acari: Ixodidae) of North East India by ITS2 and 16S rDNA sequences and morphological analysis. *Experimental and Applied Acarology*, 62, pp.253–265.
9. Bryson, N.R., Tice, G.A., Horak, I.G., Stewart, C.G. and Du Plessis, B.J.A., 2002. Ixodid ticks on indigenous goats owned by small-scale farmers in four communal

- grazing areas in South Africa. *Journal of the South African Veterinary Association*, 73(1), pp.26–30.
10. Cheng, W.Y., Zhao, G.H., Jia, Y.Q., Bian, Q.Q., Du, S.Z., Fang, Y.Q., Qi, M.Z. and Yu, S.K., 2013. Characterization of *Haemaphysalis flava* (Acari: Ixodidae) from Qingling subspecies of giant panda (*Ailuropoda melanoleuca qinlingensis*) in Qinling Mountains (Central China) by morphology and molecular markers. *Public Library of Science ONE*, 8(7), p.e69793.
 11. Cruickshank, R.H., 2002. Molecular markers for the phylogenetics of mites and ticks. *Systematic and Applied Acarology*, 7(1), pp.3–14.
 12. Dabert, M., 2006. DNA markers in the phylogenetics of the Acari. *Biological Letters*, 43(2), pp.97–107.
 13. Estrada-Peña, A., D’Amico, G., Palomar, A.M., Dupraz, M., Fonville, M., Heylen, D., Habela, M.A., Hornok, S., Lempereur, L., Madder, M. and Nuncio, M.S., 2017. A comparative test of ixodid tick identification by a network of European researchers. *Ticks and Tick-borne Diseases*, 8(4), pp.540–546.
 14. Felsenstein, J., 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. *Journal of Molecular Evolution*, 17, pp.368–376.
 15. Ghosh, P., Tikar, S.N., Gupta, M.K. and Sukumaran, D., 2020. Molecular characterization using 16S rRNA and COI gene sequence in hard ticks of Gwalior, India. *Defence Life Science Journal*, 5(3), pp.173–179.
 16. Guo, H., Moumouni, P.F.A., Thekiso, O., Gao, Y., Liu, M., Li, J., Galon, E.M., Efstratiou, A., Wang, G., Jirapattharasate, C. and Ringo, A.E., 2019. Genetic characterization of tick-borne pathogens in ticks infesting cattle and sheep from three South African provinces. *Ticks and Tick-borne Diseases*, 10(4), pp.875–882.
 17. Hall, T., 2004. BioEdit version 7.0.0. Distributed by the author. Available at: www.mbio.ncsu.edu/BioEdit/bioedit.html.
 18. Hall, T., Biosciences, I. and Carlsbad, C.J.G.B.B., 2011. BioEdit: an important software for molecular biology. *GERF Bulletin of Biosciences*, 2(1), pp.60–61.
 19. Horak, I.G., Heyne, H., Williams, R., Gallivan, G.J., Spickett, A.M., Bezuidenhout, J.D. and Estrada-Peña, A., 2018. *The ixodid ticks (Acari: Ixodidae) of southern Africa*. Springer.

20. Hornok, S., Kontschán, J., Takács, N., Heyne, H., Kovács, Á.B., Plantard, O., Keve, G., Fedorov, D., Gyuranecz, M. and Halajian, A., 2023. Molecular-phylogenetic analyses of *Ixodes* species from South Africa suggest an African origin of bird-associated exophilic ticks (subgenus *Trichotoixodes*). *Parasites & Vectors*, 16(1), p.392.
21. Iweriebor, B.C., Afolabi, K.O., Nqoro, A. and Obi, L.C., 2022. Emergence of *Theileria* species in ticks from free-ranging domestic animals in Raymond Mhlaba Local Municipality, South Africa. *Heliyon*, 8(3).
22. Iweriebor, B.C., Mmbaga, E.J., Adegborioye, A., Igwaran, A., Obi, L.C. and Okoh, A.I., 2017. Genetic profiling for *Anaplasma* and *Ehrlichia* species in ticks collected in the Eastern Cape Province of South Africa. *BMC Microbiology*, 17, pp.1–8.
23. Jackson, J., Beveridge, I., Chilton, N.B., Andrews, R.H. and Dixon, B., 2002. Morphological comparison of the adult and larval stages of the Australian ticks *Ixodes holocyclus* Neumann, 1899 and *I. cornuatus* Roberts, 1960 (Acari: Ixodoidea). *Systematic and Applied Acarology*, 7(1), pp.91–108.
24. Jongejan, F., Berger, L., Busser, S., Deetman, I., Jochems, M., Leenders, T., De Sitter, B., Van der Steen, F., Wentzel, J. and Stoltz, H., 2020. *Amblyomma hebraeum* is the predominant tick species on goats in the Mnisi Community Area of Mpumalanga Province, South Africa, and is co-infected with *Ehrlichia ruminantium* and *Rickettsia africae*. *Parasites & Vectors*, 13, pp.1–12.
25. Karim, S., Budachetri, K., Mukherjee, N., Williams, J., Kausar, A., Hassan, M.J., Adamson, S., Dowd, S.E., Apanaskevich, D., Arijo, A., Sindhu, Z.U., Kakar, M.A., Khan, R.M.D., Ullah, S., Sajid, M.S., Ali, A. and Iqbal, Z., 2017. A study of ticks and tick-borne livestock pathogens in Pakistan. *Public Library of Science Neglected Tropical Diseases*, 11(6), e0005681.
26. Khumalo, N., Chaisi, M., Magoro, R. and Mwale, M., 2024. An analysis of the gaps in the South African DNA barcoding library of ticks of veterinary and public health importance. *Genome*, 67(11), pp.392-402.
27. Kneubehl, A.R., Muñoz-Leal, S., Filatov, S., De Klerk, D.G., Pienaar, R., Lohmeyer, K.H., Bermúdez, S.E., Suriyamongkol, T., Mali, I., Kanduma, E. and Latif, A.A., 2022. Amplification and sequencing of entire tick mitochondrial genomes for a phylogenomic analysis. *Scientific Reports*, 12(1), p.19310.

28. Kumar, S., Stecher, G., Li, M., Knyaz, C. and Tamura, K., 2018. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, 35(6), pp.1547–1549.
29. Latif Abdallah, A., 2013. *Ticks and tick-borne diseases: illustrated guide to identification of African tick species*. Afrivet, Nairobi, Kenya.
30. Letunic, I. and Bork, P., 2021. Interactive Tree Of Life (iTOL) v5: An online tool for phylogenetic tree display and annotation. *Nucleic Acids Research*, 49(W1), pp.W293–W296.
31. Lew, A. and Jorgensen, W., 2005. Molecular approaches to detect and study the organisms causing bovine tick-borne diseases: babesiosis and anaplasmosis. *African Journal of Biotechnology*, 4(4), pp.292–302.
32. Lv, J., Wu, S., Zhang, Y., Chen, Y., Feng, C., Yuan, X., Jia, G., Deng, J., Wang, C., Wang, Q. and Mei, L., 2014. Assessment of four DNA fragments (*COI*, 16S rDNA, ITS2, 12S rDNA) for species identification of the Ixodida (Acari: Ixodida). *Parasites & Vectors*, 7, pp.1–11.
33. Madder, M., Horak, I. and Stoltz, H., 2013. Ticks: Tick importance and disease transmission. *Afrivip*, 1, pp.1–30.
34. McCann, K.M., Grant, W.N., Spratt, D.M. and Hedtke, S.M., 2019. Cryptic species diversity in ticks that transmit disease in Australia. *International Journal for Parasitology: Parasites and Wildlife*, 10, pp.125–131.
35. Mhlambo, K., Iweriebor, B.C., Mkolo, N., Afolabi, K.O. and Obi, L.C., 2023. Molecular evidence and phylogenetic delineation of spotted fever group *Rickettsia* species in *Amblyomma* ticks from cattle in Gauteng and Limpopo Provinces, South Africa. *Asian Pacific Journal of Tropical Medicine*, 16(8), pp.371–378.
36. Mnisi, S.S., Mphuthi, M.B., Ramatla, T., Mofokeng, L.S., Thekisoe, O. and Syakalima, M., 2022. Molecular detection and genetic characterization of *Ehrlichia ruminantium* harbored by *Amblyomma hebraeum* ticks of domestic ruminants in North West Province, South Africa. *Animals*, 12(19), p.2511.
37. Monakale, K.S., Smith, R.M., Gaorekwe, R.M., Ledwaba, M.B. and Malatji, D.P., 2024. Phenotypic and genotypic characterization of ticks and tick-borne

- pathogens from cattle in selected villages of Greater Letaba Municipality in Limpopo Province, South Africa. *Parasitology Research*, 123(8), p.292.
38. Mtshali, K., Khumalo, Z.T., Nakao, R., Grab, D.J., Sugimoto, C. and Thekiso, O.M., 2015. Molecular detection of zoonotic tick-borne pathogens from ticks collected from ruminants in four South African provinces. *Journal of Veterinary Medical Science*, 77(12), pp.1573-1579.
 39. Muruthi, C.W., Lwande, O.W., Makumi, J.N., Runo, S., Otiende, M. and Makori, W.A., 2016. Phenotypic and genotypic identification of ticks sampled from wildlife species in selected conservation sites of Kenya. *Journal of Veterinary Science and Technology*, 7(1), p.293.
 40. Nava, S., Guglielmone, A.A. and Mangold, A.J., 2009. An overview of systematics and evolution of ticks. *Frontiers in Bioscience*, 14(8), pp.2857–2877.
 41. Norval, R.A.I., 1977. Ecology of the tick *Amblyomma hebraeum* Koch in the Eastern Cape Province of South Africa. I. Distribution and seasonal activity. *The Journal of Parasitology*, pp.734–739.
 42. Nyangiwe, N., Yawa, M. and Muchenje, V., 2018. Driving forces for changes in geographic range of cattle ticks (Acari: Ixodidae) in Africa: A review. *South African Journal of Animal Science*, 48(5), pp.829–841.
 43. Omotayo, A.O., Ndhlovu, P.T., Tshwene, S.C. and Aremu, A.O., 2020. Utilization pattern of indigenous and naturalized plants among some selected rural households of North West Province, South Africa. *Plants*, 9(8), p.953.
 44. Onyiche, T.E. and MacLeod, E.T., 2023. Hard ticks (Acari: Ixodidae) and tick-borne diseases of sheep and goats in Africa: A review. *Ticks and Tick-borne Diseases*, 14(6), p.102232.
 45. Orkun, Ö., 2018. Molecular characterization based on 16S rDNA phylogeny of some ixodid ticks in Turkey. *Türkiye Parazitoloji Dergisi*, 42(2), p.122.
 46. Qin, X.C., Tian, J.H., Wang, J.B., Lu, X., Sun, Q.Z., Jin, D., Zhou, D.J., Xu, J.G. and Zhang, Y.Z., 2011. Identification of *Haemaphysalis longicornis* and *Rhipicephalus microplus*. *Zhonghua Liu Xing Bing Xue Za Zhi*, 32(6), pp.608–612.
 47. Rothen, J., Githaka, N., Kanduma, E.G., Olds, C., Pflüger, V., Mwaura, S., Bishop, R.P. and Daubenberger, C., 2016. Matrix-assisted laser desorption/ionization time

- of flight mass spectrometry for comprehensive indexing of East African ixodid tick species. *Parasites & Vectors*, 9, pp.1–15.
48. Santana, M.D.S., Hoppe, E.G.L., Carraro, P.E., Calchi, A.C., de Oliveira, L.B., do Amaral, R.B., Mongruel, A.C.B., Machado, D.M.R., Burger, K.P., Barros-Batesti, D.M. and Machado, R.Z., 2022. Molecular detection of vector-borne agents in wild boars (*Sus scrofa*) and associated ticks from Brazil, with evidence of putative new genotypes of *Ehrlichia*, *Anaplasma*, and haemoplasmas. *Transboundary and Emerging Diseases*, 69(5), pp.e2808–e2831.
 49. Senbill, H., Tanaka, T., Karawia, D., Rahman, S., Zeb, J., Sparagano, O. and Baruah, A., 2022. Morphological identification and molecular characterization of economically important ticks (Acari: Ixodidae) from North and North–Western Egypt. *Acta Tropica*, 231, p.106438.
 50. Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Söding, J. and Thompson, J.D., 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular Systems Biology*, 7(1), p.539.
 51. Spickett, A.M., Heyne, I.H. and Williams, R., 2011. Survey of the livestock ticks of the North West province, South Africa. *Onderstepoort Journal of Veterinary Research*, 78(1), pp.1–12.
 52. Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution*, 28(10), pp.2731–2739.
 53. Tamura, K., Stecher, G. and Kumar, S., 2021. MEGA11: molecular evolutionary genetics analysis version 11. *Molecular Biology and Evolution*, 38(7), pp.3022–3027.
 54. Thekiso, O., Ramatla, T., Ringo, A., Mnisi, S., Mphuthi, N., Mofokeng, L., Lekota, K. and Xuan, X., 2023. Molecular detection of *Rickettsia africae* from *Amblyomma hebraeum* ticks in Mafikeng city of North West Province, South Africa. *Research in Veterinary Science*, 164, p.105027.
 55. Waladde, S.M., Young, A.S. and Morzaria, S.P., 1996. Artificial feeding of ixodid ticks. *Parasitology Today*, 12(7), pp.272–278.

56. Walker, A.R., 2003. *Ticks of domestic animals in Africa: a guide to identification of species*. Edinburgh: Bioscience Reports.

4. CHAPTER 4

Bacterial communities harboured by *Amblyomma hebraeum* from small stock in Mahikeng villages, North West Province

4.1 Introduction

Ticks are known to harbour diverse microorganisms, which threaten the health of animals and humans (Sonenshine and Roe, 2013). These ectoparasites can negatively affect livestock health and productivity, mainly through the transmission of a number of pathogens of zoonotic and livestock importance (Hurtado and Giraldo-Ríos, 2018). Among the different tick species distributed in southern Africa, *Amblyomma hebraeum* ticks are very common key vectors of *Ehrlichia ruminantium* (Peter *et al.*, 1999; Jongejan *et al.*, 2019). *Ehrlichia ruminantium* is the causative agent of heartwater, a fatal rickettsial disease affecting ruminants, which poses a major threat to the livestock industry (Zweygarth, 2006; Allsopp, 2015). This infection pose a serious threat to small stock production of in rural, small-scale farming communities like those of Mahikeng villages in the North West Province of South Africa, where livestock is of significant importance for income and food security (Mnisi *et al.*, 2022). Additionally, *A. hebraeum* is known to host a variety of bacterial communities, including symbiotic, commensal, and potentially pathogenic microorganisms that may influence the tick's physiology, vector competence, and disease transmission ability (Kisten *et al.*, 2021).

Microbial communities found in ticks include vast amounts of symbionts and putative pathogens in addition to their role as vectors for other known pathogens (Clay and Fuqua, 2010). These mixed communities of microbes can modify the biological and epidemiological aspects of ticks and tick-borne disease (TBD) vector capabilities (Hussain *et al.*, 2022). However, despite the involvement of the microbiome in tick-pathogen interactions being increasingly established, the microbiome of *A. hebraeum* remains understudied, particularly in small-stock farming areas of rural South Africa. It has been established that understanding bacterial communities can enhance our knowledge of the ecology of ticks and the likelihood of emerging new pathogens (Estrada-Peña *et al.*, 2013).

In South Africa, recent studies have investigated the bacterial communities associated with *Amblyomma hebraeum* ticks, particularly in relation to zoonotic and livestock pathogens. *Rickettsia africae*, the causative agent of African tick bite fever, is the most frequently detected rickettsial species in this tick. For example, Thekiso *et al.* (2023) reported *R. africae* in *A. hebraeum* collected from livestock in the North West Province, highlighting its continued circulation in endemic areas. Similarly, Mtshali *et al.* (2015) detected *R. africae* DNA in *A. hebraeum* from multiple provinces, indicating a wide geographic distribution and its potential role in human infections. Furthermore, Mnisi *et al.* (2023) confirmed the role of *A. hebraeum* in transmitting *E. ruminantium*, the causative agent of heartwater in ruminants, reinforcing the veterinary importance of this tick species in South Africa. High-throughput sequencing revealed the differential abundance of potentially pathogenic and endosymbiotic bacteria in *Amblyomma* ticks, including *Rickettsia*, *Escherichia*, and *Coxiellaceae* (Chigwada *et al.*, 2022). In addition, several off-target antibiotic resistance markers were in the tick microbiome, which may affect disease management strategies (Chigwada *et al.*, 2022). These findings contribute to understanding tick-associated bacterial communities and their potential impact on pathogen transmission and treatment strategies. Investigating tick microbiomes can uncover potential symbiotic relationships and identify bacteria that might influence tick physiology and pathogen transmission.

Mahikeng, located in the North West Province of South Africa, is an important livestock farming region where small stock, such as goats and sheep, are commonly raised. The presence of *A. hebraeum* ticks in these villages raises concerns regarding their potential to harbour and transmit bacterial pathogens. However, studies focusing on the bacterial diversity within *A. hebraeum* from small stock in this region remains limited. This chapter sought to investigate the bacterial communities harboured by *A. hebraeum* collected in sheep and goats from Mahikeng using high throughput 16S *rRNA* metabarcoding. By utilizing molecular techniques, we seek to identify potential pathogenic and symbiotic bacteria, thereby contributing to the understanding of tick-borne microbial ecology in South Africa. The findings of this study will effectively guide future vector control efforts and significantly enhance disease management strategies for small-scale farmers in the region.

4.2 Materials and methods

4.2.1 Study area, sample collection and DNA extraction

A total of 168 *A. hebraeum* ticks were collected from sheep and goats in regions of Masutlhe [25°48'12.0"S 25°24'50.1"E], Ramatlabama [25°42'06.0"S 25°40'40.9"E], Dihatshwane [25°54'25.6"S 25°45'12.7"E] and Rooigrond [25°55'18.3"S 25°47'58.7"E] of Mahikeng city. Ticks were identified morphologically using the keys from Latif (2013) and further confirmed using molecular assays targeting the 16S rRNA and 18S rRNA. DNA was extracted using QIAGEN DNeasy® Blood & Tissue Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). Details of the study area, sample collection, and DNA extraction are described in the materials and methods (Chapter 3.1).

4.2.2 Library preparation and sequencing

The 16S rRNA gene sequencing for *A. hebraeum* ticks was conducted using primers based on the Illumina DNA library preparation protocol (Illumina, USA). Locus-specific primers, 341F (5'-CCT ACG GGN GGC WGC AG-3') and 805R (5'-GAC TAC HVG GGT ATC TAA TCC-3') (Klindworth *et al.*, 2013), targeting the hypervariable V3-V4 region (approximately 460 bp 34 region) of the 16S rRNA gene, were used with overhang adapters at the 5'-end of forward and reverse primers respectively (Illumina, USA). The PCR primers used were: forward primer 5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG-3' and reverse primer: 5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C-3' (Klindworth *et al.*, 2013). The library preparation workflow entailed a first-stage amplicon PCR step to amplify the region, using 12.5 ng of DNA, 12.5 µl of KAPA HiFi HotStart ready Mix [0.5 U DNA polymerase, 0.3 mM dNTPs, 2.5 mM MgCl₂] (Kapa Biosystems, Wilmington, MA, USA), a final concentration of 0.2 µM of each forward and reverse primer was used in the reaction mix, along with nuclease-free water (Thermo Fisher Scientific, USA) to a final volume of 25 µl. The PCR Thermal reaction protocol using ProFlex PCR System (Applied Biosystems, USA) was carried out as follows: denaturing at 95°C for 3 min, followed by 35 cycles of 95°C for 30 seconds, 55°C for 60 seconds, and 72°C for 30 seconds, and a final extension step at 72°C for 5 min was included.

PCR amplicons were then subjected to a PCR clean-up step using Agencourt AMPure XP magnetic beads (Beckman Coulter Genomics, USA). Following clean-up, index PCR was performed to attach dual indexes to PCR products using the Nextera XT index kit (Nextera, USA). The indexing reaction was performed using 5 µl of amplicon PCR product DNA, 5 µl of Illumina Nextera XT Index Primer 1 (N7xx), 5 µl of Nextera XT Index Primer 2 (S5xx), 25 µl of 2x KAPA HiFi HotStart Ready Mix, and 10 µl of nuclease-free water. The thermocycling using ProFlex PCR System (Applied Biosystems, USA), conditions were 95°C for 3 minutes followed by 8 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and a final elongation step of 72°C for 5 minutes. Index PCR products were subjected to a second 'PCR clean-up' step. The libraries were then quantified using a Qubit 3.0 fluorometer (Thermo Fisher Scientific, USA), normalized, pooled, and denatured in 0.2 N NaOH. Denatured libraries, along with 10% PhiX sequencing control, were then loaded onto the MiSeq flow cell using the MiSeq v3 reagent kit for a paired-end (2 × 300 bp) sequencing run. Sequencing was performed using the Illumina MiSeq platform (Illumina, USA) at the North West University (NWU) Potchefstroom campus, North West, South Africa.

4.2.3 Bioinformatics and statistical analysis

DADA2 was used to process reads obtained from the sequencing platform, after indexes and primers were removed (Callahan *et al.*, 2021), following trimming, filtering, and removal of chimeras. Reads were truncated at 250 bp with maximum expected errors at 5, allowing no ambiguous bases. Error rates for forward and reverse reads were learned independently and then merged. The operational taxonomic units (OTUs) table was constructed, and taxonomy classification was assigned to the sequence variants using the SILVA 138.1 database (Quast *et al.*, 2013). Downstream analyses and visualisation were done using the *phyloseq* package in R (McMurdie & Holmes, 2013). Alpha diversity was assessed using Observed OTUs, Chao1, Shannon, and Simpson indices. Beta diversity was evaluated using Bray–Curtis dissimilarity and Jaccard distance matrices, visualized through Principal Coordinate Analysis (PCoA) and Non-Metric Multidimensional Scaling (NMDS), respectively. Microbial abundance was visualised using *ggplot2* (Wickham, 2016). The operational taxonomic units (OTUs) table was constructed, and taxonomy classification was assigned to the sequence variants using the SILVA 138.1 database (Quast *et al.*, 2013). Downstream analyses and visualisation were done using the *phyloseq* package in R (McMurdie & Holmes, 2013). Alpha diversity was assessed using Observed OTUs, Chao1, Shannon, and Simpson indices. Beta diversity was evaluated

using Bray–Curtis dissimilarity and Jaccard distance matrices, visualized through Principal Coordinate Analysis (PCoA) and Non-Metric Multidimensional Scaling (NMDS), respectively. Microbial abundance was visualised using *ggplot2* (Wickham, 2016). The statistical significance of diversity differences was tested using the Kruskal–Wallis test.

4.2. Data availability

Raw reads obtained from the present study were deposited to the National Center of Biotechnology Information (NCBI) Sequence Read Archive (SRA) database under BioProject ID PRJNA1246162 (<http://www.ncbi.nlm.nih.gov/bioproject/1246162>).

4.3 Results

4.3.1 Taxonomic composition *Amblyomma hebraeum* ticks from sheep and goats

4.3.1.1 The bacterial composition at the phylum level

The taxonomic abundances of all OTUs at the phylum level were visualised using a stacked graph based on relative abundances (Figure 4.1). A total of 13 bacterial phyla were assigned in all *A. hebraeum* ticks from sheep and goats. In ticks collected from goats, the five most abundant bacterial phyla were Proteobacteria (66.12%), followed by Firmicutes (16.01%), Actinobacteria (8.16%), Bacteroidota (4.36%), and Cyanobacteria (1.32%). The least abundant bacterial phyla included Planctomycetota (0.002%), Acidobacteriata (0.01%) and Bdellovibrionota (0.02%), as shown in Figure 4.1. In ticks from sheep, the five most abundant bacterial phyla in *A. hebraeum* ticks were Proteobacteria (41.80%), followed by Firmicutes (19.80%), Bacteroidota (12.76%), Actinobacteriota (11.99%) and Cyanobacteria (5.09%). The remaining bacterial phyla had relatively low abundances (<1%). The least abundant bacterial phyla were Patescibacteria (0.02%), Planctomycetota (0.04%) and Acidobacteriota (0.04%) depicted in Figure 4.1.

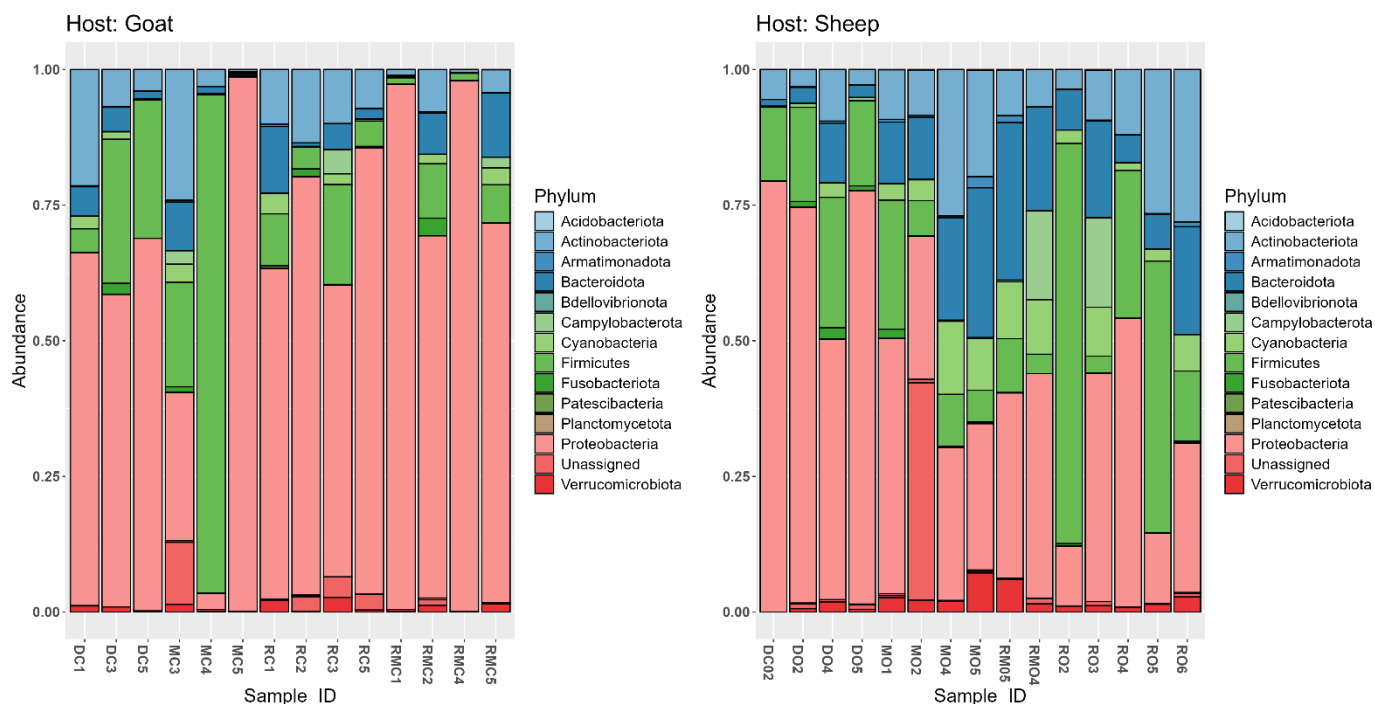


Figure 4.1: Staggered plot for taxonomic abundance of the bacterial communities in *Amblyomma hebraeum* ticks from sheep and goats at the phylum level.

4.3.1.2 The bacterial composition at the class level

A total of 16 assigned bacterial classes were identified in *A. hebraeum* ticks collected from both sheep and goats. In goat derived ticks, the dominant bacterial class level was Alphaproteobacteria (52.96%), followed by Bacilli (15.33%), Gammaproteobacteria (20.69%), Alphaproteobacteria (13.15%), and Actinobacteria (8.07%). The least abundant classes included OM190 (0.002%), Blastocatellia (0.008%) and Bdellovibrionio (0.01%) depicted in Figure 4.2. In contrast, the microbiota of *A. hebraeum* ticks from sheep was dominated by Alphaproteobacteria (21.34%), followed by Gammaproteobacteria (20.48%), Bacilli (18.41%), Bacteroidia (12.76%) and Actinobacteria (11.77 %). The least abundant classes in goat derived ticks included Saccharimonadia (0.02%), OM190 (0.03%) and Blastocatellia (0.05%), depicted in Figure 4.2.

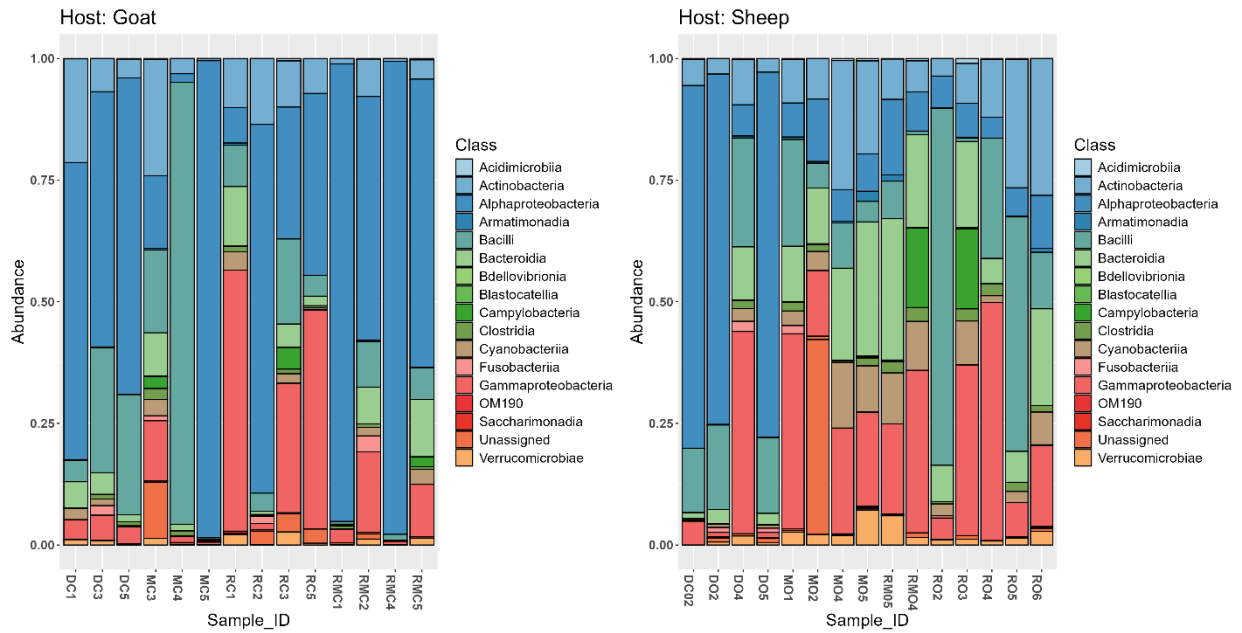


Figure 4.2: Staged plot for taxonomic abundance of the bacterial communities of *Amblyomma hebraeum* ticks from sheep and goats at the class level.

4.3.1.3 The bacterial composition at the order level

A total of 19 bacterial orders were identified in *A. hebraeum* tick samples collected from both sheep and goats. At the bacterial order level, the microbiota composition in goat-derived ticks was dominated by Rickettsiales (52.48%) followed by Bacillales (9.48%), Coxiellales (8.16%), Staphylococcales (6.18%) and Corynebacteriales (4.78%). The least assigned abundant orders included Xanthomonadales (0.35%), Sphingomonadales (0.51%) and Lactobacillales (0.70%). In contrast, the microbiota of *A. hebraeum* ticks from sheep was dominated by Rickettsiales (17.73%), followed by Staphylococcales (11.60%), Burkholderiales (10.01%), Coxiellales (7.99%) and Bacillales (7.29%). The least abundant orders in sheep-derived ticks included Verrucomicrobiales (0.56%), Peptostreptococcales-Tissierellales (1.58%) and Sphingomonadales (2.03%), depicted in Figure 4. Additionally, chloroplasts were detected in goats (1,33%) and sheep (5.85%) ticks.

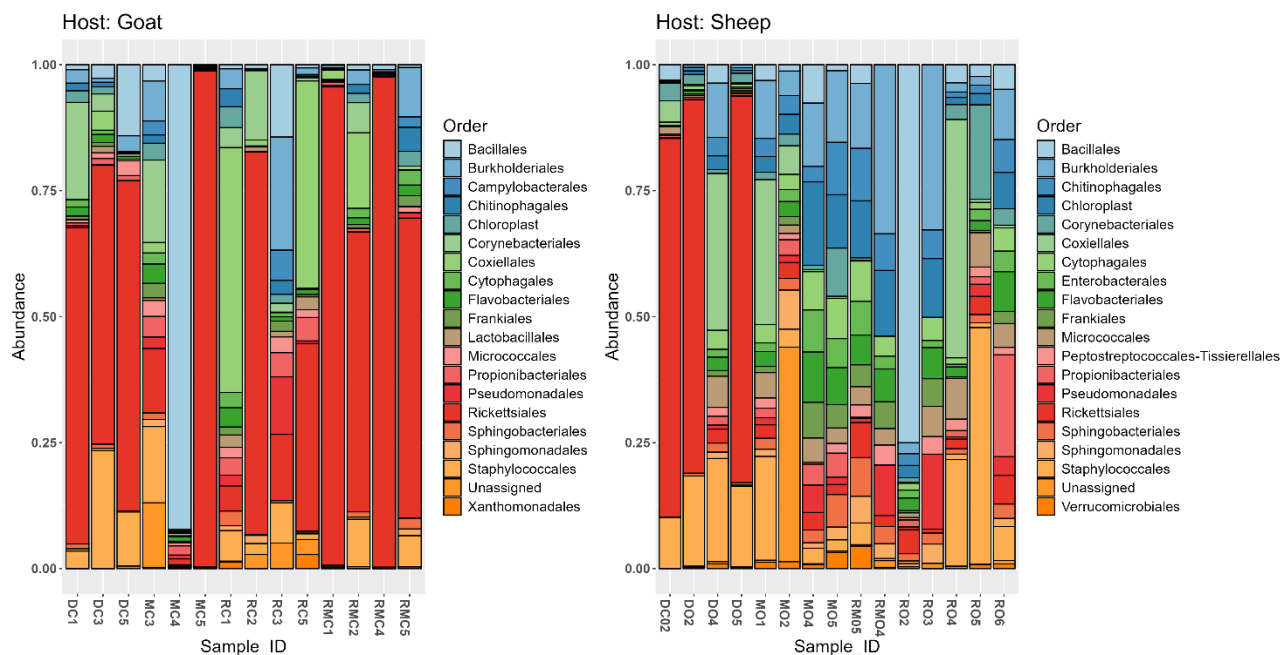


Figure 4.3: Staged plot for taxonomic abundance of the bacterial communities in *Amblyomma hebraeum* ticks from sheep and goats at the order level.

4.3.1.4 The bacterial composition at the family level

At the family level, 18 bacterial families were identified in *A. hebraeum* ticks from goats and sheep. The dominant bacterial families in goat-derived ticks included *Rickettsiaceae* (50.68%), followed by *Bacillaceae* (9.81%), *Coxiellaceae* (8.85%), *Staphylococcaceae* (6.71%) and *Corynebacteriaceae* (4.98%). The least abundant families in ticks included *Xanthomonadaceae* (0.38%), *Sphingomonadaceae* (0.55%) and *Cyclobacteriaceae* (0.59%), as shown in Figure 4.4. In sheep-derived ticks, the dominant families were *Rickettsiaceae* (18.34%) followed by *Staphylococcaceae* (12.98%), *Coxiellaceae* (7.57%), *Bacillaceae* (7.47%) and *Commandaceae* (5.46%). The least abundant family in the samples included *Intrasorangaceae* (1.22%), *Saprospiraceae* (1.43%), and *Cyclobacteriaceae* (1.95%), depicted in Figure 4.4.

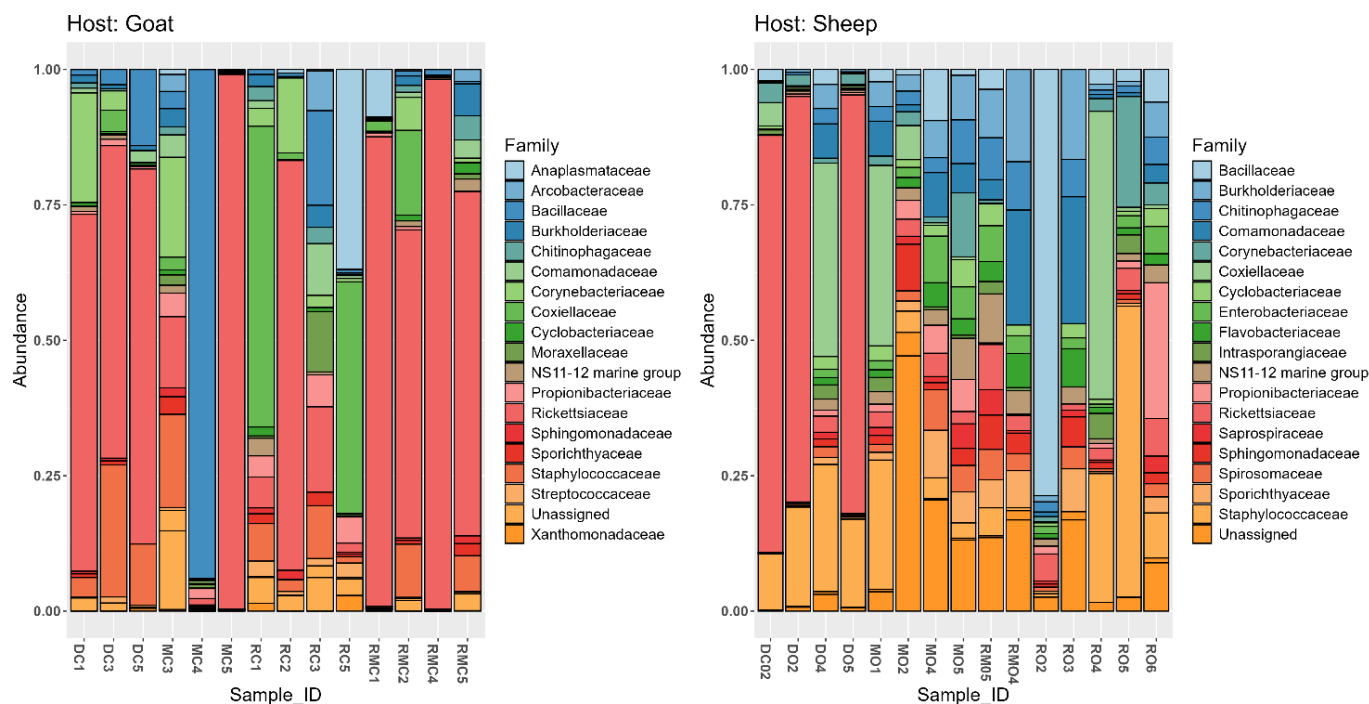


Figure 4.4: Staggered plot for taxonomic abundance of the bacterial communities in *A. hebraeum* ticks from sheep and goats at the family level.

4.3.1.5 The bacterial composition at the genus level

A total of 19 bacterial genera were identified in *A. hebraeum* ticks collected from goats and sheep. At the genus level, the microbiota composition in ticks from goats revealed the following dominant genera: *Rickettsia* (51.64%), followed by *Coxiella* (9.01%), *Bacillus* (7.77%), *Staphylococcus* (6.83%), and *Corynebacterium* (5.08%) (Figure 4.5). The least abundant genera included *Vulcaniibacterium* (0.31%), *Pseudarcicella* (0.54%), and *Algorihagus* (0.57%), depicted in Figure 5.5. In contrast, the microbiota composition in ticks from sheep showed a slightly different distribution: *Rickettsia* (18.85%) was the most predominant, followed by *Staphylococcus* (13.44%), *Coxiella* (9.97%), *Bacillus* (6.55%), and *Polynucleobacter* (5.88%). The least abundant genera in sheep-derived ticks included *Knoellia* (1.35 %), *Fluviicola* (1.48%) and *Candidatus Aquiretis* (1.61 %), depicted in Figure 5.5.

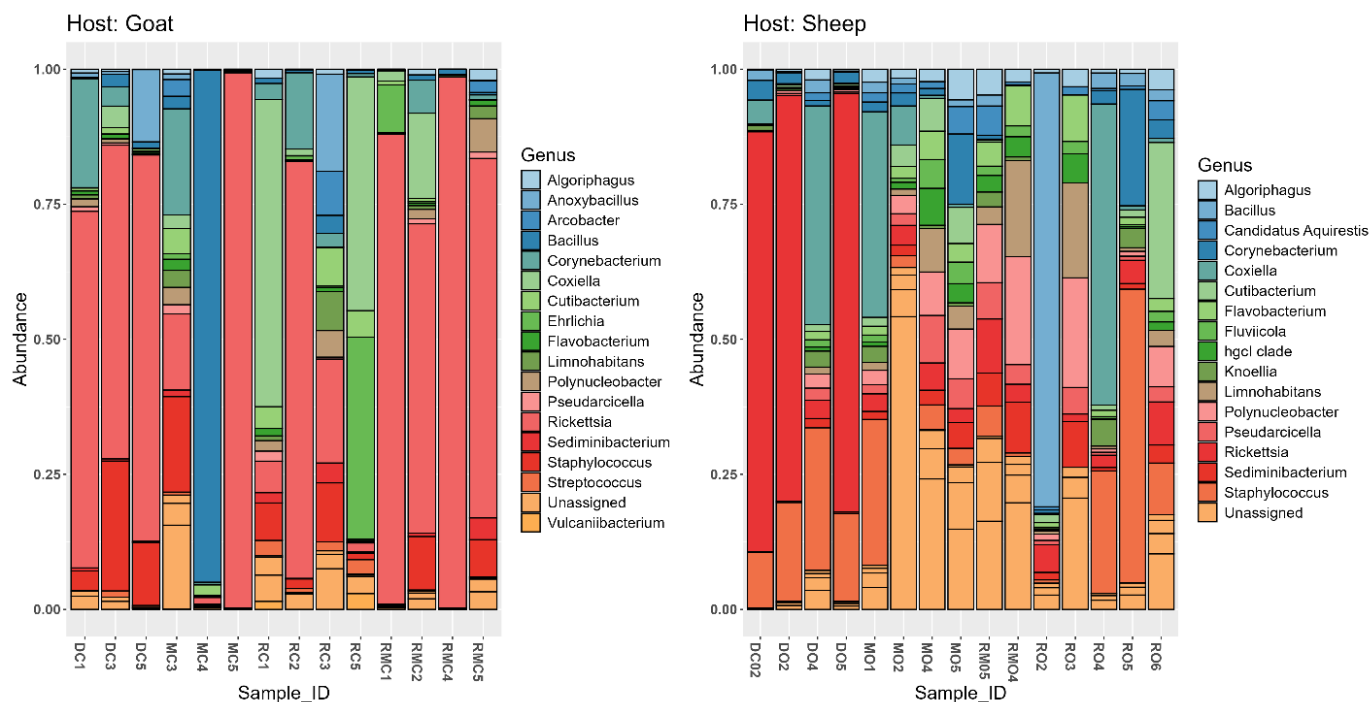


Figure 4.5: Staggered plot for taxonomic abundance of the bacterial communities in *Amblyomma hebraeum* ticks from sheep and goats at the genus level.

4.3.2 Comparative analysis of genus-level bacterial diversity in sheep and goats

The heatmap illustrates the relative abundance (log10-transformed) of the top 30 bacterial genera across *A. hebraeum* ticks collected from goats and sheep, revealing distinct host-specific patterns in microbial composition. Certain genera, such as *Rickettsia*, *Streptococcus*, and *Staphylococcus*, exhibit higher relative abundance in ticks from goats, as indicated by the warmer colours (red and orange). In contrast, *Pseudomonas* and *Roseomonas* spp. appear more prominent in ticks from sheep. Despite these variations, several genera, including *Bacillus*, *Pseudarcicella*, and *Algoriphagus*, are shared between ticks from goats and sheep, though their relative abundances vary. However, genera like *Flavobacterium* and *Escherichia-Shigella* are present across both hosts. In contrast, low-abundance genera such as *Corynebacterium*, *Cutibacterium*, and *Chryseobacterium* spp. are indicated by cooler colours (blue), likely representing less dominant members of the microbiota.

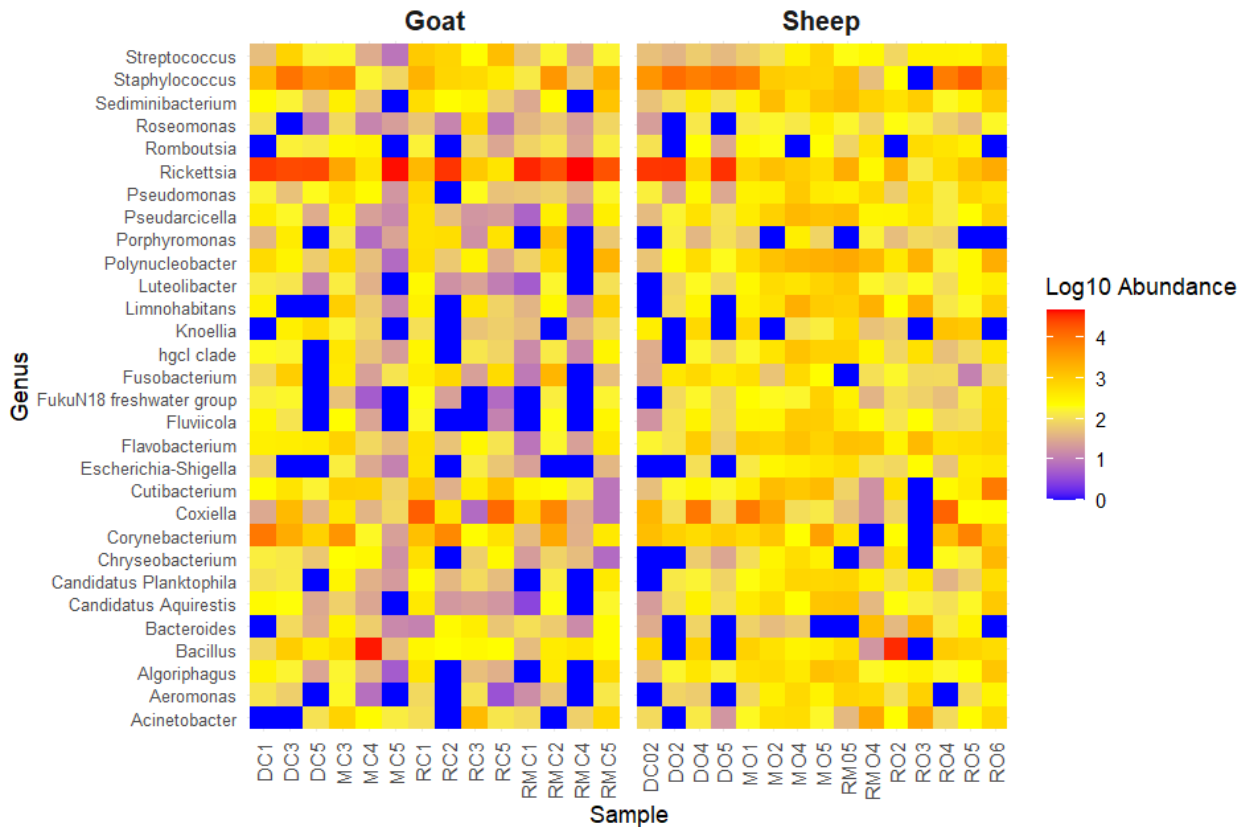


Figure 4.6: Heatmap of the top 30 genus-level bacterial taxa in *Amblyomma hebraeum* ticks from sheep and goats.

4.3.3 Alpha-diversity rarefaction curves of diversity associated with *Amblyomma hebraeum* ticks from sheep and goats

Diversity rarefaction curves were generated using operational taxonomic units (OTUs) to assess the sequencing depth and microbial diversity within the microbiomes of *A. hebraeum* ticks collected from goats and sheep. This analysis was based on the 16S rRNA amplicon sequencing data. The rarefaction curves (Figure 4.7) display species richness across varying sequence sample sizes, reflecting the diversity of bacterial communities associated with *A. hebraeum* ticks. Ticks collected from goats (represented by red curves) reached a plateau in species richness at lower sequencing depths than sheep ticks (depicted by teal curves). This indicates that the microbial diversity associated with ticks from goats is less complex and reaches saturation faster than that from sheep. In contrast, ticks collected from sheep required a greater sequencing depth to capture the full diversity of their microbiome, as their curves show a slower progression towards a plateau.

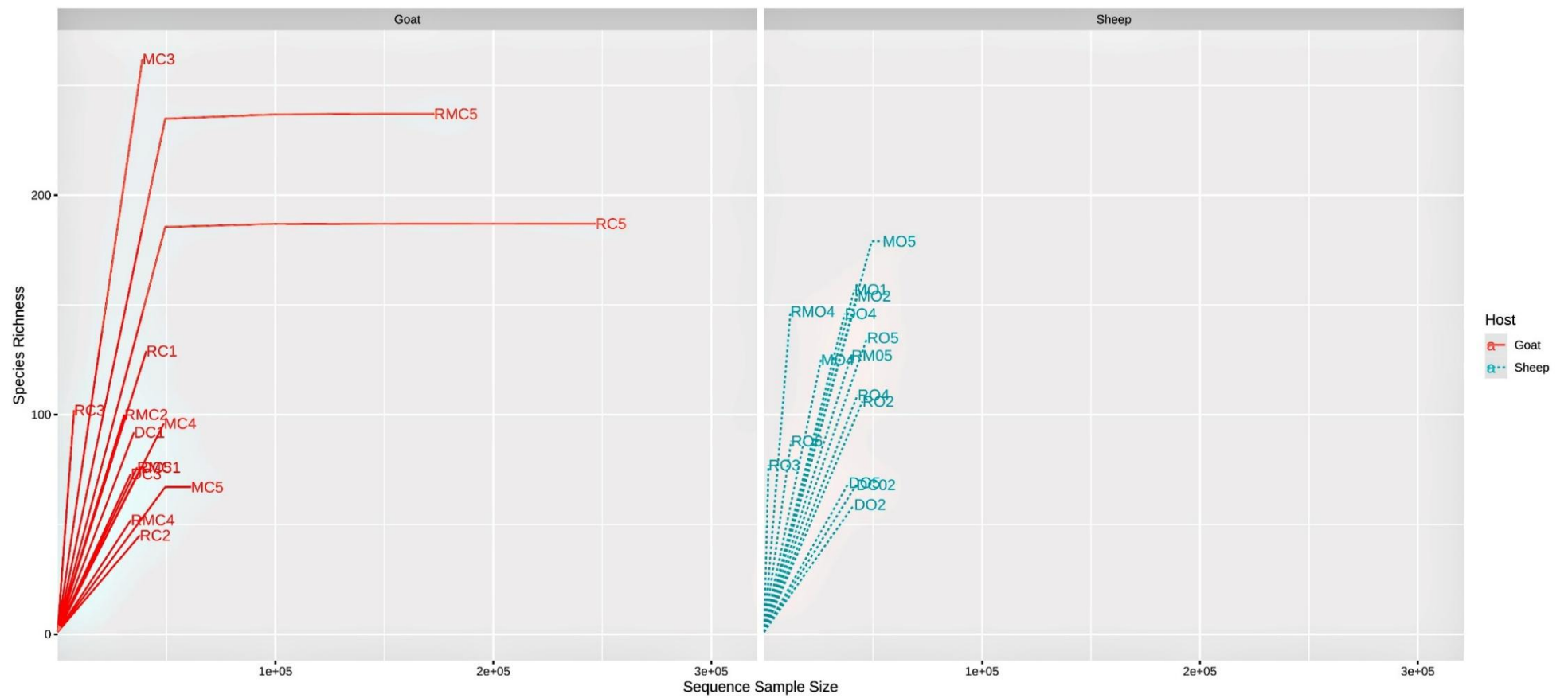


Figure 4.7 Alpha-diversity rarefaction curve to interpret the sequence depth of the bacterial diversity associated with *Amblyomma hebraeum* ticks from sheep and goats based on the 16S rRNA gene amplicon.

4.3.4 Alpha diversity estimate of microbiome associated with *A. hebraeum* ticks from sheep and goats

Illumina MiSeq sequencing generated a total of 528,482 raw sequence reads from *A. hebraeum* ticks from the goat dataset and 338,497 reads from the sheep dataset. After trimming, quality-checking, and removing chimeric sequences, as well as low-quality and non-target amplicons, the datasets were reduced to 409,037 valid reads for the goat samples and 215,319 valid reads for the sheep samples. These reads were then clustered into 16,193 OTUs for the goat dataset and 186,510 OTUs for the sheep dataset.

Alpha diversity was estimated with four indices, i.e. the richness estimators, Chao1, as well as the diversity indices, Shannon and Simpson's, using raw reads. For analysing the convergence of α -diversity estimates, the OTUs were sorted from most to least abundant. Analysis of the α -diversity convergence using the bootstrap index has shown that the proportion of the underestimated OTUs (that is, those that could be present in the biological sample but were undetected because of incomplete sequencing) among top-ranking OTUs, including 97% of reads, does not exceed 1%. Further analyses were performed on this pool of top OTUs covering 100% of reads.

The α -diversity analysis of *A. hebraeum* ticks collected from sheep and goats revealed distinct patterns of microbial diversity influenced by the host species, as depicted in plots (A-D), Figure 4.7. The p-values for each diversity metrics were determined. Observed OTUs p-value = 0.91310; Shannon index p-value 0.013832; Simpson index p-value = 0.023322, and Chao1 p-value = 0.91310. The p-values that are equal/below 0.05 are deemed significant, meaning that the null hypothesis can be discarded. In contrast, p-values greater than 0.05 indicate that no significant effect was observed.

Observed species richness (Plot A) indicates that ticks from sheep exhibit higher microbial richness compared to ticks from goats. Similarly, the Shannon diversity index (Plot B), which accounts for both richness and evenness, shows a higher Shannon diversity in ticks from sheep compared to ticks from goats, indicating that microbial taxa are not only more numerous in ticks from sheep but also more evenly distributed. The Simpson dominance index (Plot C), which emphasizes the abundance of dominant species, reveals a higher Simpson index in ticks

from sheep. The Chao1 index (Plot D), which estimates species richness including rare species, further highlights that ticks from sheep tick show higher richness, reinforcing the observed and Chao1-based predictions that microbial diversity is greater in ticks from sheep than in goats.

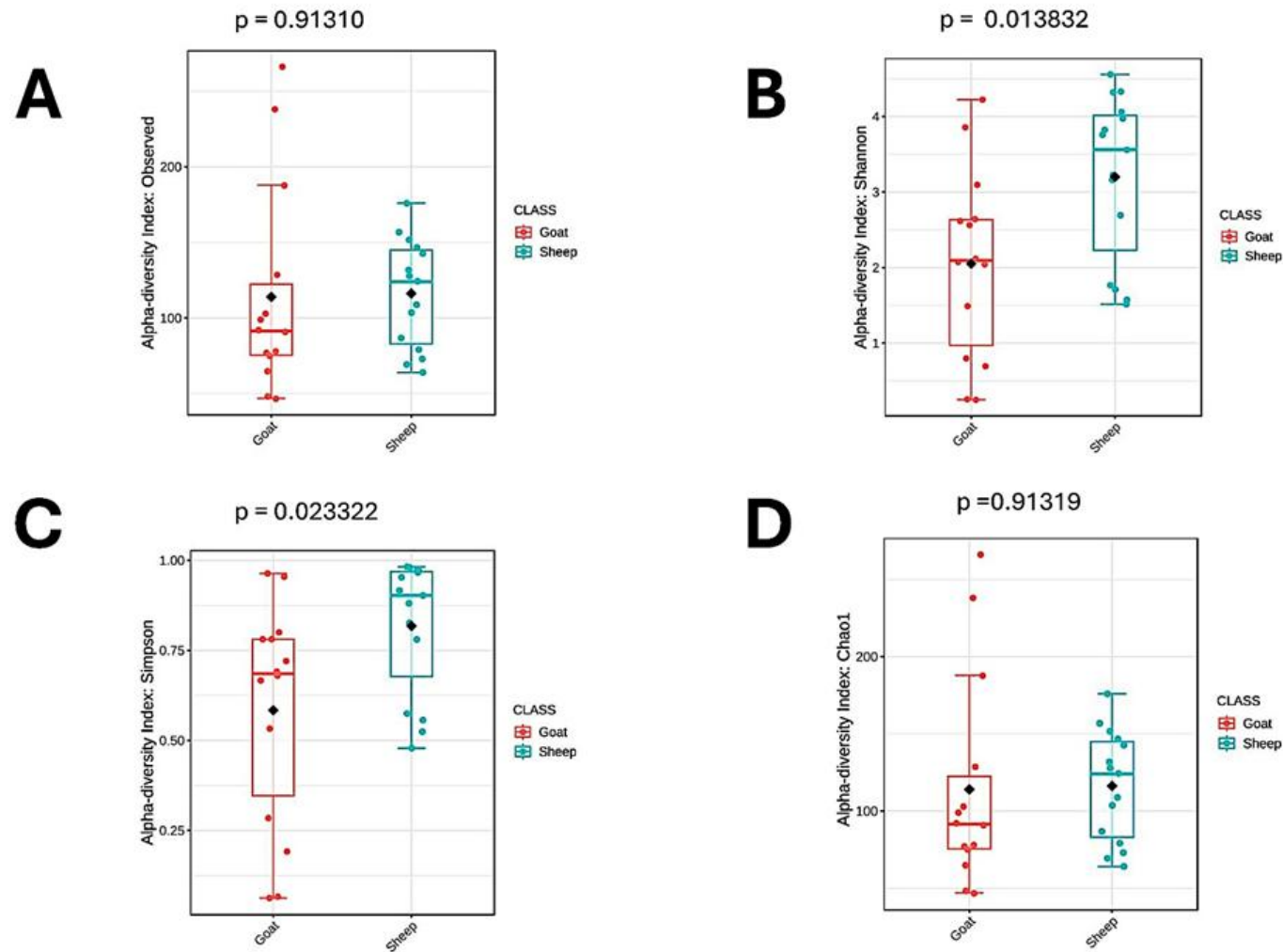
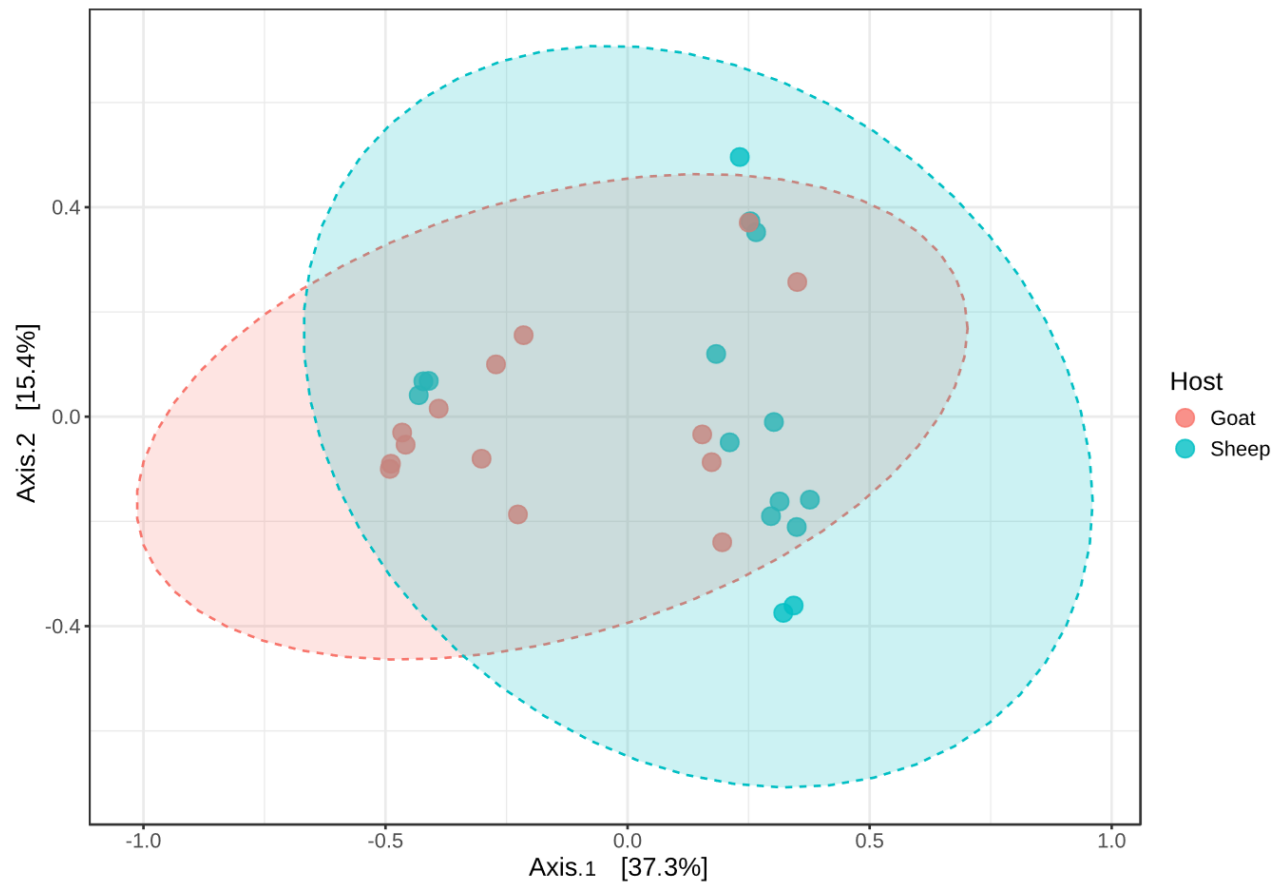


Figure 4.8: Alpha diversity analysis of boxplots (A-D) representing different alpha diversity metrics used to assess the bacterial diversity of *Amblyomma hebraeum* ticks collected from sheep and goats.

4.3.5 Beta diversity estimate of bacterial communities associated with *Amblyomma hebraeum* ticks collected from sheep and goats

The Principal Coordinate Analysis (PCoA) plot, constructed using the Bray–Curtis dissimilarity index, evaluates the beta diversity of bacterial populations in *A. hebraeum* ticks collected from goats and sheep. The first principal component (Axis 1) highlights 37.3% of the total variation, while the second principal component (Axis 2) accounts for 15.4% of the variation. The plot reveals distinct clustering patterns based on the host species. Ticks collected from goats (red) form a separate cluster from those collected from sheep (blue), suggesting that the host animal strongly influences the microbial communities in these ticks.

Although there is some overlap in the clusters, particularly along the second axis, the separation between goat- and sheep-derived ticks is evident. This distinct host-based clustering indicates that the host species primarily shapes the microbial composition of ticks. Gender influences microbial composition to a lesser extent, as suggested by the overlapping distribution of male and female ticks within each host cluster. Overall, the analysis highlights the significant role of host species in determining the microbial diversity of *A. hebraeum* ticks, with the host exerting a minor influence.



4.3.6 Jaccard-NDMS

The Non-Metric Multidimensional Scaling (NMDS) plot based on the Jaccard distance provides insights into the beta diversity of bacterial communities associated with *A. hebraeum* collected from goats and sheep (Figure 4.10). The Jaccard distance, a non-phylogenetic metric, quantifies differences in microbial composition by evaluating the presence or absence of bacterial taxa and their relative abundances across samples. In the NMDS plot, tick samples from goats (pink) and sheep (blue) exhibit distinct spatial distributions, with closer points indicating greater similarities in microbial communities and distant points reflecting higher dissimilarity.

The analysis reveals a tendency for ticks from goats and sheep to form separate clusters, albeit with some degree of overlap. Notably, ticks collected from sheep form a more tightly clustered group, indicative of a relatively homogeneous microbial composition. In contrast, ticks from goats displayed a more dispersed distribution, suggesting greater variability in their associated microbial communities. This pattern highlights the influence of the host species on the microbiota of *A. hebraeum*, with goat-associated ticks exhibiting more diverse microbiota compared to the more uniform microbial communities observed in sheep-associated ticks.

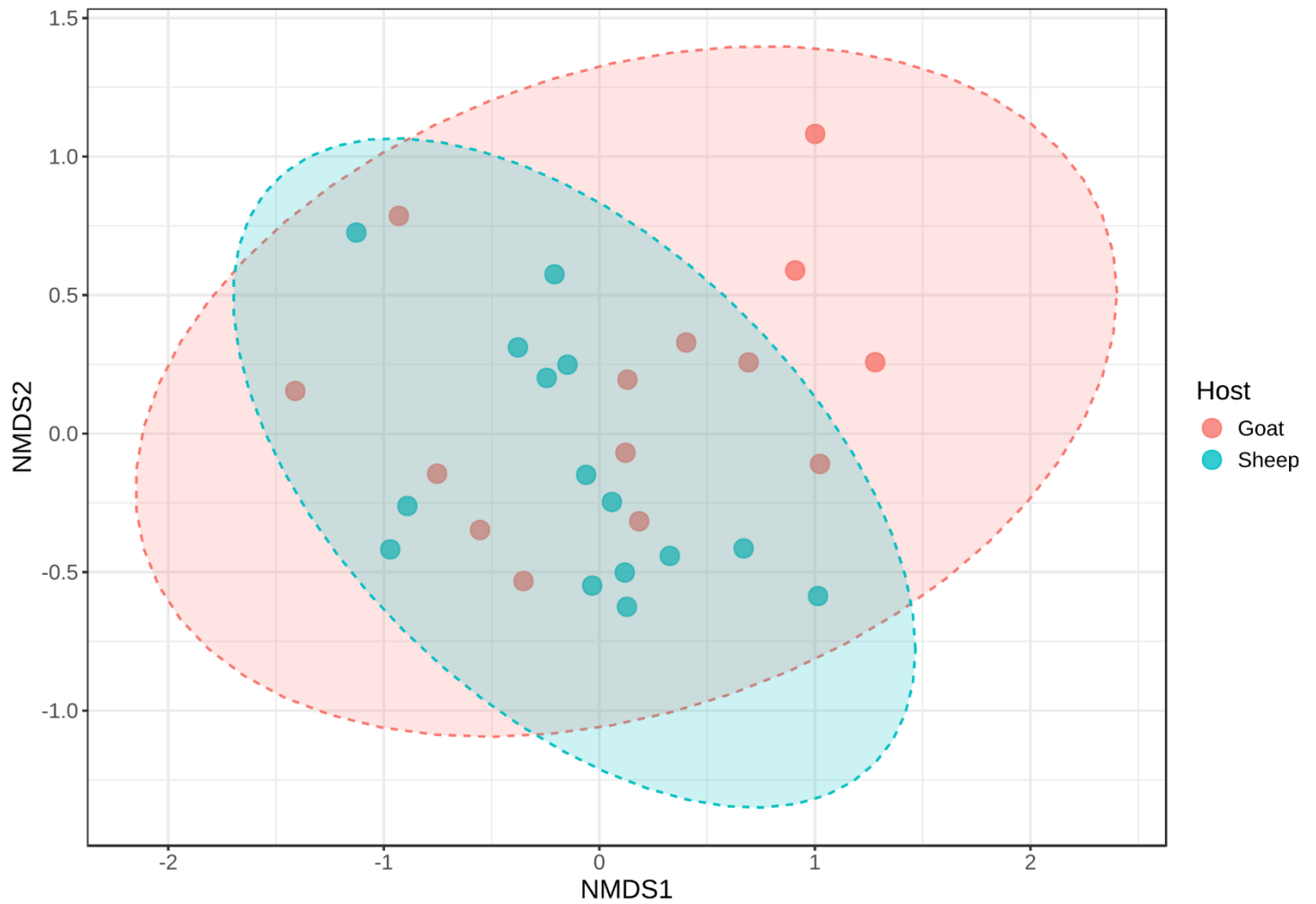


Figure 4.10: The Non-Metric Multidimensional Scaling (NMDS) in *Amblyomma hebraeum* ticks collected from sheep and goat, showing the beta diversity constructed using Bray–Curtis.

4.3.7 Determining the shared species richness between *Amblyomma hebraeum* ticks from different hosts and locations

Venn diagrams of bacterial genera were constructed to determine the shared species richness between *A. hebraeum* ticks collected from different hosts and locations. The Venn diagram reveals the distribution of bacterial genera found in *A. hebraeum* ticks collected from goats and sheep, depicted in Figure 4.11. A total of 1,374 bacterial genera are shared between ticks from both hosts, indicating a significant overlap in microbiome composition. However, ticks from goats and sheep also harbour distinct microbiota, with 1,504 and 1,372 unique bacterial genera, respectively. Overall, the combined richness of 4,250 bacterial genera highlights the diversity of microbial communities associated with *A. hebraeum* ticks in these hosts.

The second Venn diagram illustrates the distribution of bacterial genera identified in *A. hebraeum* ticks collected from four locations: Dihatshwane, Rooigrond, Ramtlabama, and Masutlhe depicted in Figure 4.12. The central region of the diagram shows that 200 bacterial genera are shared across all four locations, indicating a core microbiome that persists regardless of geographic differences. Beyond this shared core, specific overlaps highlight further relationships. For example, 376 bacterial genera are shared between Rooigrond and Ramtlabama, while 281 are shared between Ramtlabama and Masutlhe. Unique bacterial genera were observed in each location, with Dihatshwane, Rooigrond, Ramtlabama, and Masutlhe harbouring 194, 1,003, 601, and 868 exclusive genera, respectively.

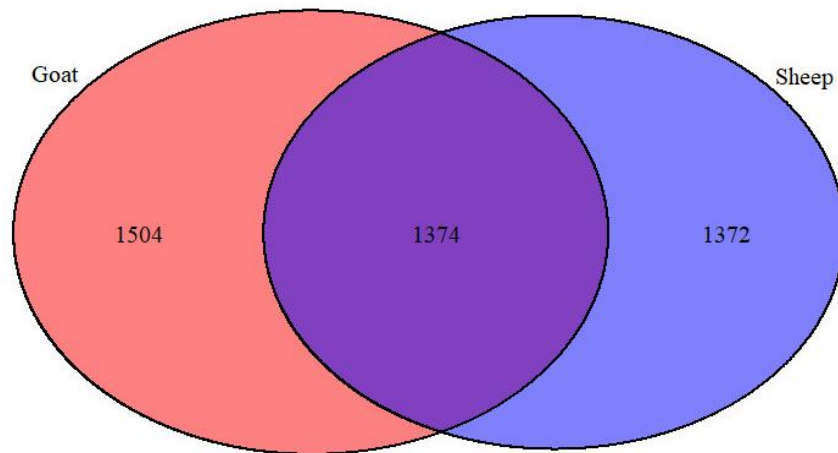


Figure 4.11: Venn diagram indicating the shared number of bacterial genera between *Amblyomma hebraeum* ticks collected from goats and sheep based on OTUs.

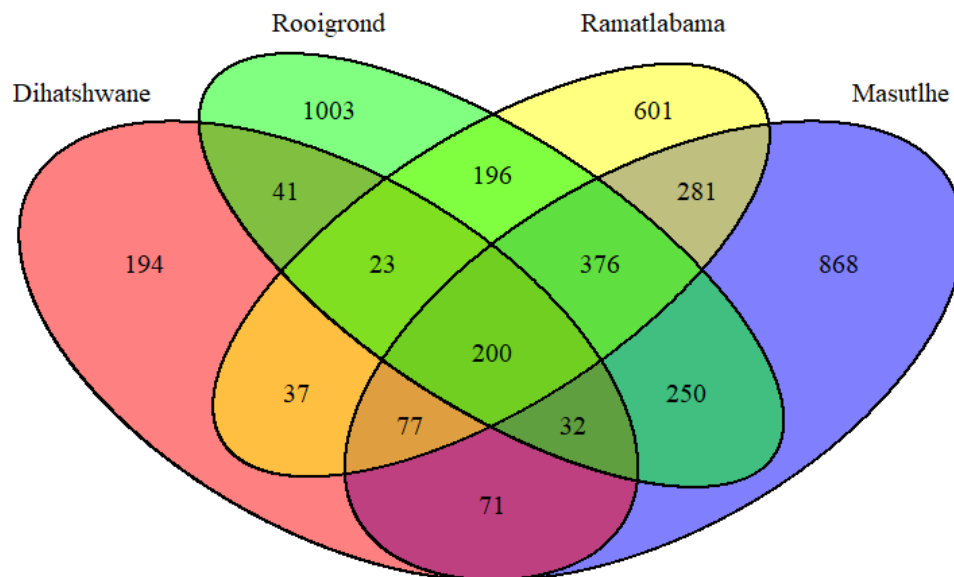


Figure 4.12: Venn diagram indicating the shared number of bacterial genera between *Amblyomma hebraeum* ticks collected from four different locations: Dihatshwane, Rooigrond, Ramtlabama, and Masutlhe based on OTUs.

4.4. Discussion

Bacterial communities were investigated in *A. hebraeum* ticks collected from sheep and goats in Mahikeng villages, North West Province, South Africa. Understanding livestock health, tick-borne pathogen transmission, and the dynamics of tick-borne diseases requires understanding the tick microbiome of ticks collected from various hosts. Despite the significance, studies on the tick microbiome of South African small stock are scarce. The study revealed a significant divergence in bacterial communities associated with *A. hebraeum* ticks from sheep and goats, suggesting a host-dependent microbial diversity.

The alpha diversity analyses, based on Observed OTUs, Shannon, Simpson, and Chao1 indices, revealed key differences in microbial community structure of the two host species. Shannon ($p = 0.013832$) and Simpson ($p = 0.023322$) indices showed significant variation, indicating that sheep-associated *A. hebraeum* ticks harbored a more diverse and evenly distributed microbiota compared to goat-associated ticks. One possible explanation is that differences in host physiology, immune responses, and skin microbiota may influence the microbial communities of ticks during feeding (Clay et al., 2008; Zolnik et al., 2016). Sheep may provide a more diverse microbial pool or a permissive environment for microbial colonization compared to goats. Additionally, variation in grazing behavior, husbandry practices, and environmental exposure between sheep and goats could contribute to differences in tick-associated microbiota (Estrada-Peña et al., 2018). The Shannon and Simpson indices are particularly relevant in assessing microbial diversity as they account for species richness and evenness, providing a more comprehensive understanding of community structure (Signorini et al., 2022). In contrast, the Observed OTUs and Chao1 indices, which did not show significant differences ($p > 0.05$), suggest that while the diversity of microbial species may differ, the overall richness of species present in ticks from both hosts remains comparable. This distinction is critical as it implies that while the composition of the microbial community varies, the number of species may not be a limiting factor in the ecological interactions occurring within these tick populations (Grabner et al., 2022).

The beta diversity analysis of *A. hebraeum* ticks from sheep and goats revealed distinct microbial community structures influenced primarily by host species. Principal Coordinate Analysis and NMDS showed clear clustering patterns, with ticks from sheep exhibiting more

homogeneous microbiota, while goat-associated ticks displayed a more dispersed pattern, reflecting a significant variability within the host. This variation could be attributed to differences in host physiology and immune responses, where sheep may provide a more stable internal and skin-associated microbial environment that promotes uniformity in tick microbiota, while goats may present more heterogeneous microbial inputs due to variations in diet, skin secretions, or management practices observed in the study area. These host-specific influences on tick-associated microbial communities align with earlier findings that vertebrate hosts play a major role in shaping the tick microbiome (Boulanger and Wikel, 2021; Narasimhan and Fikrig, 2015). Additionally, they may have significant consequences for vector control strategies aimed at mitigating tick-borne diseases. The differences in microbial community structures could influence the vector competency of *A. hebraeum*, as specific microbial taxa are known to affect tick physiology and pathogen transmission (Adegoke *et al.*, 2020; Mateos-Hernández *et al.*, 2020). For instance, specific endosymbionts can enhance ticks' survival and reproductive success, thereby affecting their role as vectors for pathogens (Portillo *et al.*, 2019; Du *et al.*, 2023). Understanding these dynamics is essential for developing targeted interventions that could disrupt the transmission of tick-borne pathogens by manipulating the tick microbiome (Abbasi, 2024).

At the phylum level, Proteobacteria was the dominant phylum in ticks from both hosts, accounting for 66.12% in goats and 41.80% in sheep. The predominance of Proteobacteria in various tick species has been consistently documented, suggesting a critical role for this phylum in shaping the microbial communities associated with these ectoparasites (Wei *et al.*, 2022). This dominance may be explained by the metabolic versatility of Proteobacteria, which enables them to thrive in diverse environments, including the nutrient-limited conditions within ticks. Additionally, several Proteobacteria taxa include symbiotic species that contribute to essential functions such as vitamin biosynthesis, reproductive fitness, and pathogen facilitation within ticks (Duron *et al.*, 2018; Bonnet *et al.*, 2017). Recent studies on tick microbiota across multiple species reveal Proteobacteria as the predominant phylum, followed by Bacteroidetes and Firmicutes (Papa *et al.*, 2020; Wei *et al.*, 2022; Nogueras *et al.*, 2024). The predominance of Proteobacteria has also been reported in the ticks from the AI Khotha region of Saudi Arabia (Alreshidi *et al.*, 2020). These findings suggest a universal trend in tick microbiota composition, where Proteobacteria may play vital roles in the ticks' interactions, influencing tick physiology, development, and pathogen transmission, with variations in their abundance

and interactions across different tick species, developmental stages, and environmental conditions (Zhang *et al.*, 2019).

At the family level, *Rickettsiaceae*, *Staphylococcaceae*, *Coxiellaceae*, and *Bacillaceae* were dominant across host species, but their relative abundances varied. Goat-derived ticks were strongly dominated by *Rickettsiaceae* (50.68%), whereas sheep-derived ticks exhibited a more balanced representation of families. This suggests host-specific enrichment and possible functional implications in microbial interactions; it further links to the findings of alpha diversity, where in sheep, there was a more balanced and even distribution of microbial communities, while in goats, their distribution was dispersed to specific microbial communities. Low-abundance families such as *Saprospiraceae*, *Cyclobacteriaceae*, and *Intrasporangiaceae*, although minor in abundance, may contribute to ecological balance and microbial succession (Kurilshikov *et al.*, 2021).

Furthermore, at the genus level, *Rickettsia* was predominant in *A. hebraeum* ticks from both hosts, although their relative abundances differed. In the *A. hebraeum* ticks from goats, *Rickettsia* accounted for 51.64% of the microbiome, whereas in ticks from sheep, it accounted for 18.85%. The high relative abundance of *Rickettsia* spp. in *Amblyomma hebraeum* ticks collected from goats suggests that, despite the goats' grooming behavior, these ticks may still serve as significant reservoirs and vectors for *Rickettsia* spp. This aligns with findings from previous studies that have reported higher tick burdens and associated pathogen prevalence in goats compared to sheep (Elelu *et al.*, 2022; Iweriebor *et al.*, 2020). The presence of *Rickettsia* spp. in these ticks raises both public and animal health concerns, as rickettsial infections can be transmitted through tick bites, causing various human diseases, including African tick bite fever. Notably, cases of tick-bite fever have been reported in South Africa, highlighting the ongoing risk of rickettsial infections in endemic regions (Musso *et al.*, 2014; Narantsatsral *et al.*, 2015; Cherry *et al.*, 2018).

In addition, the top 30 genera heatmap detected pathogenic genera including *Coxiella*, *Bacillus*, *Staphylococcus*, *Corynebacterium*, *Ehrlichia*, *Acinetobacter*, and *Streptococcus* in *A. hebraeum* ticks from sheep and goats, which has significant implications for both veterinary and public health. The presence of these pathogens in ticks can lead to increased morbidity and mortality in livestock, affecting productivity and economic stability for farmers (Estrada-Peña *et al.*, 2012). For instance, *Coxiella burnetii* has been associated with reproductive issues in

ruminants, including abortions and stillbirths, which can have devastating effects on livestock populations (Telmadarraiy *et al.*, 2023; Mangena, 2023). Additionally, pathogens such as *Staphylococcus aureus*, *Streptococcus dysgalactiae*, and *Corynebacterium bovis* have been reported to cause intramammary infections, leading to mastitis and reduced milk production in cattle (Taponen *et al.*, 2017). Moreover, emerging zoonotic pathogens like *Acinetobacter* species have been reported in various animals and food sources, posing potential public health risks (Carvalho, 2021). In Cuba, *Ehrlichia* bacteria were detected in *Dermacentor nitens* ticks, indicating the presence of a previously unreported *Ehrlichia* species in this tick host (Rodríguez *et al.*, 2009). A case study reported six goats with gangrenous mastitis were found to have *Bacillus* sp. infections, which were successfully treated, although affected udder halves were discarded (Mavangira *et al.*, 2013). In South Africa, seroprevalence studies have shown widespread distribution of *E. ruminantium* antibodies in goats, with higher prevalence in endemic regions and smallholder production systems (Mdladla *et al.*, 2016; Jongejan *et al.*, 2020; Seakamela *et al.*, 2024).

Venn diagram analyses based on genus-level OTUs provided insights into shared and unique microbial communities across hosts and sampling sites. These unique genera may reflect host-specific influences such as differences in diet, immune responses, or physiological conditions. The identification of a core microbiome, consisting of 200 bacterial genera shared across all locations, suggests a stable and resilient microbial community that is maintained despite geographic variations. This finding aligns with previous studies that emphasize the importance of core microbiomes in tick species, which can influence their biology and interactions with pathogens (Chicana *et al.*, 2019; Couper *et al.*, 2019; Adegoke *et al.*, 2020). The persistence of this core microbiome may play a critical role in the ticks' ecological fitness and their capacity to act as vectors for tick-borne diseases.

Further analysis on geographic origin, indicates localized microbial adaptations or interactions that could be influenced by environmental factors or host availability in the different regions. This observation is consistent with research indicating that environmental conditions and host interactions significantly shape the microbiome composition in ticks (Adegoke *et al.*, 2021; Ponnusamy *et al.*, 2024). The unique bacterial genera identified in each location further highlighted the ecological diversity and adaptability of tick microbiomes. Such diversity may reflect the specific ecological niches and host interactions present in each area, supporting the

idea that geographic and ecological factors are crucial in determining microbial community structure (Estrada-Peña *et al.*, 2020; Maldonado-Ruiz *et al.*, 2021).

Moreover, the high number of unique genera in Rooigrond suggests that this location may harbour a particularly rich and diverse microbial community, potentially due to specific environmental conditions such as its semi-arid climate, characterized by low and erratic rainfall and high temperatures (Spickett *et al.*, 201). This finding is in line with studies that have shown how habitat and host diversity can significantly influence the composition of tick microbiomes (Segura *et al.*, 2020; Liu *et al.*, 2024). Particularly, the presence and relative abundance of *Rickettsia* in *A. hebraeum* ticks in this study may also be linked to specific environmental conditions that favour tick survival and reproduction, and the prevalence of associated pathogens. Differences in habitat between sheep and goats may contribute to variations of *A. hebraeum* ticks *Rickettsia* prevalence and other microbiota, consistent with research highlighting that tick species distribution and their associated pathogens can vary significantly based on geographic and climatic factors (Shao *et al.*, 2020; Eljadar *et al.*, 2022). Understanding these unique microbial communities is essential, as they may contribute to the ticks' vector competence and their interactions with pathogens, which could have implications for the transmission dynamics of tick-borne diseases (Rojas-Jaimes *et al.*, 2020; Adegoke *et al.*, 2022).

This study investigated the bacterial communities associated with *Amblyomma hebraeum* ticks harboured by sheep and goats in Mahikeng, South Africa. It highlights the significant influence of host species and geographic origin on the composition of the microbiome. Based on the alpha and beta diversity analyses, it was found that ticks from sheep possess a more diverse and evenly distributed microbiome, whereas those from goats are predominantly characterized by *Rickettsia* and exhibit more variable microbial profiles. The prevalence of *Proteobacteria*, alongside the identification of several pathogenic genera, including *Rickettsia*, *Coxiella*, *Ehrlichia*, and *Staphylococcus*, underscores the importance of these ticks in veterinary and public health contexts. Geographic variation also plays a crucial role in shaping the microbiome structure, with distinct bacterial taxa identified across different locations. Moreover, the presence of shared core genera indicates a degree of ecological stability within the microbiome. These findings emphasize the potential effects of environmental factors, host species, and microbial interactions on tick microbiota and the dynamics of pathogen transmission. This study enhances our understanding of the intricate relationships between ticks, their

microbiomes, and pathogens in South African small ruminants, supporting the development of microbiome-targeted strategies for the surveillance and control of tick-borne diseases.

References

1. Abbasi, A.M., Nasir, S., Bajwa, A.A., Akbar, H., Ali, M.M. and Rashid, M.I., 2024. A comparative study of the microbiomes of the ticks *Rhipicephalus microplus* and *Hyalomma anatolicum*. *Parasite*, 31, p.74.
2. Adegoke, A., Kumar, D., Bobo, C., Rashid, M.I., Durrani, A.Z., Sajid, M.S. and Karim, S., 2020. Tick-borne pathogens shape the native microbiome within tick vectors. *Microorganisms*, 8(9), p.1299.
3. Adegoke, A., Kumar, D., Budachetri, K. and Karim, S., 2021. Changes in microbial composition, diversity, and functionality in the *Amblyomma maculatum* microbiome following infection with *Rickettsia parkeri*. *bioRxiv: The Preprint Server for Biology*.
4. Adegoke, A., Kumar, D., Budachetri, K. and Karim, S., 2022. Hematophagy and tick-borne rickettsial pathogen shape the microbial community structure and predicted functions within the tick vector, *Amblyomma maculatum*. *Frontiers in Cellular and Infection Microbiology*, 12, p.1037387.
5. Allsopp, B.A., 2015. Heartwater—*Ehrlichia ruminantium* infection. In: Coetzer, J.A.W. and Tustin, R.C., eds. *Infectious diseases of livestock*, 2nd ed. Cape Town: Oxford University Press Southern Africa, pp.1055–1071.
6. Alreshidi, M.M., Veettil, V.N., Noumi, E., Del Campo, R. and Snoussi, M., 2020. Description of microbial diversity associated with ticks *Hyalomma dromedarii* (Acari: Ixodidae) isolated from camels in Hail region (Saudi Arabia) using massive sequencing of 16S rDNA. *Bioinformation*, 16(8), p.602.
7. Bonnet, S.I., Binetruy, F., Hernández-Jarguín, A.M. and Duron, O., 2017. The tick microbiome: why non-pathogenic microorganisms matter in tick biology and pathogen transmission. *Frontiers in cellular and infection microbiology*, 7, p.236.
8. Callahan, B.J., Grinevich, D., Thakur, S., Balamotis, M.A. & Yehezkel, T.B., 2021. Ultra-accurate microbial amplicon sequencing with synthetic long reads. *Microbiome*, 9(1), p.130.

9. Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A. & Holmes, S.P., 2016. DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, 13(7), pp.581-583.
10. Cantas, H., Muwonge, A., Sareyyupoglu, B., Yardimci, H. and Skjerve, E., 2011. Q fever abortions in ruminants and associated on-farm risk factors in northern Cyprus. *BMC Veterinary Research*, 7, pp.1–7.
11. Cherry, C.C., Denison, A.M., Kato, C.Y., Thornton, K. and Paddock, C.D., 2018. Diagnosis of spotted fever group rickettsioses in US travelers returning from Africa, 2007–2016. *The American journal of tropical medicine and hygiene*, 99(1), p.136.
12. Chicana, B., Couper, L.I., Kwan, J.Y., Tahiraj, E. and Swei, A., 2019. Comparative microbiome profiles of sympatric tick species from the far-western United States. *Insects*, 10(10), p.353.
13. Chigwada, A.D., Mapholi, N.O., Ogola, H.J.O., Mbizeni, S. and Masebe, T.M., 2022. Pathogenic and endosymbiotic bacteria and their associated antibiotic resistance biomarkers in *Amblyomma* and *Hyalomma* ticks infesting Nguni cattle (*Bos spp.*). *Pathogens*, 11(4), p.432.
14. Clay, K. and Fuqua, C., 2010. The tick microbiome: diversity, distribution and influence of the internal microbial community for a blood-feeding disease vector. In: *Critical needs and gaps in understanding prevention, amelioration, and resolution of Lyme and other tick-borne diseases: the short-term and long-term outcomes* (Workshop report). Washington, DC: National Academies Press.
15. Clay, K., Fuqua, C., Lively, C. and Wade, M.J., 2006. Microbial community ecology of tick-borne human pathogens. *Disease Ecology: Community Structure and Pathogen Dynamics*. Oxford University Press: Oxford, pp.41-57.
16. Couper, L.I., Kwan, J.Y., Ma, J. and Swei, A., 2019. Drivers and patterns of microbial community assembly in a Lyme disease vector. *Ecology and Evolution*, 9(13), pp.7768-7779.
17. Du, L.F., Zhang, M.Z., Yuan, T.T., Ni, X.B., Wei, W., Cui, X.M., Wang, N., Xiong, T., Zhang, J., Pan, Y.S. and Zhu, D.Y., 2023. New insights into the impact of microbiome on horizontal and vertical transmission of a tick-borne pathogen. *Microbiome*, 11(1), p.50.

18. Duron, O., Morel, O., Noël, V., Buysse, M., Binetruy, F., Lancelot, R., Loire, E., Ménard, C., Bouchez, O., Vavre, F. and Vial, L., 2018. Tick-bacteria mutualism depends on B vitamin synthesis pathways. *Current Biology*, 28(12), pp.1896-1902.
19. Elelu, N., Ola-Fadunsin, S.D., Bankole, A.A., Raji, M.A., Ogo, N.I. and Cutler, S.J., 2022. Prevalence of tick infestation and molecular characterization of spotted fever *Rickettsia massiliae* in *Rhipicephalus* species parasitizing domestic small ruminants in north-central Nigeria. *Public Library of Science ONE*, 17(2), p.e0263843.
20. Eljadar, M.S., Sallam, N.H., Soltan, M.A., El Gawady, H.M. and Abouelhassan, E.M., 2022. Morphological and molecular studies on tick species in Ismailia Governorate in Egypt and Al Gabal Al Akhdar in Libya. *Open Veterinary Journal*, 12(6), pp.985-994.
21. Estrada-Peña, A., Ayllón, N. and De La Fuente, J., 2012. Impact of climate trends on tick-borne pathogen transmission. *Frontiers in Physiology*, 3, p.64.
22. Estrada-Peña, A., Cabezas-Cruz, A. and Obregón, D., 2020. Resistance of tick gut microbiome to anti-tick vaccines, pathogen infection and antimicrobial peptides. *Pathogens*, 9(4), p.309.
23. Estrada-Peña, A., Cabezas-Cruz, A., Pollet, T., Vayssier-Taussat, M. and Cosson, J.F., 2018. High throughput sequencing and network analysis disentangle the microbial communities of ticks and hosts within and between ecosystems. *Frontiers in Cellular and Infection Microbiology*, 8, p.236.
24. Estrada-Peña, A., Gray, J.S., Kahl, O., Lane, R.S. and Nijhof, A.M., 2013. Research on the ecology of ticks and tick-borne pathogens—methodological principles and caveats. *Frontiers in Cellular and Infection Microbiology*, 3, p.29.
25. Grabner, D., Doliwa, A., Sworobowicz, L., Wysocka, A., Weigand, A., Grabowski, M., Mamos, T. and Sures, B., 2022. Microsporidian diversity in the aquatic isopod *Asellus aquaticus*. *Parasitology*, 149(13), pp.1729-1736.
26. Hurtado, O.J.B. and Giraldo-Ríos, C., 2018. Economic and health impact of the ticks in production animals. *Ticks and Tick-borne Pathogens*, 9, pp.1-9.
27. Hussain, S., Perveen, N., Hussain, A., Song, B., Aziz, M.U., Zeb, J., Li, J., George, D., Cabezas-Cruz, A. and Sparagano, O., 2022. The symbiotic continuum within ticks: opportunities for disease control. *Frontiers in Microbiology*, 13, p.854803.

28. Iweriebor, B.C., Nqoro, A. and Obi, C.L., 2020. *Rickettsia africae*: an agent of African tick bite fever in ticks collected from domestic animals in Eastern Cape, South Africa. *Pathogens*, 9(8), p.631.
29. Jongejan, F., Berger, L., Busser, S., Deetman, I., Jochems, M., Leenders, T., De Sitter, B., Van der Steen, F., Wentzel, J. and Stoltsz, H., 2020. *Amblyomma hebraeum* is the predominant tick species on goats in the Mnisi Community Area of Mpumalanga Province South Africa and is co-infected with *Ehrlichia ruminantium* and *Rickettsia africae*. *Parasites & Vectors*, 13, pp.1-12.
30. Kisten, D., Brinkerhoff, J., Tshilwane, S.I. and Mukaratirwa, S., 2021. A pilot study on the microbiome of *Amblyomma hebraeum* tick stages infected and non-infected with *Rickettsia africae*. *Pathogens*, 10(8), p.941.
31. Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M. and Glöckner, F.O., 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic acids research*, 41(1), pp.e1-e1.
32. Liu, Z.L., Qiu, Q.G., Cheng, T.Y., Liu, G.H., Liu, L. and Duan, D.Y., 2024. Composition of the midgut microbiota structure of *Haemaphysalis longicornis* tick parasitizing tiger and deer. *Animals*, 14(11), p.1557.
33. Maldonado-Ruiz, L.P., Neupane, S., Park, Y. and Zurek, L., 2021. The bacterial community of the lone star tick (*Amblyomma americanum*). *Parasites & Vectors*, 14, pp.1-10.
34. Mangena, M.L., Gcebe, N., Thompson, P.N. and Adesiyun, A.A., 2023. Q fever and toxoplasmosis in South African livestock and wildlife: A retrospective study on seropositivity, sporadic abortion, and stillbirth cases in livestock caused by *Coxiella burnetii*. *BMC Veterinary Research*, 19(1), p.168.
35. Mateos-Hernández, L., Obregón, D., Maye, J., Borneres, J., Versille, N., de La Fuente, J., Estrada-Peña, A., Hodžić, A., Šimo, L. and Cabezas-Cruz, A., 2020. Anti-tick microbiota vaccine impacts *Ixodes ricinus* performance during feeding. *Vaccines*, 8(4), p.702.
36. Mavangira, V., Angelos, J.A., Samitz, E.M., Rowe, J.D. and Byrne, B.A., 2013. Gangrenous mastitis caused by *Bacillus* species in six goats. *Journal of the American Veterinary Medical Association*, 242(6), pp.836-843.

37. McMurdie, P.J. and Holmes, S., 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *Public Library of Science ONE*, 8(4), p.e61217.
38. Mdladla, K., Dzomba, E.F. and Muchadeyi, F.C., 2016. Seroprevalence of *Ehrlichia ruminantium* antibodies and its associated risk factors in indigenous goats of South Africa. *Preventive Veterinary Medicine*, 125, pp.99-105.
39. Mnisi, S.S., Mphuthi, M.B., Ramatla, T., Mofokeng, L.S., Thekiso, O. and Syakalima, M., 2022. Molecular detection and genetic characterization of *Ehrlichia ruminantium* harbored by *Amblyomma hebraeum* ticks of domestic ruminants in North West Province, South Africa. *Animals*, 12(19), p.2511.
40. Mnisi, S.S., Mphuthi, M.B., Ramatla, T., Mofokeng, L.S., Thekiso, O. and Syakalima, M., 2022. Molecular detection and genetic characterization of *Ehrlichia ruminantium* harbored by *Amblyomma hebraeum* ticks of domestic ruminants in North West Province, South Africa. *Animals*, 12(19), p.2511.
41. Mtshali, K., Khumalo, Z.T., Nakao, R., Grab, D.J., Sugimoto, C. and Thekiso, O.M., 2015. Molecular detection of zoonotic tick-borne pathogens from ticks collected from ruminants in four South African provinces. *Journal of Veterinary Medical Science*, 77(12), pp.1573-1579.
42. Musso, D., Brout, J., Parola, P., Raoult, D. and Fournier, P.E., 2014. Absence of antibodies to *Rickettsia* spp., *Bartonella* spp., *Ehrlichia* spp. and *Coxiella burnetii* in Tahiti, French Polynesia. *BMC Infectious Diseases*, 14, pp.1-4.
43. Narantsatsral, S., Myagmarsuren, P., Davaasuren, P., Enkhtaivan, B., Battsetseg, B. and Battur, B., 2014. Molecular biological detection of emerging tick-borne zoonotic pathogens in ixodid tick species. *Mongolian Journal of Agricultural Sciences*, 13(2), pp.3-7.
44. Narasimhan, S. and Fikrig, E., 2015. Tick microbiome: the force within. *Trends in parasitology*, 31(7), pp.315-323.
45. Nogueras, V., de la Fuente, J. and Diaz Sanchez, S., 2024. Understanding the mechanisms underlying microbiota variation in wild tick populations. *bioRxiv*, pp.2024-12.

46. Papa, A., Tsioka, K., Daskou, M.A., Minti, F., Papadopoulou, E., Melidou, A. and Giadinis, N., 2020. Application of 16S rRNA next generation sequencing in ticks in Greece. *Heliyon*, 6(7).
47. Peter, T.F., Bryson, N.R., Perry, B.D., O'Callagan, C.J., Medley, G.F., Smith, G.E., Mlambo, G., Horak, I.G., Burrridge, M.J. and Mahan, S.M., 1999. *Cowdria ruminantium* infection in ticks in the Kruger National Park. *Veterinary Record*, 145(11), pp.304-307.
48. Ponnusamy, L., Travanty, N.V., Watson, D.W., Seagle, S.W., Boyce, R.M. and Reiskind, M.H., 2024. Microbiome of invasive tick species *Haemaphysalis longicornis* in North Carolina, USA. *Insects*, 15(3), p.153.
49. Portillo, A., Palomar, A.M., de Toro, M., Santibáñez, S., Santibáñez, P. and Oteo, J.A., 2019. Exploring the bacteriome in anthropophilic ticks: To investigate the vectors for diagnosis. *Public Library of Science ONE*, 14(3), p.e0213384.
50. Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J. and Glöckner, F.O., 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic acids research*, 41(D1), pp.D590-D596.
51. Ramees, T.P., Dhama, K., Karthik, K., Rathore, R.S., Kumar, A., Saminathan, M., Tiwari, R., Malik, Y.S. and Singh, R.K., 2017. *Arcobacter*: An emerging food-borne zoonotic pathogen, its public health concerns and advances in diagnosis and control—a comprehensive review. *Veterinary Quarterly*, 37(1), pp.136-161.
52. Rojas-Jaimes, J., Lindo-Seminario, D., Correa-Núñez, G. and Diringer, B., 2021. Characterization of the bacterial microbiome of *Rhipicephalus (Boophilus) microplus* collected from *Pecari tajacu* (“Sajino”) in Madre de Dios, Peru. *Scientific Reports*, 11(1), p.6661.
53. Seakamela, E., Matle, I., Ndudane, N., Lepheana, R. and Mbizeni, S., 2024. Retrospective analysis of laboratory diagnostic data to assess the prevalence and seasonal variation of *Ehrlichia ruminantium* in commercial farms of Lephalale Municipality, Limpopo, South Africa. *International Journal for Parasitology: Parasites and Wildlife*, 24, p.100959.
54. Segura, J.A., Isaza, J.P., Botero, L.E., Alzate, J.F. and Gutiérrez, L.A., 2020. Assessment of bacterial diversity of *Rhipicephalus microplus* ticks from two

- livestock agroecosystems in Antioquia, Colombia. *Public Library of Science ONE*, 15(7), p.e0234005.
55. Shao, J.W., Zhang, X.L., Li, W.J., Huang, H.L. and Yan, J., 2020. Distribution and molecular characterization of rickettsiae in ticks in Harbin area of Northeastern China. *PLOS Neglected Tropical Diseases*, 14(6), p.e0008342.
 56. Sidi-Boumedine, K., Rousset, E., Henning, K., Ziller, M., Niemczuck, K., Roest, H.I.J. and Thiéry, R., 2010. Development of harmonised schemes for the monitoring and reporting of Q-fever in animals in the European Union. *EFSA Supporting Publications*, 7(5), p.48E.
 57. Signorini, M., Midolo, G., Cesco, S., Mimmo, T. and Borruso, L., 2023. A matter of metals: Copper but not cadmium affects the microbial alpha-diversity of soils and sediments—a meta-analysis. *Microbial Ecology*, 86(2), pp.1071-1081.
 58. Sonenshine, D.E. and Roe, R.M., 2013. Ticks, people, and animals. In: *Biology of Ticks*, Vol. 1, pp.1.
 59. Taponen, S., Liski, E., Heikkilä, A.M. and Pyörälä, S., 2017. Factors associated with intramammary infection in dairy cows caused by coagulase-negative *Staphylococci*, *Staphylococcus aureus*, *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Corynebacterium bovis*, or *Escherichia coli*. *Journal of Dairy Science*, 100(1), pp.493-503.
 60. Telmadarraiy, Z., Kooshki, H., Edalat, H., Vatandoost, H., Bakhshi, H., Faghihi, F., Hosseini-Chegeni, A. and Oshaghi, M.A., 2022. Study on hard and soft ticks of domestic and wild animals in Western Iran. *Journal of Arthropod-Borne Diseases*, 16(3), p.225.
 61. Thekiso, O., Ramatla, T., Ringo, A., Mnisi, S., Mphuthi, N., Mofokeng, L., Lekota, K. and Xuan, X., 2023. Molecular detection of *Rickettsia africae* from *Amblyomma hebraeum* ticks in Mafikeng City of North West Province, South Africa. *Research in Veterinary Science*, 164, p.105027.
 62. Wei, N., Lu, J., Dong, Y. and Li, S., 2022. Profiles of microbial community and antibiotic resistome in wild tick species. *mSystems*, 7(4), pp.e00037-22.
 63. Wickham, H., 2016. Data analysis. In *ggplot2: elegant graphics for data analysis* (pp. 189-201). Cham: Springer international publishing.

64. Zhang, R., Yu, G., Huang, Z. and Zhang, Z., 2019. Microbiota assessment across different developmental stages of *Dermacentor silvarum* (Acari: Ixodidae) revealed stage-specific signatures. *Ticks and Tick-borne Diseases*, p.101321.
65. Zolnik, C.P., Prill, R.J., Falco, R.C., Daniels, T.J. and Kolokotronis, S.O., 2016. Microbiome changes through ontogeny of a tick pathogen vector. *Molecular ecology*, 25(19), pp.4963-4977.
66. Zweggarth, E.P., 2006. *In vitro cultivation of Ehrlichia ruminantium and development of an attenuated culture-derived vaccine* (Doctoral dissertation, Utrecht University).

5. CHAPTER 5

Bacterial communities harboured by *Rhipicephalus evertsi evertsi* ticks from small stock in Matlwang village, Potchefstroom

5.1 Introduction

Ticks are vectors of a wide range of pathogens, making them critical hematophagous ectoparasites that impact both animal and human health (Boulanger *et al.*, 2019). One of the most widely distributed tick species in sub-Saharan Africa, *Rhipicephalus evertsi evertsi*, plays a significant role in transmitting multiple pathogens to livestock (Jongejan and Uilenberg, 2004). To better understand tick biology and vector competence, it is essential to examine complex interactions and their surrounding microbial communities, including symbiotic and pathogenic bacteria (Narasimhan and Fikrig, 2015).

Recent advancements in molecular methods, particularly high-throughput sequencing, have provided comprehensive insights into the microbiota associated with ticks (Bonnet and Pollet, 2021). The 16S rRNA gene metabarcoding approach targets conserved bacterial regions, enabling detailed profiling of bacterial communities and offering insight into microbial populations' quantity, diversity and composition (Caporaso *et al.*, 2011). Several studies have used 16S rRNA metabarcoding to explore bacterial communities within ticks, revealing a wider variety of microbiomes across different tick species (Williams-Newkirk *et al.*, 2014; Rynkiewicz *et al.*, 2015; Kurilshikov *et al.*, 2015; Sperling *et al.*, 2017; Varela-Stokes *et al.*,

2017; Egan *et al.*, 2019; Lim *et al.*, 2019; Egan *et al.*, 2021). These studies identified a range of bacterial phyla, with *Proteobacteria* being the most dominant (Portillo *et al.*, 2019; Papa *et al.*, 2020; Mutai *et al.*, 2022). Commonly observed genera include *Rickettsia*, *Anaplasma* and *Coxiella* species, alongside additional endosymbionts such as “*Candidatus* Midichloria mitochondrii” (Cerutti *et al.*, 2018; Papa *et al.*, 2020; Egan *et al.*, 2021). Variations in tick microbiomes depend on factors such as host, species, gender, and geographic location (Kurilshikov *et al.*, 2015).

Next-generation sequencing (NGS) has revolutionized our understanding of tick-borne pathogens (TBPs), uncovering a previously hidden world of microbial diversity and host–pathogen interactions (Egan *et al.*, 2020; Rodino *et al.*, 2021). By shedding light on the complex interplay between ticks, their microbial symbionts, and the pathogens they harbour, NGS offers a powerful lens through which to explore tick ecology and disease transmission dynamics. In South Africa, emerging studies have illuminated the diverse bacterial landscapes of veterinary and public health concern harboured by various tick species. Notably, *Rickettsia africae* has been detected in *Amblyomma hebraeum* (Kisten *et al.*, 2021; Thekisoe *et al.*, 2023), while *Ehrlichia* spp. have been identified in multiple tick genera infesting ruminants (Mtshali *et al.*, 2015; Iweriebor *et al.*, 2017; Mnisi *et al.*, 2022).

Adding to this body of work, Khoza *et al.* (2024) profiled tick distributions and their associated bacterial pathogens in cattle, identifying eight tick species with *Rhipicephalus evertsi evertsi* as the most dominant and highlighting the prevalence of *Proteobacteria* in the blood microbiota. These findings underscore tick microbiomes' ecological and epidemiological importance, which may be key to understanding microbial symbiosis, vector competence, and pathogen persistence. Despite these advances, there remains a critical surveillance gap regarding the microbial communities of ticks infesting livestock in rural regions such as Matlwang village, North West. To address this, the present chapter investigates the bacterial communities of *R. e. evertsi* using high-throughput 16S rRNA metabarcoding, aiming to uncover known and novel bacterial associations that may shape this tick species' biology and vector potential.

5.2 Materials and methods

5.2.1 Study area, sample collection and DNA extraction

A total of 273 *Rhipicephalus evertsi evertsi* tick samples were collected from sheep (n = 192) and goats (n = 80) in Matlwang village, Potchefstroom city [26.4451°S; 26.5552° E]. Ticks were identified morphologically using the keys from Latif (2013) and further confirmed using molecular assays targeting the 16S rRNA and 18S rRNA. DNA was extracted using QIAGEN DNeasy® Blood & Tissue Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). Details of the study area, sample collection, and DNA extraction are described in the materials and methods (Chapter 3.1).

5.2.2 Library preparation and sequencing

The 16S rRNA gene sequencing for *R. e. evertsi* ticks was executed using primers based on the Illumina DNA library preparation protocol (Illumina, USA). Locus-specific primers, 341F (5'-CCT ACG GGN GGC WGC AG-3') and 805R (5'-GAC TAC HVG GGT ATC TAA TCC-3') (Klindworth et al., 2013), targeting the hypervariable V3-V4 region (approximately 460 bp 34 region) of the 16S rRNA gene, were used with overhang adapters at the 5'-end of forward and reverse primers, respectively (Illumina, USA). The PCR primers used were: forward primer 5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG-3' and reverse primer: 5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C-3' (Klindworth *et al.*, 2013). . The library preparation workflow entailed a first-stage amplicon PCR step to amplify the region, using 12.5 ng of DNA, 12.5 µl of KAPA HiFi HotStart ready Mix [0.5 U DNA polymerase, 0.3 mM dNTPs, 2.5 mM MgCl₂] (Kapa Biosystems, Wilmington, MA, USA), 0.2 µM final concentration of 0.2 µM of each forward and reverse primer was used in the reaction mix, a long with nuclease-free water (Thermo Fisher Scientific, USA) to a final volume of 25 µl. The PCR Thermal reaction protocol using ProFlex PCR System (Applied Biosystems, USA) was carried out as follows: denaturing at 95°C for 3 min, followed by 35 cycles of 95°C for 30 seconds, 55°C for 60 seconds, and 72°C for 30 seconds, and a final extension step at 72°C for 5 min was included.

PCR amplicons were then subjected to a PCR clean-up step using Agencourt AMPure XP magnetic beads (Beckman Coulter Genomics, USA). Following clean-up, index PCR was performed to attach dual indexes to PCR products using the Nextera XT index kit (Nextera,

USA). The indexing reaction was performed using 5 µl of amplicon PCR product DNA, 5 µl of Illumina Nextera XT Index Primer 1 (N7xx), 5 µl of Nextera XT Index Primer 2 (S5xx), 25 µl of 2x KAPA HiFi HotStart Ready Mix, and 10 µl of nuclease-free water. The thermocycling using ProFlex PCR System (Applied Biosystems, USA), conditions were 95°C for 3 minutes followed by 8 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and a final elongation step of 72°C for 5 minutes. Index PCR products were subjected to a second ‘PCR clean-up’ step. The libraries were then quantified using a Qubit 3.0 fluorometer (Thermo Fisher Scientific, USA), normalized, pooled, and denatured in 0.2 N NaOH. Denatured libraries and 10% PhiX sequencing control were loaded onto the MiSeq flow cell using the MiSeq v3 reagent kit for a paired-end (2 × 300 bp) sequencing run. Sequencing was performed using the Illumina MiSeq platform (Illumina, USA, at the North West University (NWU) Potchefstroom campus, South Africa).

5.2.3 Bioinformatics and statistical analysis

DADA2 was used to process reads obtained from the sequencing platform, after indexes and primers were removed (Callahan *et al.*, 2021), following trimming, filtering, and removal of chimeras. Reads were truncated at 250 bp with maximum expected errors at 5, allowing no ambiguous bases. Error rates for forward and reverse reads were learned independently and then merged. The operational taxonomic units (OTUs) table was constructed, and taxonomy classification was assigned to the sequence variants using the SILVA 138.1 database (Quast *et al.*, 2013). Downstream analyses and visualisation were done using the *phyloseq* package in R (McMurdie & Holmes, 2013). Alpha diversity was assessed using Observed OTUs, Chao1, Shannon, and Simpson indices. Beta diversity was evaluated using Bray–Curtis dissimilarity and Jaccard distance matrices, visualized through Principal Coordinate Analysis (PCoA) and Non-Metric Multidimensional Scaling (NMDS), respectively. Microbial abundance was visualised using *ggplot2* (Wickham, 2016). The operational taxonomic units (OTUs) table was constructed, and taxonomy classification was assigned to the sequence variants using the SILVA 138.1 database (Quast *et al.*, 2013). Downstream analyses and visualisation were done using the *phyloseq* package in R (McMurdie & Holmes, 2013). Alpha diversity was assessed using Observed OTUs, Chao1, Shannon, and Simpson indices. Beta diversity was evaluated using Bray–Curtis dissimilarity and Jaccard distance matrices, visualized through Principal Coordinate Analysis (PCoA) and Non-Metric Multidimensional Scaling (NMDS),

respectively. Microbial abundance was visualised using *ggplot2* (Wickham, 2016). The statistical significance of diversity differences was tested using the Kruskal–Wallis test.

5.3 Results

5.3.1 Taxonomic composition *Rhipicephalus evertsi evertsi* ticks from sheep and goats

5.3.1.1 The bacterial composition at the phylum level

The taxonomic abundances of all OTUs at the phylum level were visualised using a stacked graph based on relative abundances (Figure 5.1). A total of 18 bacterial phyla were identified and assigned in all *R. e. evertsi* ticks from sheep and goats. In ticks from goats, the five most abundant bacterial phyla were Actinobacteriota (61.64%), followed by Firmicutes (31.41%), Proteobacteria (30.22%), Verrucomicrobiota (12.77%), and Bacteroidota (9.62%). The least abundant bacterial phyla included Chloroflexi (0.05%), Armatimonadota (0.53%) and Synergistota (0.04%) as shown in Figure 5.1. In ticks from sheep, the five most abundant bacterial phyla in *R. e. evertsi* ticks were Proteobacteria (37.80%), followed by Firmicutes (25.25%), Actinobacteriota (20.94%), Bacteroidota (8.46%), and Cyanobacteria (1.50%). The remaining bacterial phyla had relatively low abundances. The least abundant bacterial phyla were Bdellovibrionota (0.01%), Acidobacteriota (0.01%), and Desulfobacterota (0.02%), depicted in Figure 5.1.

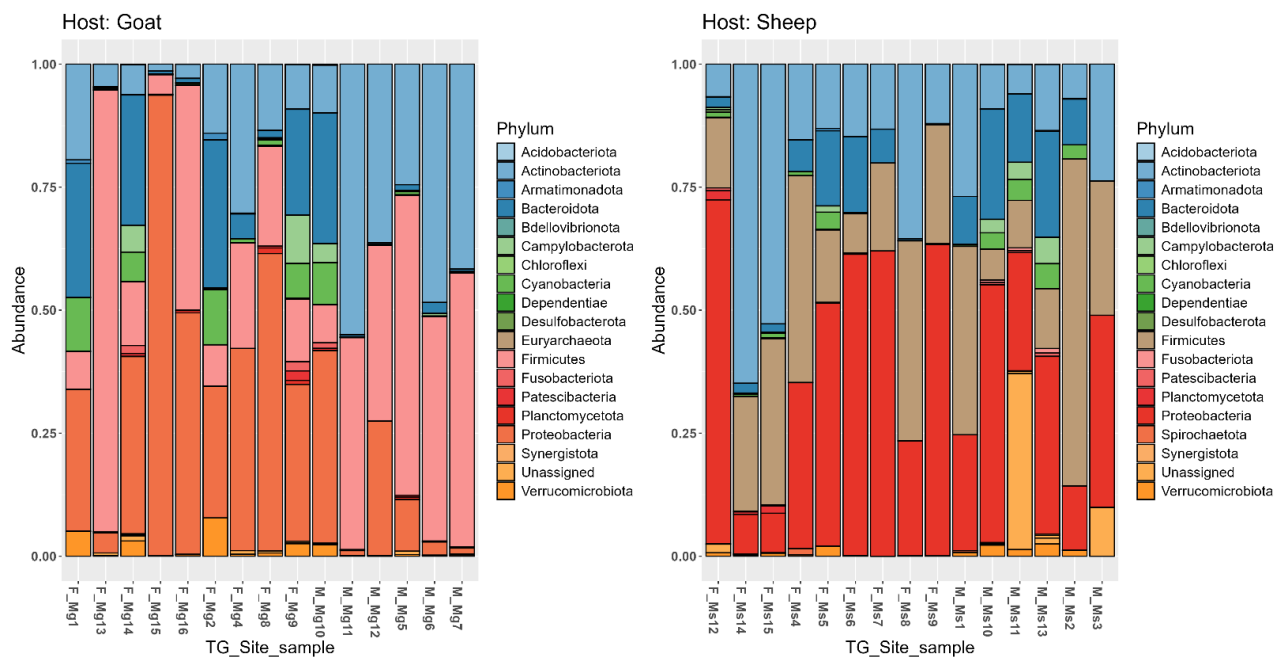


Figure 5.1: Stagged plot for taxonomic abundance of the bacterial communities in *Rhipicephalus evertsi evertsi* ticks from sheep and goats at the phylum level.

5.3.1.2 The bacterial composition at the class level

A total of 19 assigned bacterial classes were identified in *R. e. evertsi* ticks collected from both sheep and goats. In goat derived ticks, the dominant bacterial class level was Gammaproteobacteria (48.57%), followed by Bacilli (27.88%), Actinobacteria (20.69%), Alphaproteobacteria (13.11%), and Bacteroidia (9.65%). The least abundant classes included Fusobacteriia (0.01%), Vampirivibrionia (0.07%) and Gracilibacteria (0.06%) depicted in Figure 3. In contrast, the microbiota of *R. e. evertsi* ticks from sheep was dominated by Gammaproteobacteria (33.25%), followed by Bacilli (22.38%), Actinobacteria (20.89%), Bacteroidia (8.49%) and Alphaproteobacteria (4.71%). The least abundant classes in sheep-derived ticks included Coriobacteriia (0.04%), Anaerolineae (0.04%) and Vampirivibrionia (0.05%), depicted in Figure 5.2.

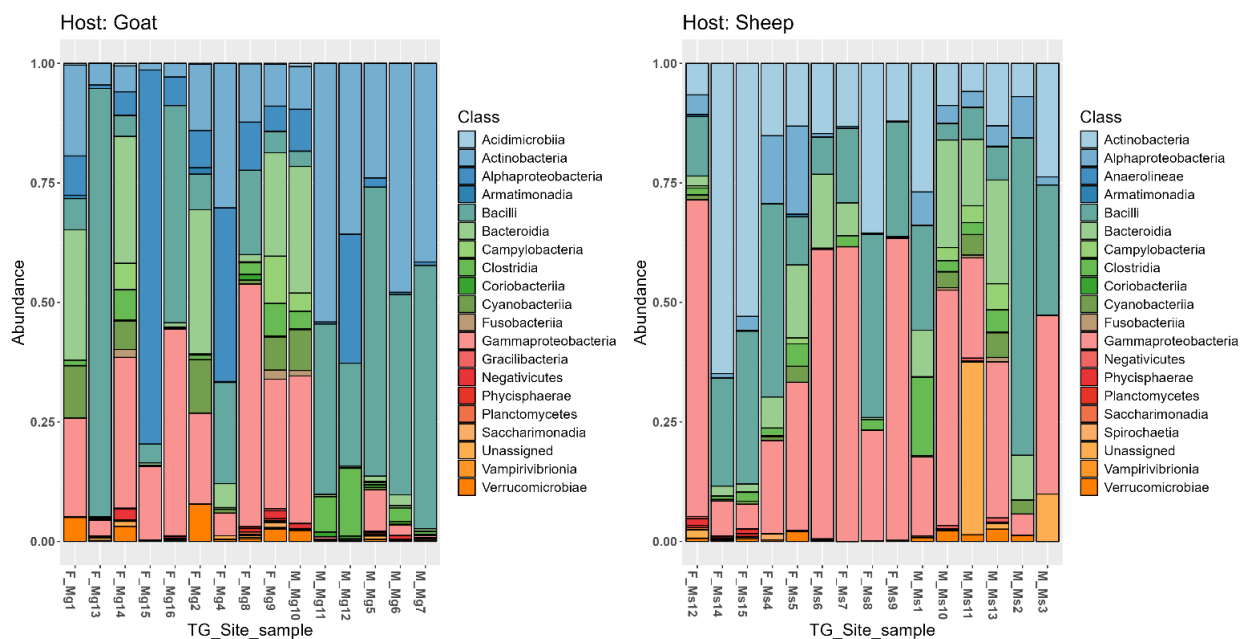


Figure 5.2: Stagged plot for taxonomic abundance of the bacterial communities in *Rhipicephalus evertsi evertsi* ticks from sheep and goats at the class level.

5.3.1.3 The bacterial composition at the order level

A total of 19 bacterial orders were identified in the *R. e. evertsi* tick from goats. At the bacterial order level, the microbiota composition in goat-derived ticks was dominated by Staphylococcales (25.25%), followed by Bacillales (15.73%), Corynebacteriales (13.69%),

Lactobacillales (11.55%) and Sphingomonadales (10.00%). The least abundant orders included Bacteroidales (1.74%), Actinomycetales (1.70%) and Flavobacteriales. In contrast, 18 bacterial orders were identified in the *R. e. evertsi* tick collected from sheep. The sheep-derived ticks were dominated by Pseudomonadales (23.51%), followed by Corynebacteriales (16.33%), Staphylococcales (13.68%), Bacillales (5.17%) and Micrococcales (4.61%). The least abundant orders in sheep-derived ticks included Rhizobiales (0.56%), Caulobacteriales (0.60%) and Cytophagales (1.08%), depicted in Figure 5.3. Additionally, chloroplasts were detected in tick samples from goats (3,70%) and sheep (1.56%).

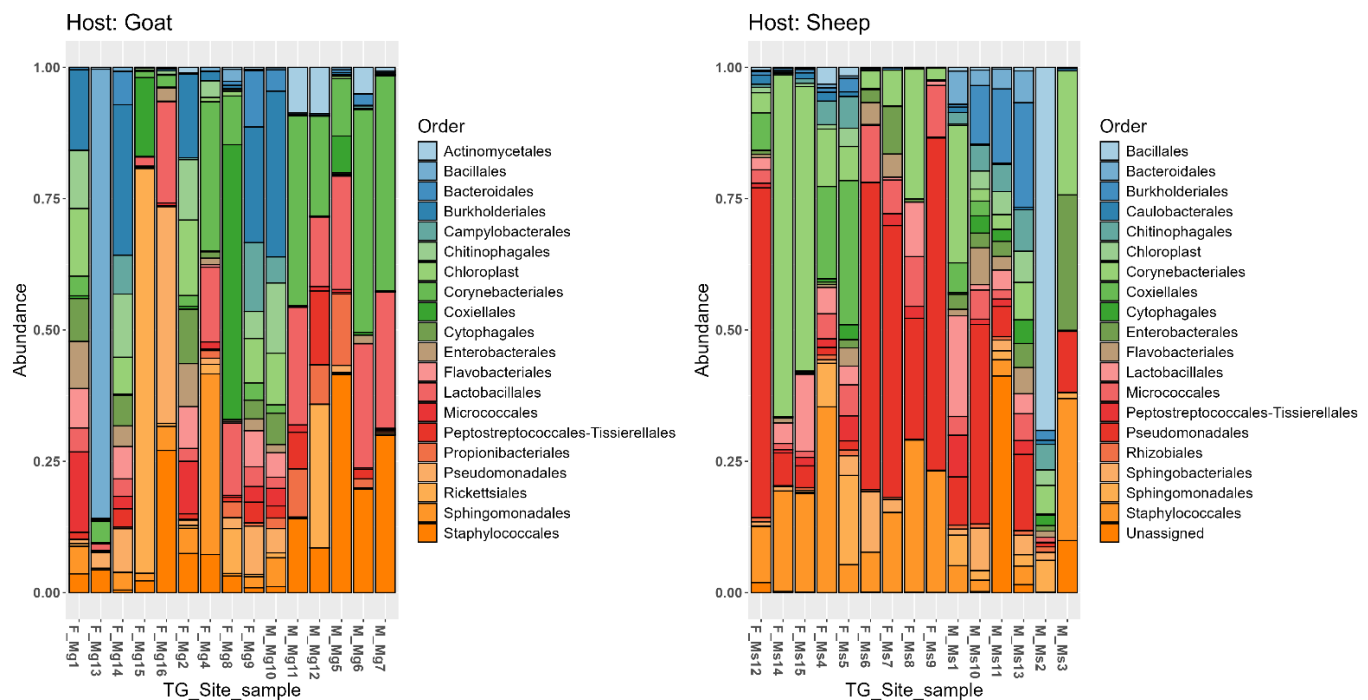


Figure 5.3: Staggered plot for taxonomic abundance of the bacterial communities in *Rhipicephalus evertsi evertsi* ticks from sheep and goats at the order level.

5.3.1.4 The bacterial composition at the family level

At a family level, 19 bacterial families were identified in *R. e. evertsi* ticks from goats and sheep. The dominant bacterial families in goat-derived ticks included *Aerococcaceae* (26.33%), followed by *Corynebacteriaceae* (14.2%), *Coxiellaceae* (13.80%), *Staphylococcaceae* (12.08%) and *Sphingomonadaceae* (10.49%). The least abundant families in ticks included *Streptococcaceae* (0.87%), *Family XI* (1.66%) and *Arcobacteraceae* (2.41%), as shown in Figure 5.4. In sheep-derived ticks, the dominant families were *Corynebacteriaceae* (16.87%), followed by *Pseudomonadaceae* (15.35%), *Staphylococcaceae* (14.80%), *Moraxellaceae* (10.58%) and *Bacillaceae* (5.44%). The least abundant family in the samples

included *Dietziaceae* (0.70%), *Family XI* (1.25%), and *Dermabacteraceae* (1.81%), depicted in Figure 5.4.

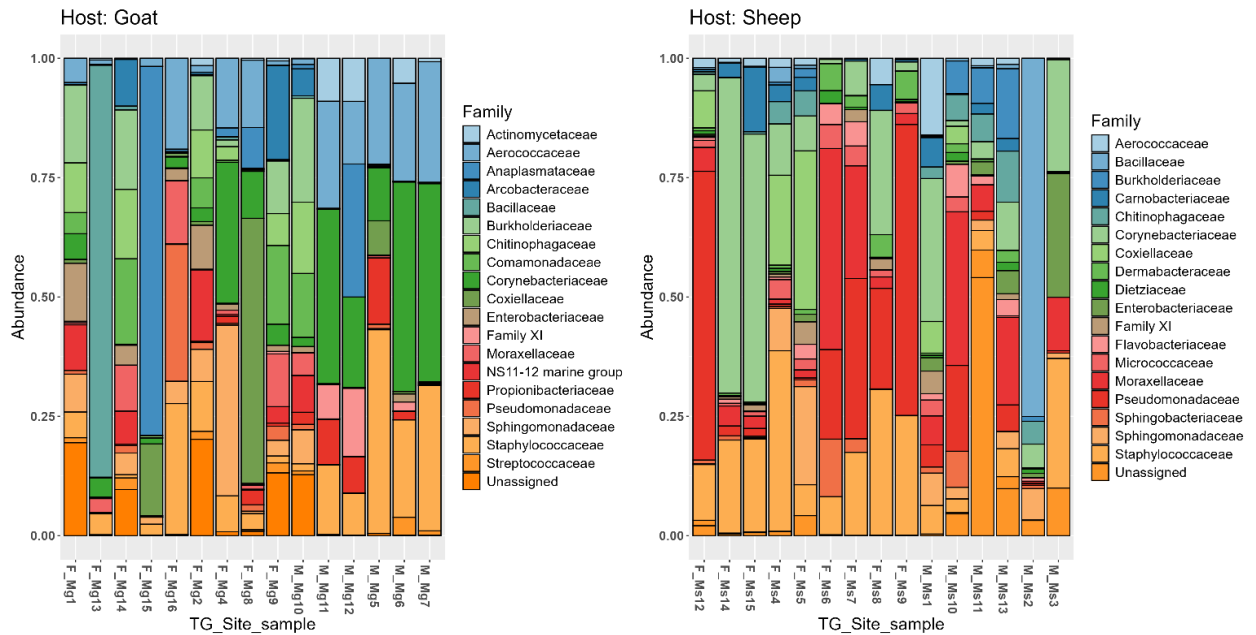


Figure 5.4: Staggered plot for taxonomic abundance of the bacterial communities in *Rhipicephalus evertsi evertsi* ticks from sheep and goats at the family level.

5.3.1.5 The bacterial composition at the genus level

A total of 19 bacterial genera were identified in *R. e. evertsi* ticks collected from goats and sheep. At the genus level, the microbiota composition in ticks from goats revealed the following dominant genera: *Corynebacterium* (15.05%), followed by *Staphylococcus* (12.26%), *Aerococcus* (11.49%), *Ehrlichia* (7.26%), and *Polynucleobacter* (6.10%) (Figure 7). The least abundant genera included *Anaplasma* (0.79%), *Streptococcus* (1.00%), and *Arcanobacterium* (1.35%), depicted in Figure 5.5. In contrast, the microbiota composition in ticks from sheep showed a slightly different distribution: *Corynebacterium* (17.48%) was the most predominant, followed by *Pseudomonas* (16.37%), *Acinetobacter* (11.20%), *Staphylococcus* (10.29%), and *Bacillus* (5.66%) (Figure 7). The least abundant genera in sheep-derived ticks included *Glutamicibacter* (1.21%), *Sphingobacterium* (1.28%) and *Salinicoccus* (1.36 %), depicted in Figure 5.5.

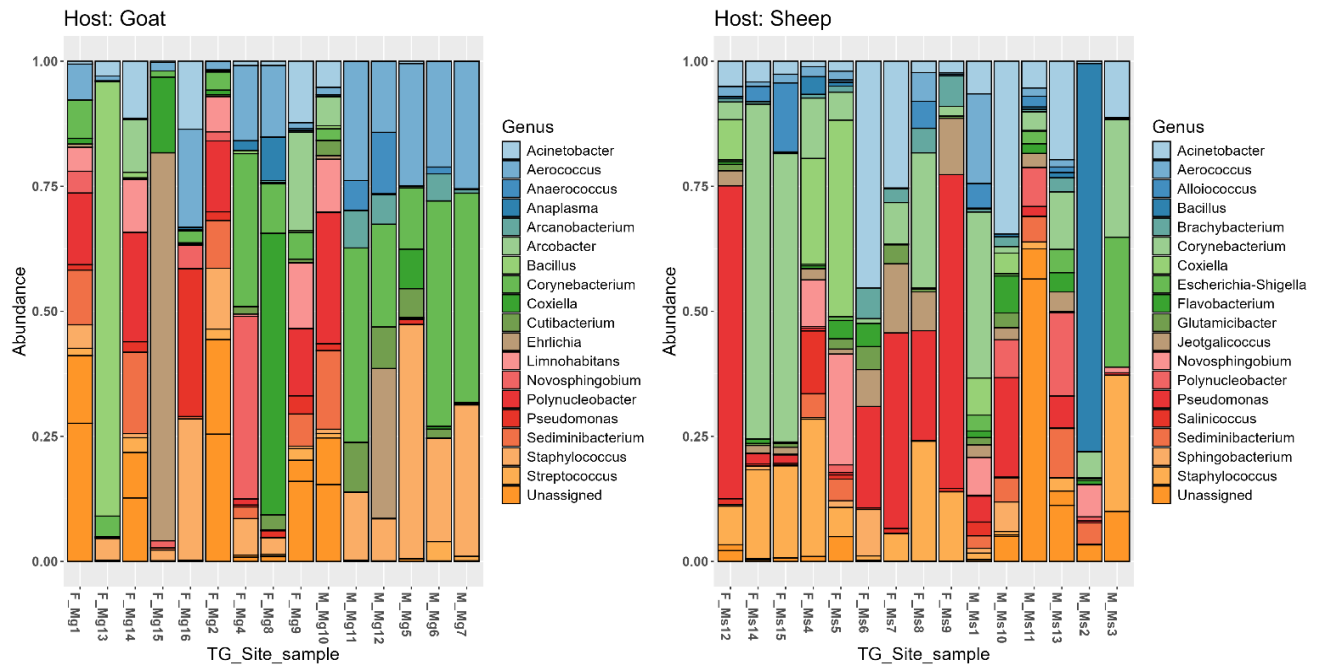


Figure 5.5: Staggered plot for taxonomic abundance of the bacterial communities in *Rhipicephalus evertsi evertsi* ticks from sheep and goats at the genus level.

5.3.2 Host-based differences in bacterial genera

The heatmap (Figure 5.6) illustrates the relative abundance of the top genus-level bacterial taxa in *R. e. evertsi* ticks collected from goats and sheep, highlighting distinct host-specific microbial patterns. *Corynebacterium* species were identified as the dominant species, occurring at a significantly high rate of 90% in both ticks collected from sheep and goats. In ticks from goats, genera such as *Corynebacterium*, *Staphylococcus*, and *Aerococcus* species show higher prevalence (>80%), as indicated by the warmer colours (green to yellow), suggesting these taxa are more dominant in goat-associated tick bacterial communities. In contrast, ticks from sheep display higher relative abundances of *Pseudomonas* and *Acinetobacter* species. Unassigned taxa or bacterial species were also observed in the sheep-derived ticks. Despite these variations, several genera, including *Algoriphagus*, *Flavobacterium*, and *Escherichia-Shigella*, are shared between both hosts, although their relative abundances differ. For instance, *Algoriphagus* shows ~60 % prevalence in goat samples but is lower in sheep (~20%). Notably, *Sediminibacterium* and *Novosphingobium* species are prevalent in ticks from both hosts, indicating potential resilience across different host environments. The *Cutibacterium*, *Candidatus Aquarickettsia*, and *Sphingobacterium* are exclusively detected in ticks from goats.

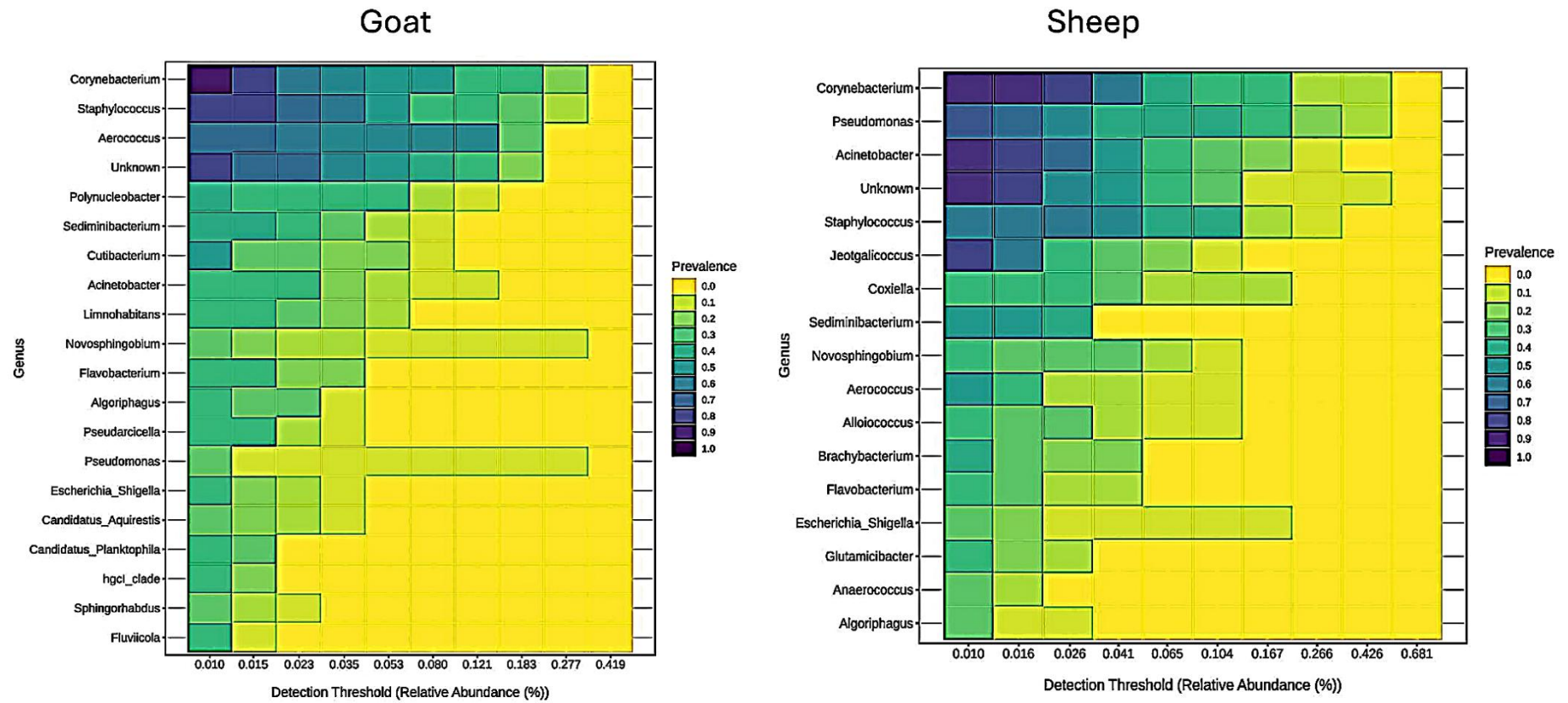


Figure 5.6: Heatmap of the top genus-level bacterial taxa in *Rhipicephalus evertsi evertsi* ticks from sheep and goats.

5.3.3 Alpha-diversity rarefaction curves of diversity associated with *Rhipicephalus evertsi evertsi* ticks from sheep and goats

The rarefaction curves, generated using operational taxonomic units (OTUs), assess the sequencing depth and microbial diversity within the microbiomes of *R. e. evertsi* ticks collected from goats and sheep. Based on 16S rRNA amplicon sequencing, the curves illustrate species richness across varying sequence sample sizes, providing insight into the diversity of bacterial communities associated with ticks from different hosts. Ticks collected from goats (represented by red curves) reached a plateau in species richness at lower sequencing depths, indicating that their microbial communities are less diverse and that sequencing saturation is achieved more rapidly. In contrast, ticks collected from sheep (depicted by teal curves) show a slower progression toward a plateau, requiring greater sequencing depth to capture the full extent of microbial diversity. The variation in curve heights among individual samples highlights differences in microbial richness between individual ticks within each host group. Notably, some goat-associated ticks (e.g., Mg9 and Mg10) exhibit higher species richness than others, while sheep-associated ticks display more consistent but diverse microbial profiles.

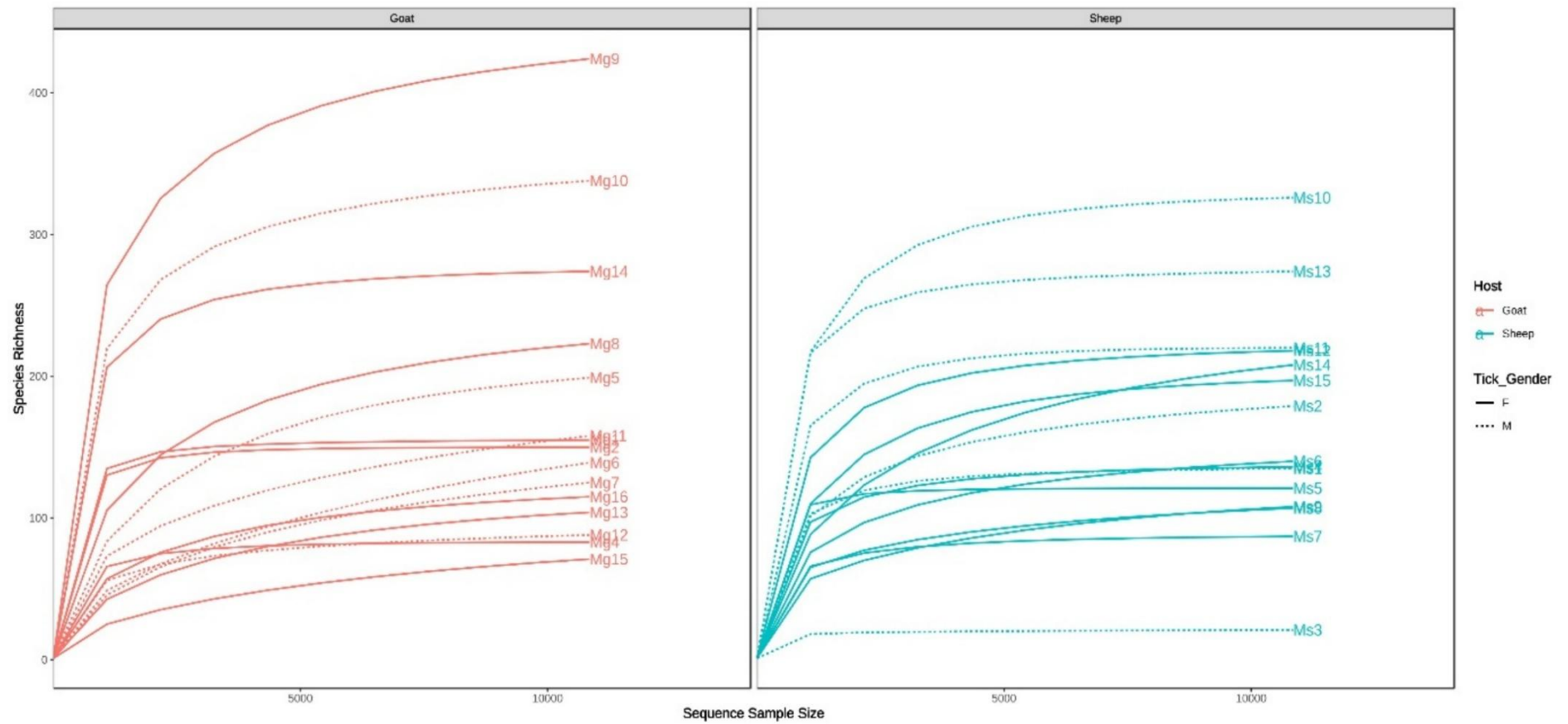


Figure 5.7: Alpha-diversity rarefaction curve to interpret the sequence depth of the microbial diversity associated with *Rhipicephalus evertsi evertsi* ticks from sheep and goats based on the 16S rRNA gene amplicon.

5.3.4 Alpha diversity estimate of bacterial communities associated with *Rhipicephalus evertsi evertsi* ticks from sheep and goats

Illumina MiSeq sequencing generated 996,689 sequence reads from *R. e. evertsi* ticks collected from goats and 791,038 reads from sheep. Following trimming, quality-control, removal of chimeric, low-quality and non-target sequences, the datasets were reduced to 758,806 valid reads for goat tick samples and 688,092 sheep tick samples. These sequences were clustered into 18,087 for goat ticks and 18,006 OTUs for sheep ticks.

Alpha diversity was assessed using Observed OTUs and Chao1 (richness estimators) and Shannon and Simpson indices (diversity and evenness metrics). Statistical comparison of the metrics revealed distinct patterns in microbial diversity influenced by host species (Figure 5.8). The Kruskal-Wallis test was used to evaluate the significance. Observed OTUs and Chao1 indices demonstrated a statistically significant difference ($p < 0.05$), indicating a greater diversity and even distribution of the microbial taxa in ticks from the sheep host. In contrast, the non-significant differences in Shannon and Simpson indices suggest that the overall richness, including rare taxa, was similar between the host-associated tick populations. The p-values for each diversity metric were determined as follows: Observed OTUs p-value = 0.38; Shannon index p-value = 0.72; Simpson index p-value = 0.85; and Chao1 p-value = 0.23.

The alpha diversity analysis of *R. e. evertsi* ticks from sheep and goats, as depicted in Figure 5.8 (A-D), revealed clear patterns of bacterial diversity based on ticks' gender and host species. In plot A, species richness is highest in female ticks from goats, followed by male ticks from the same host. In contrast, both male and female ticks from sheep exhibited lower species richness than goats. Similarly, the Shannon diversity index (plot B), which considers both richness and evenness, showed that female ticks from goats had the highest diversity, indicating a richer and more uniform microbial community. Male ticks from goats also had high diversity, but the bacterial communities of male and female sheep ticks were less diverse and more unevenly distributed. The Simpson dominance index (plot C), which emphasizes the abundance of dominant species, showed generally low dominance across all ticks, though male ticks from sheep exhibited slightly higher dominance values. Lastly, the Chao1 index (plot D), an estimator of species richness that includes rare species, confirmed that female ticks from goats had the highest estimated richness, followed by male ticks from goats. In contrast, the

Chao1 values for ticks from sheep were lower, particularly for female ticks, indicating that the microbial communities in sheep ticks were less diverse.

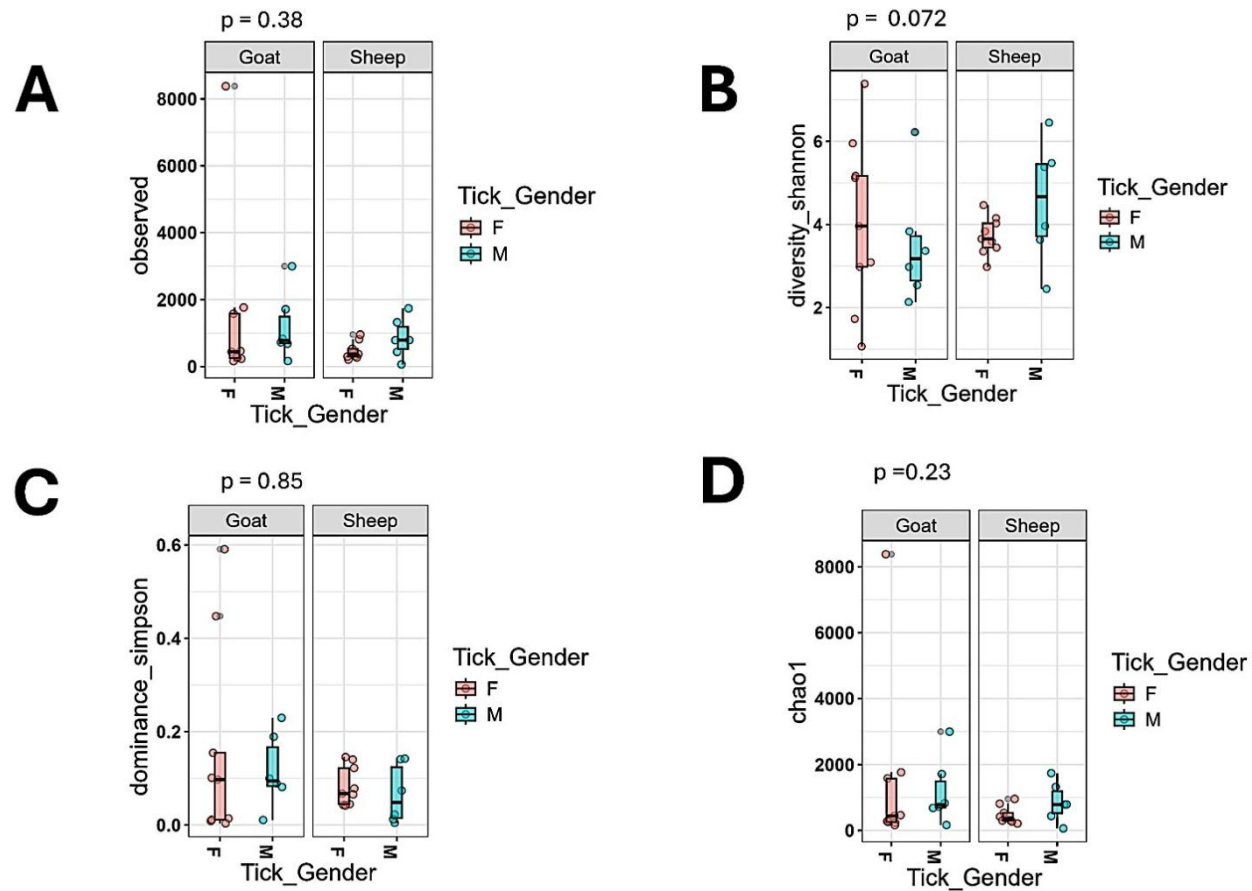


Figure 5.8: Alpha diversity analysis of boxplots (A-D) representing different alpha diversity metrics used to assess the microbial diversity of *Rhipicephalus evertsi evertsi* ticks collected from sheep and goats, separated by tick gender (female: F, male: M). (A) Observed OTUs, (B) Shannon diversity index, (C) Simpson diversity index, and (D) Chao1 richness estimator. Significant differences were observed for Observed OTUs and Chao1 's indices ($p < 0.05$), indicating higher microbial diversity and evenness in ticks from sheep.

5.3.4 Beta diversity estimate of tick bacterial composition

The Principal Coordinate Analysis (PCoA) plot, constructed using the Bray–Curtis dissimilarity index, assessed the beta diversity of bacterial populations in *R. e. evertsi* ticks collected from goats and sheep. The analysis was conducted at the genus level and aimed to explore variations in microbial community composition between the two host animals and across tick genders. The PCoA plot revealed that the first principal component (Axis 1) accounts for 18% of the total variation, while the second principal component (Axis 2) accounts for 12.9% of the variation. A clear clustering pattern is observed, with ticks from goats (represented by red circles and triangles) and sheep (represented by blue circles and triangles) forming separate groups (Figure 5.9).

Regarding the tick gender, the PCoA plot demonstrates some overlap between male and female ticks within their respective host clusters. However, the clustering appears to be primarily influenced by the host species rather than gender. This finding suggests that, while gender may have a minor effect on microbial diversity, the host species is a more significant determinant of the microbial composition in these ticks.

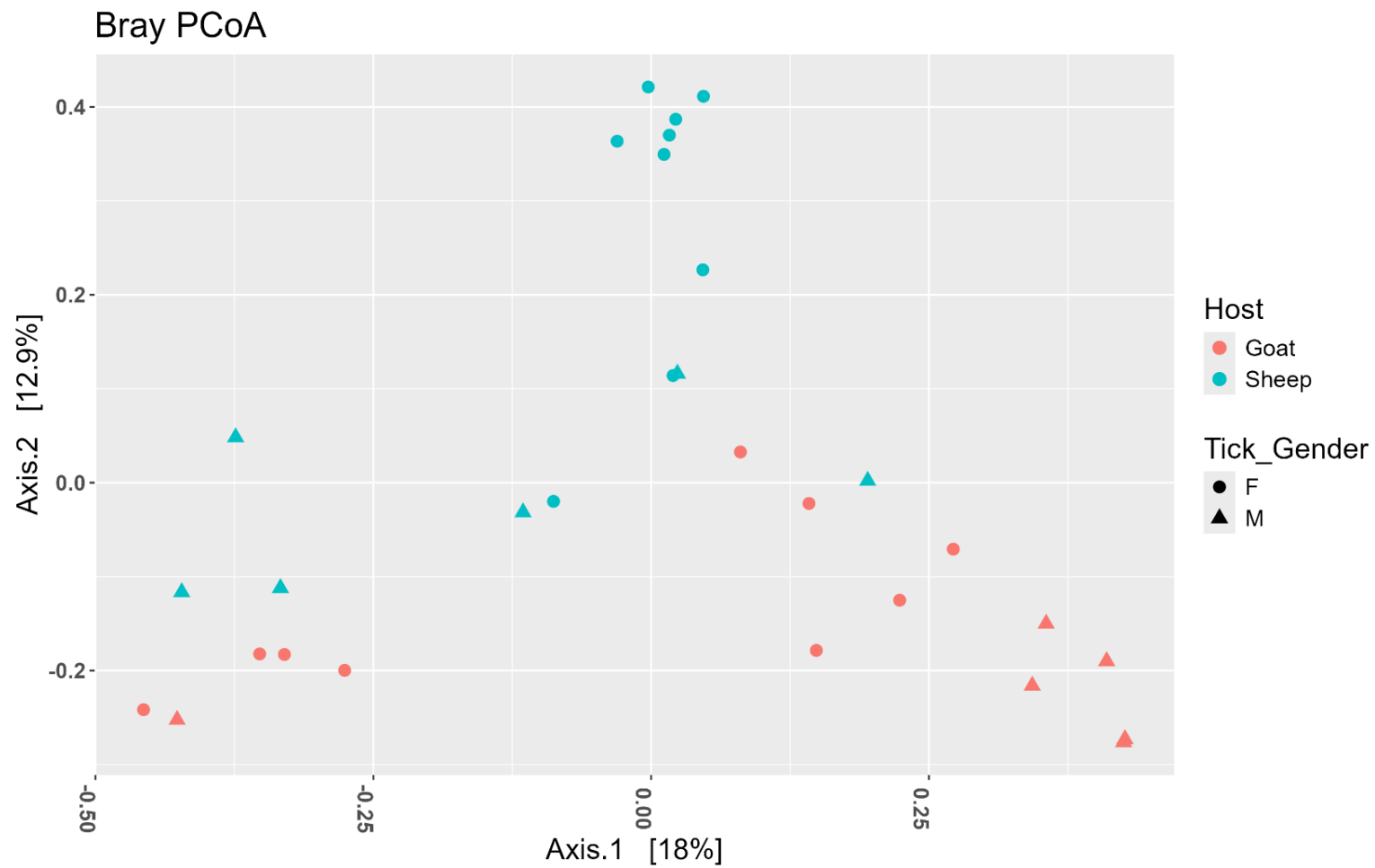


Figure 5.9: Principal Coordinate Analysis (PCoA) in *Rhipicephalus evertsi evertsi* ticks collected from sheep and goat showing the beta diversity constructed using Bray–Curtis.

A Non-Metric Multidimensional Scaling (NMDS) plot was created based on the Jaccard distance to assess the beta diversity of bacterial populations in *R. e. evertsi* ticks collected from goats and sheep. The Jaccard distance, a non-phylogenetic metric, focuses on the presence or absence of bacterial taxa as well as their relative abundance, allowing for an examination of variations in microbial composition across different samples. In the NMDS plot, tick samples are displayed according to their microbial communities. Samples from goats are represented by pink circles and triangles, while samples from sheep are shown as blue circles and triangles. The positioning of these points on the plot indicates the degree of similarity or dissimilarity among the microbial communities; points that are closer together suggest greater similarity, while those that are farther apart represent larger differences. The analysis revealed that ticks from goats and sheep tend to form distinct clusters, although some overlap is observed. This clustering suggests that the microbial communities differ somewhat between ticks from the two host animals. Additionally, ticks from goats are more dispersed, indicating greater variability in their microbial communities compared to those from sheep.

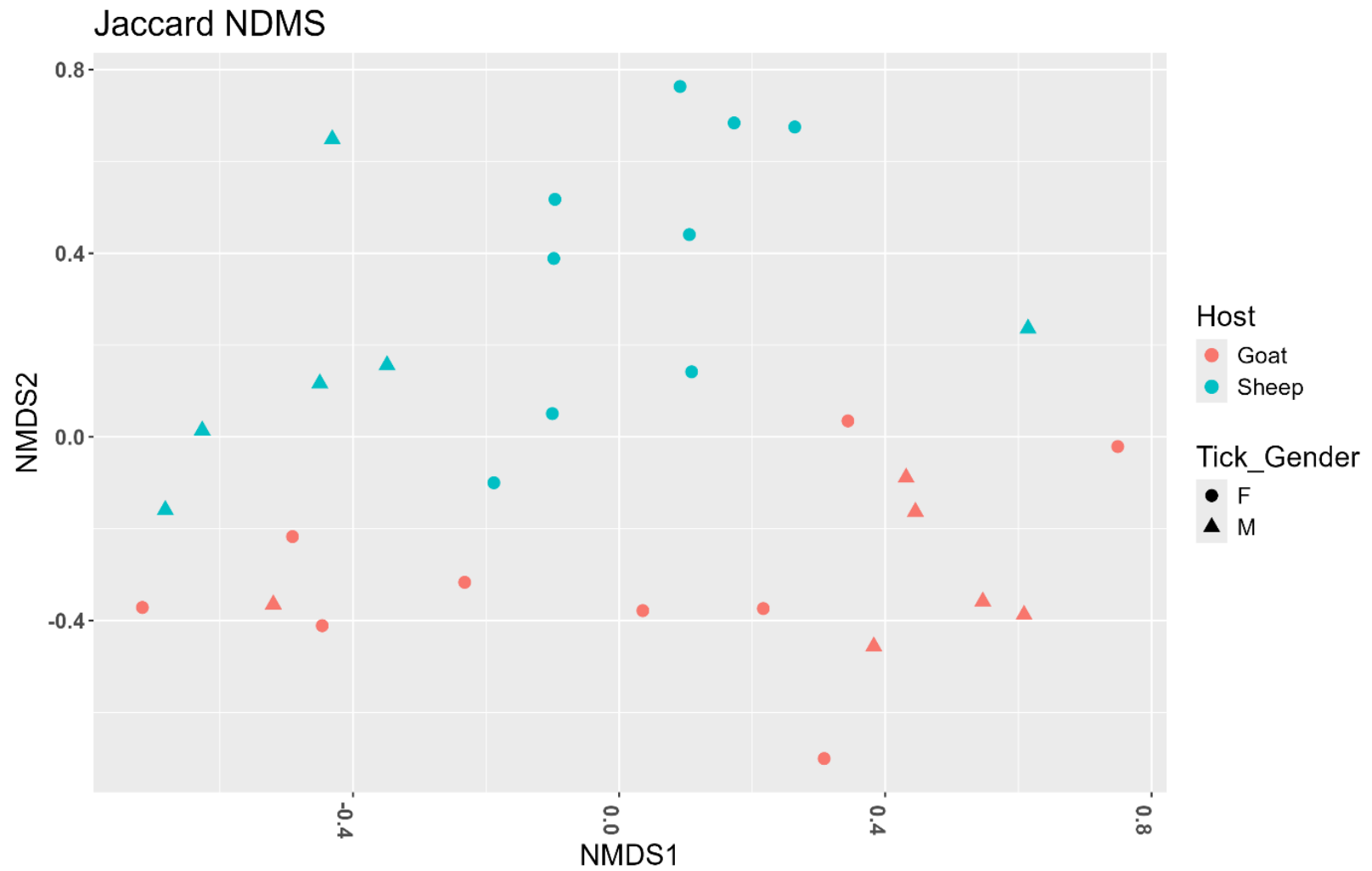


Figure 5.10: Principal Coordinate Analysis (PCoA) in *Rhipicephalus evertsi evertsi* ticks collected from goats and sheep showing the Beta diversity constructed using Jaccard NDMS distance.

5.4 Discussion

This study explored the bacterial communities harboured by *Rhipicephalus evertsi evertsi* ticks collected from sheep and goats in Matlwang village, Potchefstroom, North West Province, South Africa. Understanding the microbial community of ticks infesting livestock is essential for unravelling the complex dynamics of tick-borne diseases and their impact on animal health. Despite the growing concern around vector-borne pathogens, studies focusing exclusively on the tick-associated microbiomes of South African small ruminants remain scarce. Our findings revealed a striking divergence in bacterial community composition between ticks collected from sheep and those from goats, underscoring a clear host-associated microbial signature. These results not only enrich the current understanding of the tick microbial community but also emphasize the influence of host species on shaping microbial diversity within tick vectors. In this study, *Actinobacteria* (61.64%) were the predominant phylum identified in ticks from goat, while *Proteobacteria* (37.80%) dominated the microbiota of ticks from sheep. It has been reported that *Actinobacteria* were found to be less predominant in various tick species (*Ixodes*, *Rhipicephalus*, *Dermacentor*, and *Haemaphysalis*) collected from goats and sheep in Greece, whereas *Proteobacteria* were consistently the most abundant phylum in most ticks (Papa *et al.*, 2020).

Proteobacteria play a crucial role in the microbiome of ticks, influencing the ecology and transmission dynamics of tick-borne pathogens (Bonnet and Pollet, 2021). This phylum encompasses a diverse array of bacteria, including significant veterinary pathogens such as *Rickettsia*, *Anaplasma*, and *Ehrlichia*, which are frequently associated with ticks and diseases in livestock (Narasimhan and Fikrig, 2015; Yan *et al.*, 2019; Carvajal-Agudelo *et al.*, 2022). The predominance of *Proteobacteria* in tick microbiota is well-documented across various tick species and geographical regions (Clayton *et al.*, 2015; Van Treuren *et al.*, 2015; Chicana *et al.*, 2019; Grandi *et al.*, 2023; Kamani *et al.*, 2024). This high abundance highlights its potential role in tick physiology and pathogen interactions, suggesting that the presence of *Proteobacteria* is integral to tick biology (Clayton *et al.*, 2015; Bonnet *et al.*, 2017). These bacteria may influence tick development, reproduction, and immune responses, thereby impacting the tick's ability to transmit pathogens (Portillo *et al.*, 2019; Carvajal-Agudelo *et al.*, 2022). Additionally, the dominance of *Proteobacteria* may impact metabolic processes within

ticks, such as carbohydrate fermentation and the maintenance of microecological balance (Carvajal-Agudelo *et al.*, 2022; Zhang *et al.*, 2023).

The observed dominant phylum in this study, *Actinobacteriota*, is known for its diverse metabolic functions, and also plays a significant role in the tick microbiome (Liu *et al.*, 2024). Recent studies have highlighted the complexity of microbiomes alongside other phyla such as *Proteobacteria*, *Firmicutes*, and *Bacteroidota*, *Actinobacteriota* is one of the predominant bacterial groups in ticks (Adegoke *et al.*, 2020). The presence of TBPs does not significantly alter the alpha and beta diversity of the tick microbiome, indicating a stable microbial community structure despite pathogen presence (Adegoke *et al.*, 2020). This stability may reflect the contribution of *Actinobacteriota* to the resilience of the tick microbiome, potentially enhancing the tick's adaptability to various environments (Jiang *et al.*, 2024). Moreover, the composition of the gut microbiota in ticks can vary significantly depending on the host species they parasitize.

Furthermore, in this study, at the genus level, *Corynebacterium* and *Staphylococcus* were prominent in ticks from both sheep and goats, although their relative abundances differed. Among the compared top 20% genera compared across, the *R. e. evertsi* ticks from goats, *Corynebacterium* accounted for (15.05%) and *Staphylococcus* for (12.26%), whereas ticks from sheep showed (17.48%) and (10.29%), respectively. The prevalence of *Corynebacterium* and *Staphylococcus* species in ticks from livestock has been reported at high relative abundance, up to (57.6%) and (41.5%), respectively, in ticks from sheep (Perveen *et al.*, 2022). This high prevalence suggests that these genera play an important role in the tick's biology, potentially influencing tick fitness, survival, and interactions with pathogens (Bonnet *et al.*, 2017; Hussain *et al.*, 2022; Zhong *et al.*, 2024). Variation in their abundance across different host species could reflect the adaptability of the tick microbiome to environmental conditions and host interactions. For instance, differences in microbial composition have been linked to factors such as geographical location, host species, and even the sex of the ticks (Zhang *et al.*, 2019; Rojas-Jaimes *et al.*, 2021). This adaptability might affect pathogen transmission, as certain microbial communities could either facilitate or inhibit the colonization of pathogens within the ticks (Karim *et al.*, 2017; Perveen *et al.*, 2022). Furthermore, interactions between commensal bacteria like *Corynebacterium* spp. and *Staphylococcus* spp. and pathogenic organisms may influence the transmission dynamics of tick-borne diseases such as Lyme disease or anaplasmosis (Karim *et al.*, 2017; Perveen *et al.*, 2022).

The detection of bacteria, such as *Ehrlichia* spp. (7.26%), and *Anaplasma* spp. (0.79%) in *R. e. evertsi* ticks from goats, alongside *Pseudomonas* spp. (16.37%), *Bacillus* spp. (5.66%), *Acinetobacter* spp. (11.20%) in ticks from sheep, has significant implications for both veterinary health and public health. These findings indicate a potential risk of TBD transmission, which could adversely affect livestock health and productivity. The detection of *Ehrlichia* spp. and *Anaplasma* spp., suggests that these ticks may act as reservoirs or vectors of these pathogens to livestock, leading to significant morbidity in livestock (Addo, 2023). Furthermore, the presence of *Acinetobacter* spp., *Pseudomonas* spp. and *Bacillus* spp. in sheep ticks indicates complex interactions between the tick microbiome and the pathogens they may carry, further influencing disease transmission (Adegoke *et al.*, 2020).

The alpha diversity analysis of *R. e. evertsi* ticks collected from sheep and goats assessed using four diversity indices (Observed OTUs, Shannon, Simpson, and Chao1), revealed distinct patterns in microbial diversity influenced by the host species and tick gender. The p-values obtained for each diversity index were as follows: Observed OTUs ($p = 0.38$), Shannon index ($p = 0.72$), Simpson index ($p = 0.85$), and Chao1 ($p = 0.23$). Since all p-values exceed 0.05, there is no statistically significant difference in microbial diversity between ticks from the two host species, indicating that the null hypothesis cannot be rejected. Despite the lack of statistical significance, graphical representations of the data indicate observable trends in microbial diversity. Female ticks from goats exhibited the highest species richness (Observed OTUs) and estimated richness (Chao1 index), followed by male ticks from the same host. In contrast, both male and female ticks from sheep harboured lower microbial richness. Similarly, the Shannon diversity index, which accounts for both species richness and evenness, suggests that goat-associated ticks, particularly females, have more diverse and evenly distributed microbial communities than those from sheep. The Simpson index, which emphasizes the dominance of specific microbial species, shows relatively low dominance across all groups, although male ticks from sheep displayed slightly higher values, suggesting the presence of a few dominant species.

This study offers the first detailed examination of the bacterial communities associated with *R. e. evertsi* ticks that parasitize sheep and goats in Matlwang village, South Africa. Although no significant differences in overall bacterial alpha diversity were observed between ticks from

the two host species, bacterial diversity was notably influenced by tick sex, with female ticks from goats exhibiting the highest species richness. This pattern may reflect differences in host blood composition, immune responses, or tick feeding behaviour. Goats are known to browse more diversely than sheep, which may expose ticks to a wider range of microbial communities through blood meals (van Soest, 1994). In contrast, sheep exhibit a more uniform grazing behaviour, potentially limiting microbial acquisition. Furthermore, sex-related physiological differences, such as larger blood meal volumes and longer feeding periods in females, could increase the likelihood of acquiring diverse bacterial taxa (Oliver, 1989; Arthur, 1965), while differences in reproductive investment and hormonal status may also influence microbial community composition (Zhang *et al.*, 2014; Obregón *et al.*, 2019). In addition, both male and female ticks from sheep displayed lower diversity and a more uneven distribution of bacterial communities. Ticks collected from goats exhibited a greater abundance of *Actinobacteria*, particularly *Corynebacterium* and *Staphylococcus* species. In contrast, ticks from sheep displayed a higher prevalence of *Proteobacteria*, including potential pathogens such as *Pseudomonas* and *Bacillus* species. The identification of *Ehrlichia* and *Anaplasma* species in ticks derived from goats suggests their potential role as vectors for critical tick-borne diseases affecting livestock. Additionally, the presence of a high number of commensal bacteria, which may interact with pathogens, emphasizes the intricate ecology of tick microbiomes. This complexity could significantly influence pathogen transmission dynamics. Overall, the findings underscore that host identity plays a crucial role in shaping the microbiome of *R. e. evertsi*, indicating that these bacterial communities are vital for tick fitness, adaptability, and vector competence. This study lays the groundwork for future investigations into tick-pathogen-host-microbiome interactions and highlights the need for integrated tick surveillance strategies within South African small ruminant production systems.

References

1. Abdelbaset, A.E., Nonaka, N. and Nakao, R., 2022. Tick-borne diseases in Egypt: a one health perspective. *One Health*, 15, p.100443.
2. Addo, S.O., Bentil, R.E., Baako, B.O.A., Addae, C.A., Behene, E., Asoala, V., Mate, S., Oduro, D., Dunford, J.C., Larbi, J.A. and Baidoo, P.K., 2023. First record of *Babesia* and *Theileria* parasites in ticks from Kassena-Nankana, Ghana. *Medical and Veterinary Entomology*, 37(4), pp.878-882.
3. Adegoke, A., Kumar, D., Bobo, C., Rashid, M.I., Durrani, A.Z., Sajid, M.S. and Karim, S., 2020. Tick-borne pathogens shape the native microbiome within tick vectors. *Microorganisms*, 8(9), p.1299.
4. Arthur, D.R., 1965. Feeding in ectoparasitic Acari with special reference to ticks. *Advances in Parasitology*, 3, pp.249-298.
5. Barraza-Guerrero, S.I., Meza-Herrera, C.A., García-De la Peña, C., González-Álvarez, V.H., Vaca-Paniagua, F., Díaz-Velásquez, C.E., Sánchez-Tortosa, F., Ávila-Rodríguez, V., Valenzuela-Núñez, L.M. and Herrera-Salazar, J.C., 2020. General microbiota of the soft tick *Ornithodoros turicata* parasitizing the bolson tortoise (*Gopherus flavomarginatus*) in the Mapimi Biosphere Reserve, Mexico. *Biology*, 9(9), p.275.
6. Beninati, T., Lo, N., Sacchi, L., Genchi, C., Noda, H. and Bandi, C., 2004. A novel alpha-Proteobacterium resides in the mitochondria of ovarian cells of the tick *Ixodes ricinus*. *Applied and Environmental Microbiology*, 70(5), pp.2596-2602.
7. Bonnet, S.I. and Pollet, T., 2021. Update on the intricate tango between tick microbiomes and tick-borne pathogens. *Parasite Immunology*, 43(5), p.e12813.
8. Bonnet, S.I., Binetruy, F., Hernández-Jarguín, A.M. and Duron, O., 2017. The tick microbiome: why non-pathogenic microorganisms matter in tick biology and pathogen transmission. *Frontiers in Cellular and Infection Microbiology*, 7, p.236.
9. Boulanger, N., Boyer, P., Talagrand-Reboul, E. and Hansmann, Y., 2019. Ticks and tick-borne diseases. *Médecine et Maladies Infectieuses*, 49(2), pp.87-97.
10. Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh, P.J., Fierer, N. and Knight, R., 2011. Global patterns of 16S rRNA

- diversity at a depth of millions of sequences per sample. *Proceedings of the National Academy of Sciences*, 108(supplement_1), pp.4516-4522.
11. Carvajal-Agudelo, J.D., Ramírez-Chaves, H.E., Ossa-López, P.A. and Rivera-Páez, F.A., 2022. Bacteria related to tick-borne pathogen assemblages in *Ornithodoros cf. hasei* (Acari: Argasidae) and blood of the wild mammal hosts in the Orinoquia region, Colombia. *Experimental and Applied Acarology*, 87(2), pp.253-271.
 12. Cerutti, F., Modesto, P., Rizzo, F., Cravero, A., Jurman, I., Costa, S., Giammarino, M., Mandola, M.L., Gorla, M., Radovic, S. and Cattonaro, F., 2018. The microbiota of hematophagous ectoparasites collected from migratory birds. *Public Library of Science ONE*, 13(8), e0202270.
 13. Chicana, B., Couper, L.I., Kwan, J.Y., Tahiraj, E. and Sweit, A., 2019. Comparative microbiome profiles of sympatric tick species from the far-western United States. *Insects*, 10(10), p.353.
 14. Clayton, K.A., Gall, C.A., Mason, K.L., Scoles, G.A. and Brayton, K.A., 2015. The characterization and manipulation of the bacterial microbiome of the Rocky Mountain wood tick, *Dermacentor andersoni*. *Parasites & Vectors*, 8, pp.1-5.
 15. Egan, S.L., Loh, S.M., Banks, P.B., Gillett, A., Ahlstrom, L., Ryan, U.M., Irwin, P.J. and Oskam, C.L., 2020. Bacterial community profiling highlights complex diversity and novel organisms in wildlife ticks. *Ticks and Tick-borne Diseases*, 11(3), p.101407.
 16. Egan, S.L., Taylor, C.L., Banks, P.B., Northover, A.S., Ahlstrom, L.A., Ryan, U.M., Irwin, P.J. and Oskam, C.L., 2021. The bacterial biome of ticks and their wildlife hosts at the urban-wildland interface. *Microbial Genomics*, 7(12), p.000730.
 17. Elbir, H., Almathen, F. and Alhumam, N.A., 2019. A glimpse of the bacteriome of *Hyalomma dromedarii* ticks infesting camels reveals human *Helicobacter pylori* pathogen. *The Journal of Infection in Developing Countries*, 13(11), pp.1001-1012.
 18. Estrada-Peña, A., Cabezas-Cruz, A. and Obregón, D., 2020. Resistance of tick gut microbiome to anti-tick vaccines, pathogen infection and antimicrobial peptides. *Pathogens*, 9(4), p.309.
 19. Estrada-Peña, A., Cabezas-Cruz, A., Pollet, T., Vayssier-Taussat, M. and Cosson, J.F., 2018. High-throughput sequencing and network analysis disentangle the

- microbial communities of ticks and hosts within and between ecosystems. *Frontiers in Cellular and Infection Microbiology*, 8, p.236.
20. Estrada-Peña, A., Gray, J.S., Kahl, O., Lane, R.S. and Nijhof, A.M., 2013. Research on the ecology of ticks and tick-borne pathogens—methodological principles and caveats. *Frontiers in Cellular and Infection Microbiology*, 3, p.29.
 21. Glöckner, F.O., 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic acids research*, 41(D1), pp.D590-D596.
 22. Grandi, G., Chiappa, G., Ullman, K., Lindgren, P.E., Olivieri, E., Sassera, D., Östlund, E., Omazic, A., Perissinotto, D. and Söderlund, R., 2023. Characterization of the bacterial microbiome of Swedish ticks through 16S rRNA amplicon sequencing of whole ticks and of individual tick organs. *Parasites & Vectors*, 16(1), p.39.
 23. Guizzo, M.G., Neupane, S., Kucera, M., Perner, J., Frantová, H., da Silva Vaz, I., Kopáček, P. and Zurek, L., 2020. Poor unstable midgut microbiome of *Amblyomma sculptum* and *Amblyomma aureolatum* is not essential for their development. *Frontiers in Cellular and Infection Microbiology*, 10, p.601417.
 24. Gupta, A., Nair, D.T. and Tripathi, T., 2020. Unraveling the interactions of ticks, their microbiome, and pathogens. *Frontiers in Cellular and Infection Microbiology*, 10, p.313.
 25. Kamani, J., Gonçalves-Oliveira, J., Janssen, J.N., Quesada, J.R., Nachum-Biala, Y., Harrus, S. and Gutiérrez, R., 2024. Microbiome of two adult tick species and their laboratory-reared offspring shows intra-and inter-species differences. *Acta Tropica*, 257, p.107315.
 26. Karim, S., Budachetri, K., Mukherjee, N., Williams, J., Kausar, A., Hassan, M.J., Adamson, S., Dowd, S.E., Apanskevich, D., Arijo, A. and Sindhu, Z.U., 2017. A study of ticks and tick-borne livestock pathogens in Pakistan. *PLoS Neglected Tropical Diseases*, 11(6), p.e0005681.
 27. Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M. and Glöckner, F.O., 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic acids research*, 41(1), pp.e1-e1.

28. Kurilshikov, A., Livanova, N.N., Fomenko, N.V., Tupikin, A.E., Rar, V.A., Kabilov, M.R., Livanov, S.G. and Tikunova, N.V., 2015. Comparative metagenomic profiling of symbiotic bacterial communities associated with *Ixodes persulcatus*, *Ixodes pavlovskyi* and *Dermacentor reticulatus* ticks. *Public Library of Science ONE*, 10(7), e0131413.
29. Kwan, J.Y., Griggs, R., Chicana, B., Miller, C., Swei, A. and Wu, S., 2022. Investigating the effects of host identity and environmental factors on the tick microbiome. *Microbial Ecology*, 83(4), pp.779-790.
30. Lavhelani, F.G., 2021. Quantifying the use of firewood as a source of energy in North West Province: a case study for Matlwang. *Doctoral dissertation, North-West University (South Africa)*.
31. Lim, F.S., Khoo, J.J., Tan, K.K., Zainal, N., Loong, S.K., Khor, C.S. and AbuBakar, S., 2020. Bacterial communities in *Haemaphysalis*, *Dermacentor* and *Amblyomma* ticks collected from wild boar of an Orang Asli Community in Malaysia. *Ticks and Tick-borne Diseases*, 11(2), p.101352.
32. Liu, Z.L., Qiu, Q.G., Cheng, T.Y., Liu, G.H., Liu, L. and Duan, D.Y., 2024. Composition of the midgut microbiota structure of *Haemaphysalis longicornis* tick parasitizing tiger and deer. *Animals*, 14(11), p.1557.
33. Mateos-Hernández, L., Obregón, D., Wu-Chuang, A., Maye, J., Bornères, J., Versillé, N., de La Fuente, J., Díaz-Sánchez, S., Bermúdez-Humarán, L.G., Torres-Maravilla, E. and Estrada-Peña, A., 2021. Anti-microbiota vaccines modulate the tick microbiome in a taxon-specific manner. *Frontiers in Immunology*, 12, p.704621.
34. McMurdie, P.J. and Holmes, S., 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *Public Library of Science ONE*, 8(4), e61217.
35. Mnisi, S.S., Mphuthi, M.B., Ramatla, T., Mofokeng, L.S., Thekiso, O. and Syakalima, M., 2022. Molecular detection and genetic characterization of *Ehrlichia ruminantium* harbored by *Amblyomma hebraeum* ticks of domestic ruminants in North West Province, South Africa. *Animals*, 12(19), 2511.
36. Mota, T.F., Fukutani, E.R., Martins, K.A., Salgado, V.R., Andrade, B.B., Fraga, D.B. and Queiroz, A.T., 2023. Another tick bites the dust: exploring the association of

- microbial composition with a broad transmission competence of tick vector species. *Microbiology Spectrum*, 11(6), pp.e02156-23.
37. Mutai, B., Njaanake, K., Gathii, K., Estambale, B.B. and Waitumbi, J.N., 2022. Bacteriome in ticks collected from domestic livestock in Kenya. *Advances in Microbiology*, 12(2), pp.67-82.
 38. Narasimhan, S. and Fikrig, E., 2015. Tick microbiome: the force within. *Trends in Parasitology*, 31(7), pp.315-323.
 39. Obregón, D., Bard, E., Abrial, D., Estrada-Peña, A. and Cabezas-Cruz, A., 2019. Sex-specific linkages between taxonomic and functional profiles of tick gut microbiomes. *Frontiers in cellular and infection microbiology*, 9, p.298.
 40. Oduniyi, O.S. and Tekana, S.S., 2019. Adoption of agroforestry practices and climate change mitigation strategies in North West province of South Africa. *International Journal of Climate Change Strategies and Management*, 11(5), pp.716-729.
 41. Oliver, J.H., 1989. Biology and systematics of ticks (Acari: Ixodida). *Annual review of Ecology and Systematics*, pp.397-430.
 42. Omotayo, A.O. and Aremu, A.O., 2020. Evaluation of factors influencing the inclusion of indigenous plants for food security among rural households in the North West Province of South Africa. *Sustainability*, 12(22), p.9562.
 43. Omotayo, A.O., Ndhlovu, P.T., Tshwene, S.C. and Aremu, A.O., 2020. Utilization pattern of indigenous and naturalized plants among some selected rural households of North West Province, South Africa. *Plants*, 9(8), p.953.
 44. Papa, A., Tsioka, K., Daskou, M.A., Minti, F., Papadopoulou, E., Melidou, A. and Giadinis, N., 2020. Application of 16S rRNA next-generation sequencing in ticks in Greece. *Heliyon*, 6(7).
 45. Perveen, N., Muzaffar, S.B., Vijayan, R. and Al-Deeb, M.A., 2022. Microbial composition in *Hyalomma anatolicum* collected from livestock in the United Arab Emirates using next-generation sequencing. *Parasites & Vectors*, 15(1), p.30.
 46. Portillo, A., Palomar, A.M., de Toro, M., Santibáñez, S., Santibáñez, P. and Oteo, J.A., 2019. Exploring the bacteriome in anthropophilic ticks: To investigate the vectors for diagnosis. *Public Library of Science ONE*, 14(3), e0213384.

47. Rodino, K.G., Wolf, M.J., Sheldon, S., Kingry, L.C., Petersen, J.M., Patel, R. and Pritt, B.S., 2021. Detection of tick-borne bacteria from whole blood using 16S ribosomal RNA gene PCR followed by next-generation sequencing. *Journal of Clinical Microbiology*, 59(5), pp.10-1128.
48. Rojas-Jaimes, J., Lindo-Seminario, D., Correa-Núñez, G. and Diringer, B., 2021. Characterization of the bacterial microbiome of *Rhipicephalus (Boophilus) microplus* collected from *Pecari tajacu* in Peru. *Scientific Reports*, 11(1), p.6661.
49. Rymaszewska, A., 2007. Symbiotic bacteria in oocyte and ovarian cell mitochondria of the tick *Ixodes ricinus*: biology and phylogenetic position. *Parasitology Research*, 100, pp.917-920.
50. Rynkiewicz, E.C., Hemmerich, C., Rusch, D.B., Fuqua, C. and Clay, K., 2015. Concordance of bacterial communities of two tick species and blood of their shared rodent host. *Molecular Ecology*, 24(10), pp.2566-2579.
51. Sanchez-Vicente, S., Tagliafierro, T., Coleman, J.L., Benach, J.L. and Tokarz, R., 2019. Polymicrobial nature of tick-borne diseases. *mBio*, 10(5), pp.e02055-19.
52. Sperling, J.L., Silva-Brandão, K.L., Brandão, M.M., Lloyd, V.K., Dang, S., Davis, C.S., Sperling, F.A.H. and Magor, K.E., 2017. Comparison of bacterial 16S rRNA variable regions for microbiome surveys of ticks. *Ticks and Tick-borne Diseases*, 8(4), pp.453-461.
53. Thekiso, O., Ramatla, T., Ringo, A., Mnisi, S., Mphuthi, N., Mofokeng, L., Lekota, K. and Xuan, X., 2023. Molecular detection of *Rickettsia africae* from *Amblyomma hebraeum* ticks in Mafikeng city of North West Province, South Africa. *Research in Veterinary Science*, 164, p.105027.
54. Van Soest, P.J., 1994. *Nutritional ecology of the ruminant*. 2nd ed. Ithaca, NY: Cornell University Press.
55. Van Treuren, W., Ponnusamy, L., Brinkerhoff, R.J., Gonzalez, A., Parobek, C.M., Juliano, J.J., Andreadis, T.G., Falco, R.C., Ziegler, L.B., Hathaway, N. and Keeler, C., 2015. Variation in the microbiota of *Ixodes* ticks with regard to geography, species, and sex. *Applied and Environmental Microbiology*, 81(18), pp.6200-6209.
56. Varela-Stokes, A.S., Park, S.H., Kim, S.A. and Ricke, S.C., 2017. Microbial communities in North American ixodid ticks of veterinary and medical importance. *Frontiers in Veterinary Science*, 4, p.179.

57. Wickham, H., 2016. Data analysis. In *ggplot2: elegant graphics for data analysis* (pp. 189-201). Cham: Springer international publishing.
58. Williams-Newkirk, A.J., Rowe, L.A., Mixson-Hayden, T.R. and Dasch, G.A., 2014. Characterization of the bacterial communities of life stages of free-living lone star ticks (*Amblyomma americanum*). *PLoS One*, 9(7), p.e102130.
59. Xiang, Y., Zhou, J., Yu, F., Zhang, Y., Li, S., Hu, Y., Liang, W. and Liu, Q., 2023. Characterization of bacterial communities in ticks parasitizing cattle in a touristic location in southwestern China. *Experimental and Applied Acarology*, 90(1), pp.119-135.
60. Yan, P., Qiu, Z., Zhang, T., Li, Y., Wang, W., Li, M., Yu, Z. and Liu, J., 2019. Microbial diversity in the tick *Argas japonicus* (Acari: Argasidae) with a focus on *Rickettsia* pathogens. *Medical and Veterinary Entomology*, 33(3), pp.327-335.
61. Zhang, L., Miao, G., Hou, X., Wang, P., Guo, Y., Duan, C., Yang, X. and Hao, Q., 2023. Spotted fever group *Rickettsiae* were the dominant pathogens in parasitic *Rhipicephalus microplus* in Yunnan Province, China. *bioRxiv*, pp.2023-09.
62. Zhang, R., Huang, Z., Yu, G. and Zhang, Z., 2019. Characterization of microbiota diversity of field-collected *Haemaphysalis longicornis* (Acari: Ixodidae) with regard to sex and blood meals. *Journal of Basic Microbiology*, 59(2), pp.215-223.
63. Zhang, X.C., Yang, Z.N., Lu, B., Ma, X.F., Zhang, C.X. and Xu, H.J., 2014. The composition and transmission of microbiome in hard tick, *Ixodes persulcatus*, during blood meal. *Ticks and tick-borne diseases*, 5(6), pp.864-870.
64. Zhong, Z., Wang, K. and Wang, J., 2024. Tick symbiosis. *Current Opinion in Insect Science*, p.101163.
65. Zolnik, C.P., Prill, R.J., Falco, R.C., Daniels, T.J. and Kolokotronis, S.O., 2016. Microbiome changes through ontogeny of a tick pathogen vector. *Molecular Ecology*, 25(19), pp.4963-4977.

Comparison of bacterial communities harboured by *Amblyomma hebraeum* and *Rhipicephalus evertsi evertsi* ticks from the North West Province, South Africa

6.1 Introduction

Ticks are known to carry a wide range of bacteria, which includes symbionts, opportunistic pathogens, and important tick-borne pathogens (TBPs) like *Borrelia*, *Rickettsia*, and *Anaplasma* (Andreotti *et al.*, 2011; Adegoke *et al.*, 2020). Several crucial elements, such as tick species, life stages, types of hosts, and environmental factors, influence these microbial ecosystems' complexity. *Amblyomma hebraeum* is a tick vector for various pathogens, including *Rickettsia* and *Ehrlichia* species. Research suggests that this tick species is frequently found on various host animals, highlighting its ecological versatility and potential for supporting diverse microbial communities (Bournez *et al.*, 2015; Magaia *et al.*, 2020; Makwarela *et al.*, 2024). Similarly, *Rhipicephalus evertsi evertsi* has been associated with the spread of multiple tick-borne pathogens due to its common occurrence in livestock and its ability to serve as a vector for pathogens like *Babesia*, *Anaplasma* and *Theileria* (Matsimbe *et al.*, 2017; Magzoub *et al.*, 2020; Booysen *et al.*, 2017; Makwarela *et al.*, 2024). Additionally, the biotic association of these tick species with their microbes may aid in the understanding of the ecological and evolutionary epidemiology of these pathogens in different settings and hosts (Ledger *et al.*, 2021; Mahlobo-Shwabede *et al.*, 2021).

Research indicates that the bacterial diversity harboured in these tick species may vary considerably due to host species, ecological niches, and geographical distribution. For instance, different climatic conditions and alternative host species have been shown to affect the distribution and microbial composition of ticks (Sungirai *et al.*, 2016; Etiang *et al.*, 2023). The interaction between *A. hebraeum* and *R. e. evertsi* may also contribute to their respective microbial profiles by influencing which species establish within them (Mapholi *et al.*, 2022). Understanding these relationships sheds light on the ecology of these ticks and helps devise strategies for controlling tick populations and managing the pathogens they transmit. These

interactions not only provide information on the biology of the ticks but are also essential in formulating measures to reduce tick infestation and the associated diseases.

The comparative analysis of the bacterial communities of *A. hebraeum* and *R. e. evertsi* ticks collected from sheep and goats in the North West Province will yield valuable information regarding their ecological roles and the potential health risks associated with the pathogens they harbor. This chapter contributes to the broader understanding of TBDs and highlights the importance of monitoring bacterial communities within tick populations to enhance disease surveillance and control in South Africa.

6.2 Materials and methods

6.2.1 Study area, sample collection and DNA extraction

Details of the sample number, study area, sample collection, and DNA extraction are described in the materials and methods (Chapter 3.1).

6.2.2 Library preparation and sequencing

The 16S rRNA gene sequencing for *A. hebraeum* ticks was executed using primers based on Illumina DNA library preparation protocol (Illumina, USA). Locus-specific primers, 341F (5'-CCT ACG GGN GGC WGC AG-3') and 805R (5'-GAC TAC HVG GGT ATC TAA TCC-3') (Klindworth *et al.*, 2013), targeting the hypervariable V3-V4 region (approximately 460 bp 34 region) of the 16S rRNA gene, were used with overhang adapters at the 5'-end of forward and reverse primers respectively (Illumina, USA). The PCR primers used were: forward primer 5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG-3' and reverse primer: 5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C-3' (Klindworth *et al.*, 2013). . The library preparation workflow entailed a first-stage amplicon PCR step to amplify the region, using 12.5 ng of DNA, 12.5 µl of KAPA HiFi HotStart ready Mix [0.5 U DNA polymerase, 0.3 mM dNTPs, 2.5 mM MgCl₂] (Kapa Biosystems, Wilmington, MA, USA), 0.2 µM final concentration of 0.2 µM of each forward and reverse primer was used in the reaction mix, along with nuclease-free water (Thermo Fisher Scientific, USA) to a final volume of 25 µl. The PCR Thermal reaction protocol using ProFlex PCR System (Applied Biosystems, USA) was carried out as

follows: denaturing at 95°C for 3 min, followed by 35 cycles of 95°C for 30 seconds, 55°C for 60 seconds, and 72°C for 30 seconds, and a final extension step at 72°C for 5 min was included.

PCR amplicons were then subjected to a PCR clean-up step using Agencourt AMPure XP magnetic beads (Beckman Coulter Genomics, USA). Following clean-up, index PCR was performed to attach dual indexes to PCR products using the Nextera XT index kit (Nextera, USA). The indexing reaction was performed using 5 µl of amplicon PCR product DNA, 5 µl of Illumina Nextera XT Index Primer 1 (N7xx), 5 µl of Nextera XT Index Primer 2 (S5xx), 25 µl of 2x KAPA HiFi HotStart Ready Mix, and 10 µl of nuclease-free water. The PCR Thermal reaction protocol using ProFlex PCR System (Applied Biosystems, USA), conditions were 95°C for 3 minutes followed by 8 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and a final elongation step of 72°C for 5 minutes. Index PCR products were subjected to a second 'PCR clean-up' step. The libraries were then quantified using a Qubit 3.0 fluorometer (Thermo Fisher Scientific, USA), normalized, pooled, and denatured in 0.2 N NaOH. Denatured libraries and 10% PhiX sequencing control were then loaded onto the MiSeq flow cell using the MiSeq v3 reagent kit for a paired-end (2 × 300 bp) sequencing run. Sequencing was performed using the Illumina MiSeq platform (Illumina, USA), at the North West University (NWU) Potchefstroom campus, North West, South Africa.

6.2.3 Bioinformatics and statistical analysis

Microbiome bioinformatics analysis was conducted on the demultiplexed paired-end sequence reads utilising the QIIME 2 platform (Bolyen *et al.* 2019). The quality of the sequences was assessed using the FastQC software (Andrews, 2010) and subsequently trimmed at both the 5'- and 3'- ends to eliminate poor quality nucleotides. The sequences were denoised, merged, and depleted of chimeric sequences before clustering into Operational Taxonomic Units (OTUs) using the DADA2 denoiser (Callahan *et al.* 2016), which is integrated into QIIME 2. The OTU count table resulting from this process was depleted of singletons. The taxonomic assignment of the sequences was based on a 97% identity threshold using the q2-feature classifier (Bokulich *et al.*, 2018) with a default confidence threshold of 0.7% against the SILVA v132 reference database for bacterial V3 - V4 (Quast *et al.*, 2013). Finally, bacterial OTUs were filtered to remove archaea, mitochondrial, and chloroplast sequences, and the resulting OTU tables were subsequently imported into R software (R Core Team, 2023) for further analysis.

Biostatistical analysis and visualisation were conducted using R statistical software version 4.2.1 (R Core Team, 2022), primarily with the Phyloseq package (McMurdie and Holmes, 2013) and its dependencies, unless otherwise specified. The descriptive statistics, including counts, mean, median, percentage, and range, were employed to describe the basic features of the sequences. Kruskal-Wallis analysis of variance was employed to evaluate differences in microbial richness (i.e., Chao1) and diversity (i.e., Shannon and Simpson) indices. Differences among groups were determined using Dunn's multiple comparison post-hoc test at alpha levels of 0.001, 0.01, and 0.05 presented as annotated boxplots. The Analysis of Group Similarities statistics and the Bray-Curtis distance method (Bray and Curtis, 1957) were used to assess the overall variance in microbial composition of the ticks from goat and sheep, and the results were visualised as Principal Component Ordinate Analysis (PCoA) plots. Linear discriminant analysis (LDA) Effect Size (LEfSe) was carried out using the *lefser* package (Khleborodova *et al.*, 2024) at alpha level of 0.1. Co-occurrence of bacterial genera was determined using the NetCoMi package (Peschel *et al.*, 2021) and the Spearman correlation method (Spearman, 1904), with a filter threshold of 0.01. Undirected network modularity was calculated using the igraph cluster fast greedy function (Csardi and Nepusz, 2006). The resulting network and network attributes were imported and visualised on Gephi software version 0.10.1 (Bastian, Heymann and Jacomy, 2009), except for taxa roles, which was visualised using the R package *ggplot2* (Wickham, 2016). The functional traits of the genus grouped in each module on the network were calculated as percentages by querying against the FAPROTAX database (Louca, Parfrey and Doebeli, 2016) without considering the abundance of the genus within each module.

6.2.4 Validation of the microbiome analysis on selected taxa

In order to validate the microbiome findings, the tick DNA samples were screened for the presence of Rickettsial agents using a *Rickettsiaceae* family-specific qPCR, using primers, Ma16SF: (5'-ACA GAA GAA GTC CCG GCA AA-3'), Ma16SR: (5'-TTG CCC CCT CCG TAT TAC C-3') (Inqaba Biotech, South Africa) and a TaqMan MGBTM probe, Ma16SP: (FAM-5'-CCG TGC CAG CAG C-3'-MGB) (Thermo Fisher Scientific, South Africa) targeting a 64 bp fragment in the V3 hypervariable region of the 16S rRNA gene that is conserved between all known *Anaplasma* and *Ehrlichia* species species. Reactions were performed in a final volume of 20 µl according to Makgabo *et al.* (2023) and Makgabo *et al.* (2024). DNA extracted from the *A. centrale* and *E. ruminantium* vaccine strain (Onderstepoort

Biological Products (OBP), Pretoria, South Africa) were used as positive controls and molecular grade water as a negative control. Data was analyzed using the StepOnePlus™ software version 2.2.

DNA samples were further subjected to the *A. marginale* and *A. centrale* species specific duplex qPCR assay targeting the *msp1β* gene of *A. marginale* and the *groEL* gene of *A. centrale* as previously described (Chaisi *et al.*, 2017) with modification of the *A. centrale* probe, AC-Pb (NED- ATC ATC ATT CTT CCC CTT TAC CTC GT- NFQ-MGB) (Thermo Fisher Scientific, South Africa) for use on an Applied Biosystem StepOnePlus™ Real-time PCR System 2.3 (Applied Biosystems, Life Technologies, South Africa). Reactions were prepared in a 25 µl final reaction volume comprising 12.5 µl TaqMan® Universal PCR Master Mix (LTC Tech South Africa (Pty) Ltd), 0.6 µM of the *A. marginale*-specific primers, 0.9 µM of the *A. centrale*-specific primers, 0.2 µM of each probe and 2.5 µl of target DNA (approximately 200 ng). Positive control for the *A. centrale* assay was DNA extracted from the *A. centrale* vaccine strain obtained from OBP, Pretoria, South Africa. The positive control for the *A. marginale* assay was provided by the Department of Veterinary Tropical Diseases, Faculty of Veterinary Sciences, University of Pretoria, which was confirmed by sequencing of the *A. marginale msp1α* gene. Nuclease-free water was used as a negative control for the assay. Cycling conditions were as follows: UNG incubation at 50°C for 2 min and activation of AmpliTaq Gold at 95°C for 10 min, followed by 40 cycles of 1 min at 90°C, 1 min at 60°C. Data was analyzed with the StepOnePlus™ Real-time PCR System Software version 2.3. Samples were run in triplicate for both assays for reproducibility and repeatability and average Ct values recorded.

6.3 Results

6.3.1 Comparison of the bacterial communities in *Amblyomma hebraeum* and *Rhipicephalus evertsi evertsi* at the phylum level

The bar plot illustrates the microbial composition at the phylum level across *R. e. evertsi* and *A. hebraeum* ticks collected from sheep and goats, depicted in Figure 6.1. The microbial diversity shows distinct variations between tick species and host animals. The most dominant bacterial phyla identified include Proteobacteria (54.32%), Firmicutes (16.77%),

Actinobacteriota (13%), *Bacteroidota* (10.34%), and *Campilobacterota* (1.48%), with noticeable differences in relative abundance across samples.

Proteobacteria is the most abundant phylum in many samples, followed by *Actinobacteriota* and *Firmicutes*, while *Bacteroidota* is found in lower proportions. The remaining phyla are present in varying but lesser abundances, contributing to microbial diversity. Particularly, *A. hebraeum* ticks from sheep exhibit a distinct dominance of *Proteobacteria* (82.81%), whereas *R. e. evertsi* from goats and sheep show a more balanced representation of *Firmicutes* (27.82% and 19.48%, respectively) and *Actinobacteriota* (23.47% and 19.74%, respectively). The presence of additional phyla such as *Cyanobacteria* (0.27%), *Spirochaetota* (0.06%), *Myxococcota* (0.024%) and *Desulfobacterota* (0.06%) suggests a complex microbiome structure influenced by both tick species and host factors.

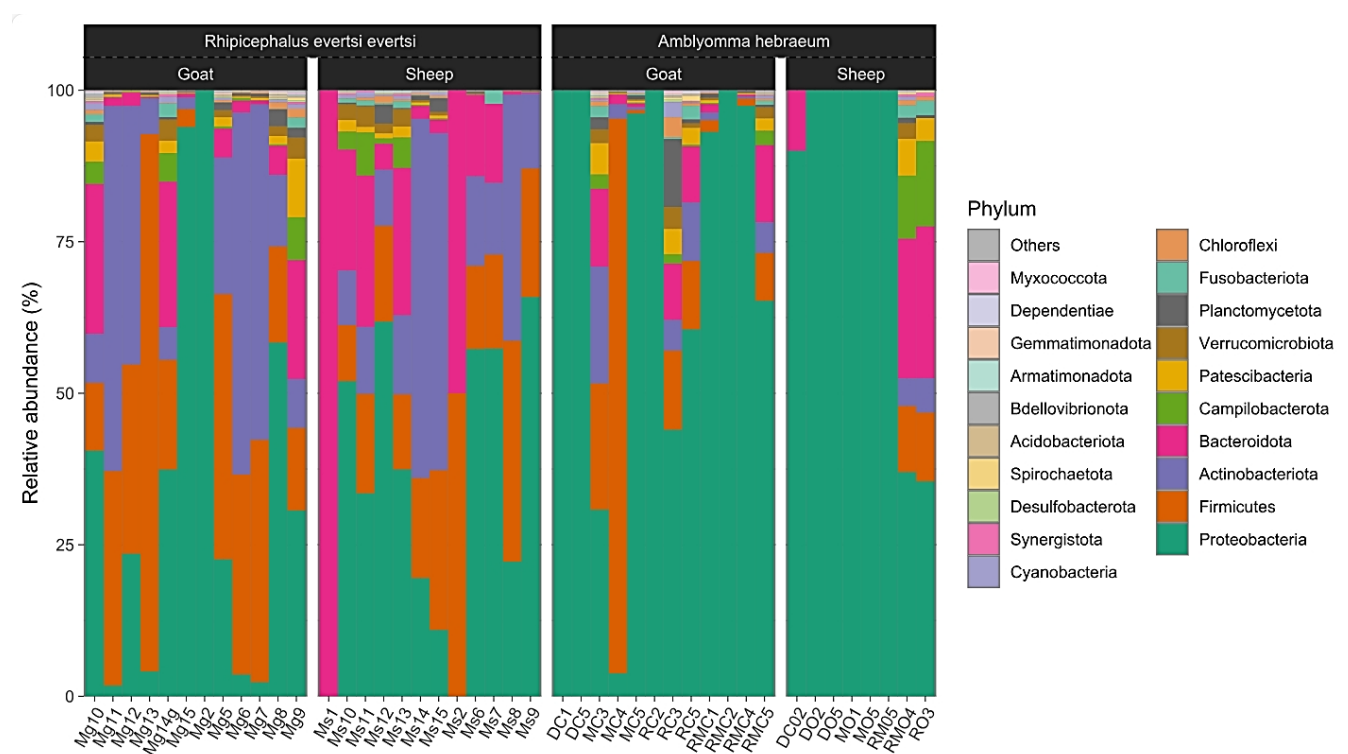


Figure 6.1: Stacked bar chart representing the taxonomic bacterial composition of *Rhipicephalus evertsi evertsi* and *Amblyomma hebraeum* ticks from sheep and goats at the phylum level.

6.3.2 Comparison of the bacterial communities in *Amblyomma hebraeum* and *Rhipicephalus evertsi evertsi* at the genus level

The genus-level microbiota composition of *R. e. evertsi* and *A. hebraeum* ticks collected from goats and sheep reveals distinct differences in bacterial abundance across tick species and host associations depicted in Figure 6.2. The most dominant genus across all groups is *Rickettsia* (27.98%), with significantly higher abundance (ANOVA: $p < 0.05$) in *A. hebraeum* from sheep (61.42%) and goats (61.29%) than in *R. e. evertsi* from goats (0.21%) and sheep (0.1%). Other highly abundant genera include *Corynebacterium* (8.56%), *Bacillus* (5.24%), *Staphylococcus* (4.19%), and *Sphingorhabdus* (4.72%), though their distributions vary depending on the tick species and host. Significantly, *Bacillus* is more enriched in *R. e. evertsi* from goats (7.05%). Statistical differences in bacterial abundance among groups are indicated by the letters above the bars, where *Rickettsia* shows significant enrichment in *A. hebraeum* from sheep and goats, while *Bacillus* and *Corynebacterium* exhibit host-specific variations. Significant differences can be observed in *R. e. evertsi* from sheep, which have a relatively high abundance of *Acinetobacter* (2.8%), *Coxiella* (2.2%), and *Pseudomonas* species (3.9%). In contrast, *Ehrlichia* (2.9%) and *Aerococcus* species (2.49%) comprise a high proportion of the bacterial composition in *R. e. evertsi* ticks from goats.

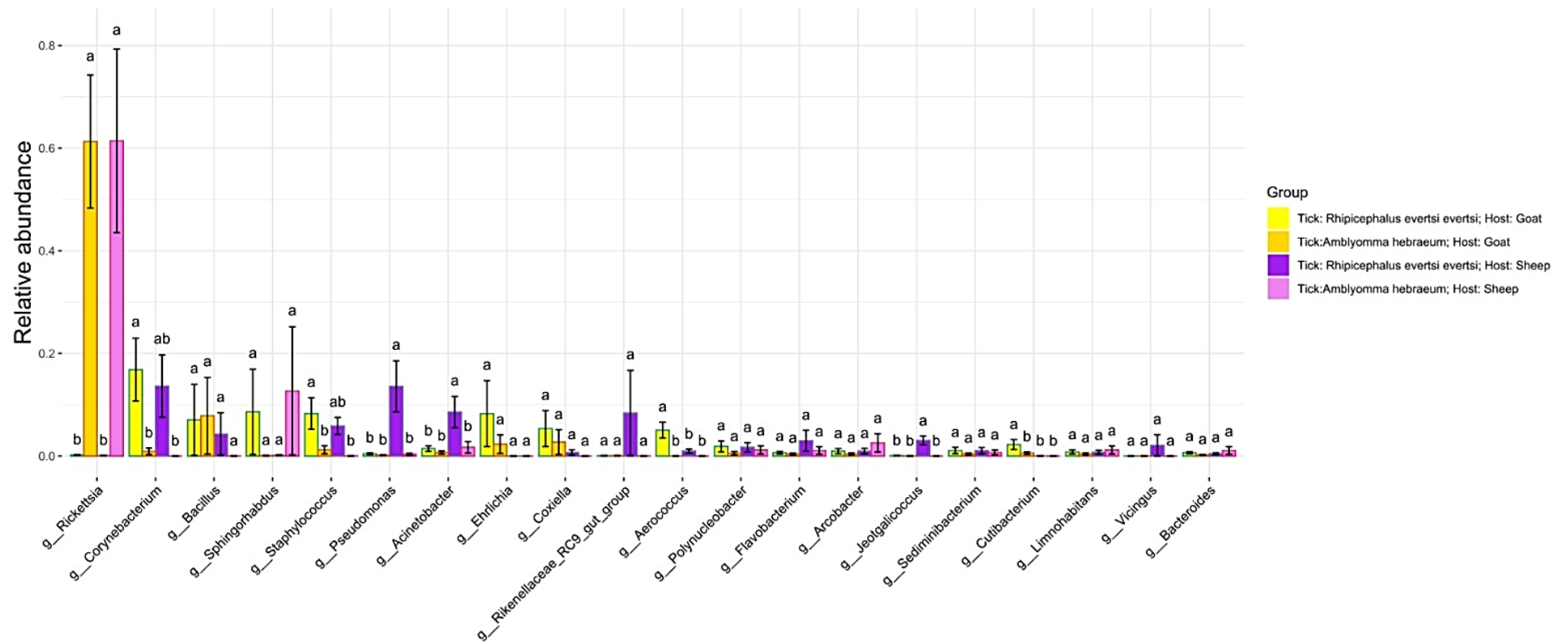


Figure 6.2: Stacked bar chart representing the taxonomic bacterial composition in *Rhipicephalus evertsi evertsi* and *Amblyomma hebraeum* ticks from goats and sheep at genus level. A significant difference is indicated by a, b or ab.

6.3.3 Comparative top fifty genus-level microbial diversity in *Rhipicephalus evertsi evertsi* and *Amblyomma hebraeum* from sheep and goats

The heatmap presents the core microbiome composition at the genus level for *R. e. evertsi* and *A. hebraeum* ticks collected from goats and sheep, as depicted in Figure 6.3. The relative abundance of bacterial genera is illustrated using a colour gradient, where blue signifies low abundance and red indicates high abundance. Across all tick species and host animals, certain bacterial genera such as *Rickettsia*, *Bacillus*, *Staphylococcus*, *Corynebacterium*, *Acinetobacter*, and *Pseudomonas* species are consistently observed. However, the abundance of these genera differs among tick species and host animals. Notably, *Rickettsia* is found to be particularly abundant in *A. hebraeum* ticks, particularly those collected from sheep. Moreover, *Acinetobacter* and *Pseudomonas* species are dominant in sheep-derived *R. e. evertsi* ticks. Other genera, including *Coxiella*, *Ehrlichia*, *Anaplasma*, and *Pseudarcicella*, display irregular distributions. Furthermore, genera such as *Algoriphagus* and *Flavobacterium* are sporadically present across the ticks. *Anaplasma* species, a notable veterinary pathogen, was initially presumed to be widely distributed across various tick species. However, the heatmap analysis revealed its presence at low relative abundance, predominantly in *R. e. evertsi* ticks, specifically in samples Mg10, Mg12, and Mg8 collected from goats, and MSI and MS8 from sheep. Only a single *A. hebraeum* tick sample (MC3) exhibited a low-level presence of the pathogen. In *A. hebraeum*, *Ehrlichia* spp. were detected at low abundance from goat-associated samples DC3, RC2, RC3, and RC5, as well as in sheep-associated samples DC3, RC2, and RC3. Similarly, *R. e. evertsi* ticks collected from goats, *Ehrlichia* spp. were detected in samples Mg15 and Mg12. Overall, *R. e. evertsi* ticks show a significant bacterial taxon at the genus level compared to *A. hebraeum*.

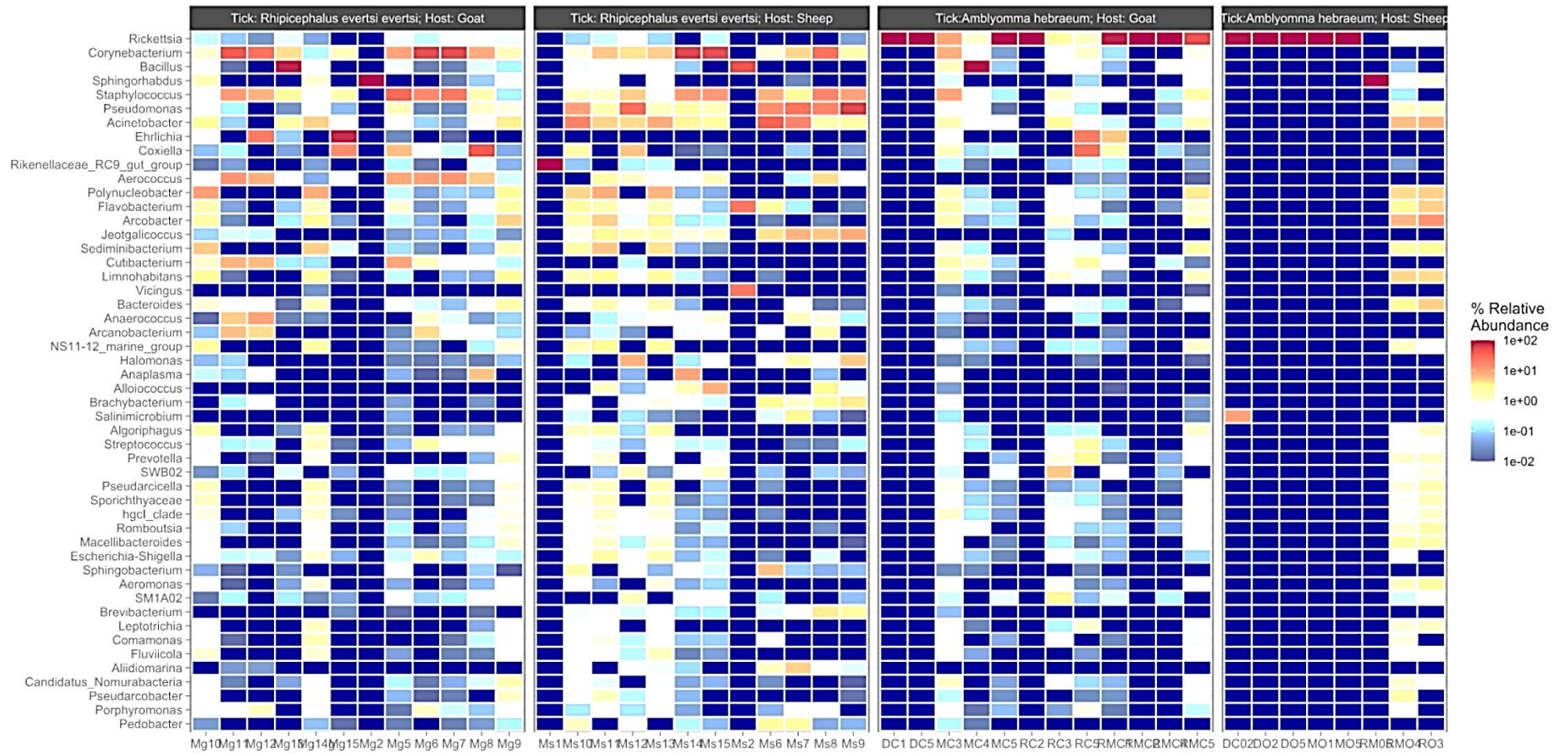


Figure 6.3: Heatmap of the top fifty genus-level bacterial taxa in *Rhipicephalus evertsi evertsi* and *Amblyomma hebraeum* ticks from sheep and goats.

6.3.4 Validation of the presence of *Anaplasma/Ehrlichia* species in the ticks on qPCR

The *Anaplasma/Ehrlichia* group-specific qPCR assay based on the 16S rRNA gene revealed the presence of *Anaplasma/Ehrlichia* spp. in 37.1% (13/ 35) and 31.4% (11/35) *R. e. evertsi* ticks collected from goat and sheep, respectively. The group-specific qPCR assay furthered the presence of *Anaplasma/Ehrlichia* spp. in 25.7% (9/35) and 5.7% (2/35) *A. hebraeum* ticks collected from a goat and sheep, respectively, confirming the microbiome data observed. Notably, the pathogenic *A. marginale* and less pathogenic *A. centrale* were not detected in the two tick species included in this study (qPCR results for individual samples are shown in Supplementary Table S1).

6.3.5 Core microbiome and biomarker identification in *Rhipicephalus evertsi evertsi* and *Amblyomma hebraeum* ticks from sheep and goats

The cladogram presents a phylogenetic analysis of bacterial taxa associated with *R. e. evertsi* and *A. hebraeum* from goats and sheep. The colour-coded sections indicate different bacterial groups significantly associated with each tick-host combination. *Rhipicephalus evertsi evertsi* from goats (green) and sheep (purple) share several bacterial taxa regardless of the host. However, *A. hebraeum* exhibits distinct bacterial communities when collected from goats (orange) and sheep (pink). The *Proteobacteria* phylum, particularly *Alphaproteobacteria* and *Gammaproteobacteria*, is dominant across all groups, with *Rickettsiales* (including *Rickettsia* and *Ehrlichia*) being significantly enriched. *Firmicutes*, including *Bacillales* and *Lactobacillales*, are prominent, particularly in *R. evertsi evertsi*. *Actinobacteria*, including *Corynebacteriales* and *Propionibacteriales*, are particularly enriched in *A. hebraeum*.

clusters. The prediction ellipses enclose individual tick groups, with some ellipses overlapping. The observed beta-diversity differences highlight species-specific microbiome variations, with potential bacterial co-occurrence across tick species and hosts.

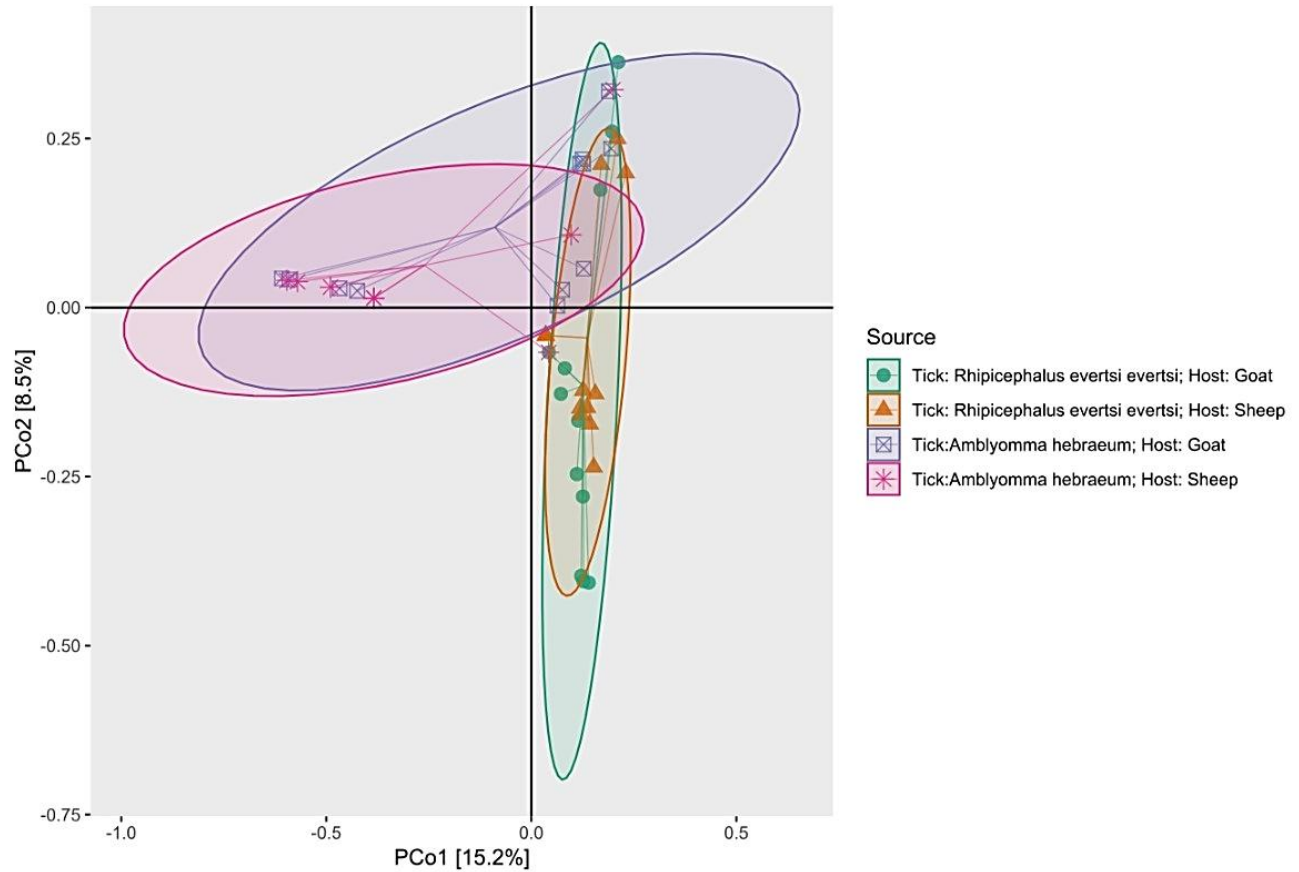


Figure 6.5: Principal coordinate analysis (PCoA) plot showing relatedness between *Rhipicephalus evertsi evertsi* and *Amblyomma hebraeum* tick bacterial community structures at the genus level.

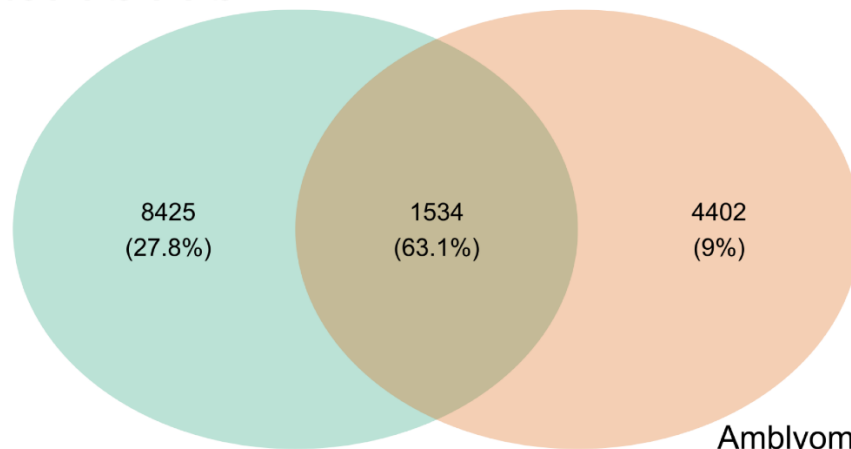
6.3.7 Determining the shared species richness between *Rhipicephalus evertsi evertsi* and *Amblyomma hebraeum* ticks collected from sheep and goats

The Venn diagram illustrates the shared and unique microbiome composition between *R. e. evertsi* and *A. hebraeum* as depicted in Figure 6.6. The overlapping region represents 1,534 bacterial taxa, which constitute 63.1% of the total microbiome, indicating a significant core microbiome shared

between both tick species. *R. e. evertsi* harbours 8,425 unique taxa (27.8%), while *A. hebraeum* possesses 4,402 unique taxa (9%).

The second Venn diagram illustrates the shared and unique bacterial taxa among *R. e. evertsi* and *A. hebraeum* collected from goats and sheep as depicted in Figure 6.7. The analysis reveals that *R. e. evertsi* from goats harbours the highest number of unique bacterial taxa (6,883 taxa, 12.8%), followed by *R. e. evertsi* from sheep (4,181 taxa, 9%), whilst *A. hebraeum* has significantly fewer unique taxa in both goats (1,266 taxa, 6.7%) and sheep (212 taxa, 0.1%). Interestingly, 211 bacterial taxa (17.5%) are shared among all four groups. There is substantial intra-species microbiome similarity in *R. e. evertsi*, with 676 taxa (13.3%) shared between individuals from goats and sheep. In contrast, *A. hebraeum* from goats and sheep share only three taxa (0.02%). Moreover, microbial overlap between *R. e. evertsi* from sheep and *A. hebraeum* from goats is minimal (72 taxa, 0.6%), while *R. e. evertsi* from goats and *A. hebraeum* from sheep share no bacterial taxa (0 taxa). These findings highlight that tick species and host type significantly influence microbial community composition.

Rhipicephalus evertsi evertsi



Amblyomma hebraeum

Figure 6.6: Venn diagram indicating the shared number of bacterial genera between *Rhipicephalus evertsi evertsi* and *Amblyomma hebraeum* ticks collected from sheep and goats based on OTUs.

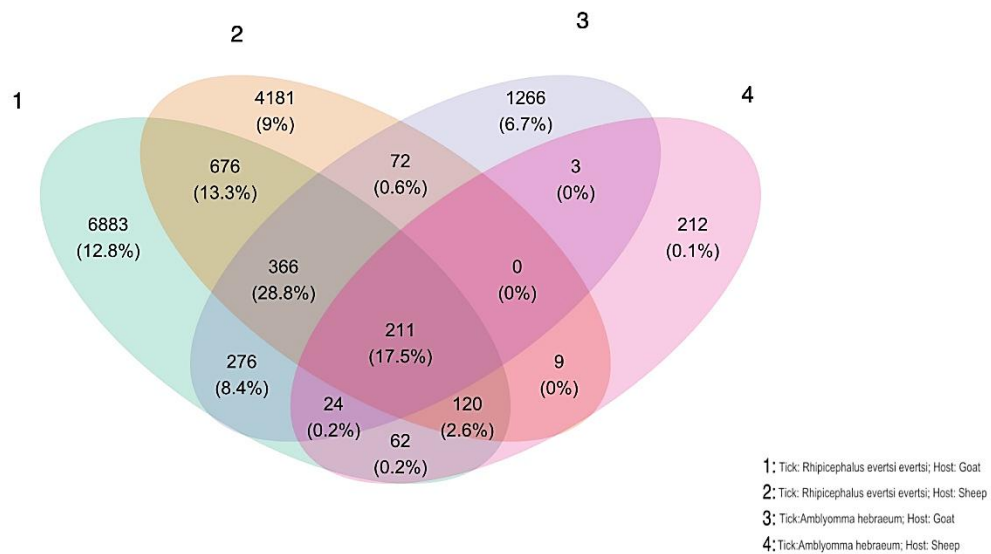


Figure 6.7: Venn diagram indicating the shared number of bacterial genera between *Rhipicephalus evertsi evertsi* and *Amblyomma hebraeum* ticks collected from sheep and goats based on OTUs.

6.3.8 Network connectivity of bacterial taxa within *Rhipicephalus evertsi evertsi* and *Amblyomma hebraeum*

Due to the core-microbiome structure of the tick species, both ticks were investigated to understand their functional potential. The network connectivity of bacterial taxa within the tick microbiome at the phylum level, illustrating their ecological roles in microbial community structure, is depicted in Figure 6.8 and Figure 6.9. The x-axis displays different bacterial phyla, while the y-axis represents connectivity, divided into within-module and among-module connectivity (Figure 6.9). Within-module connectivity indicates how strongly a taxon is linked to others within own community, while among-module connectivity reflects its influence across multiple microbial clusters.

Most taxa are represented as peripheral nodes (denoted by asterisks). In contrast, a few taxa are identified as module hubs (denoted by open circles), indicating their central role in maintaining microbiome structure. *Proteobacteria*, *Firmicutes*, and *Bacteroidota* appear to be highly represented. The presence of *Cyanobacteria*, *Verrucomicrobiota*, and *Fusobacteriota* with fewer connections suggests they play a more specialized role. Taxa abundance is depicted by the size of the points, with some phyla showing higher representation. *Proteobacteria* and *Firmicutes* exhibit the highest number of highly connected taxa, indicating their potential role in core microbiome functions. This connectivity analysis provides insights into microbial community dynamics and potential functional contributions to tick bacterial diversity.

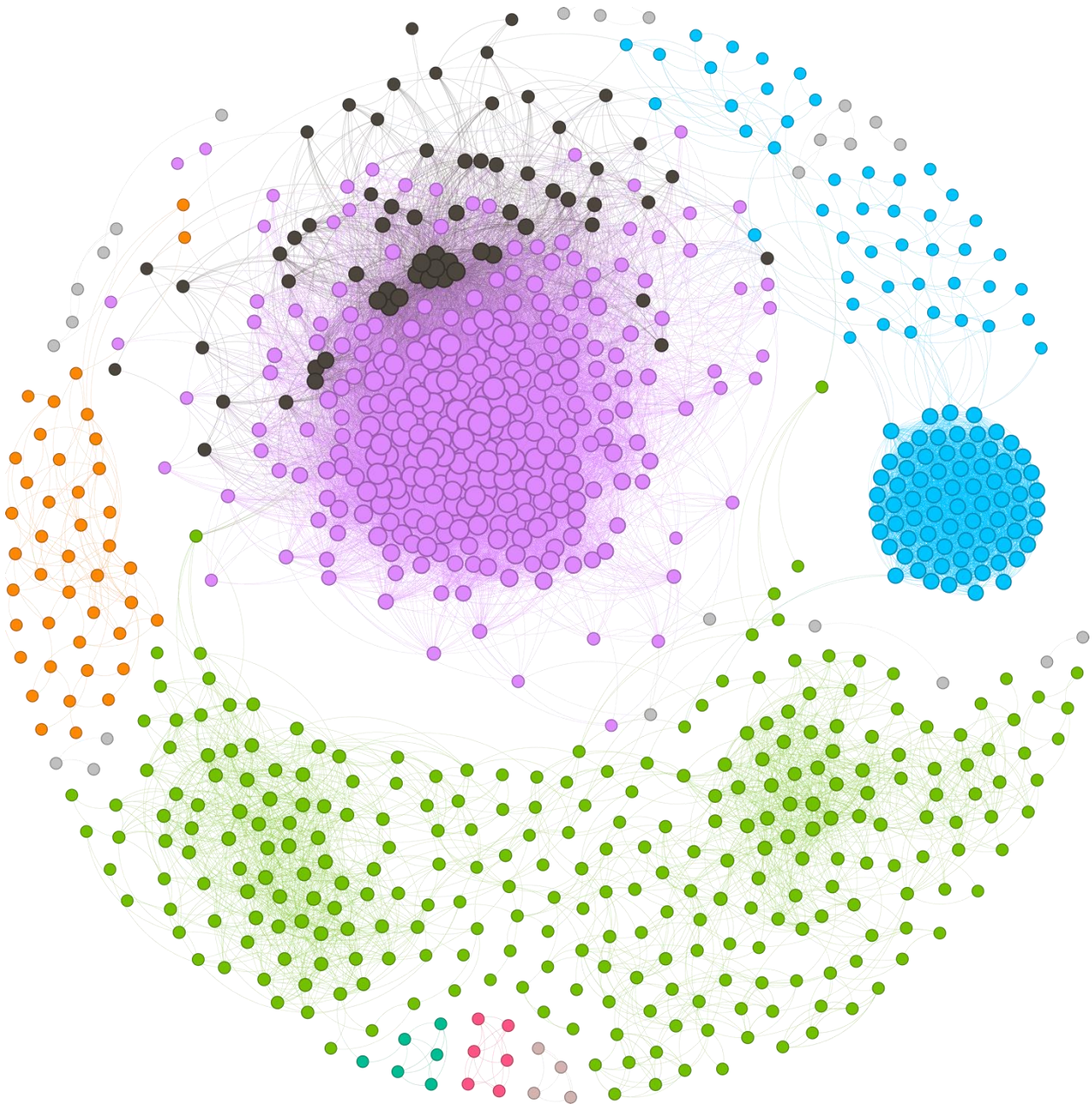


Figure 6.8: Network connectivity of bacterial taxa within the tick microbiome of *R. e. evertsi* and *A. hebraeum* ticks. Key: **M1: Firmicutes**, **M2: Proteobacteria**, **M3: Actinobacteriota**, **M4: Campilobacterota**, **M5: Patescibacteria**, **M6: Verrucomicrobiota**, **M7: Fusobacteriota**, **M8: Cyanobacteria**, **M9: Planctomycetota**, **M10: Bacteroidota**, **M11: Chloroflexi**.

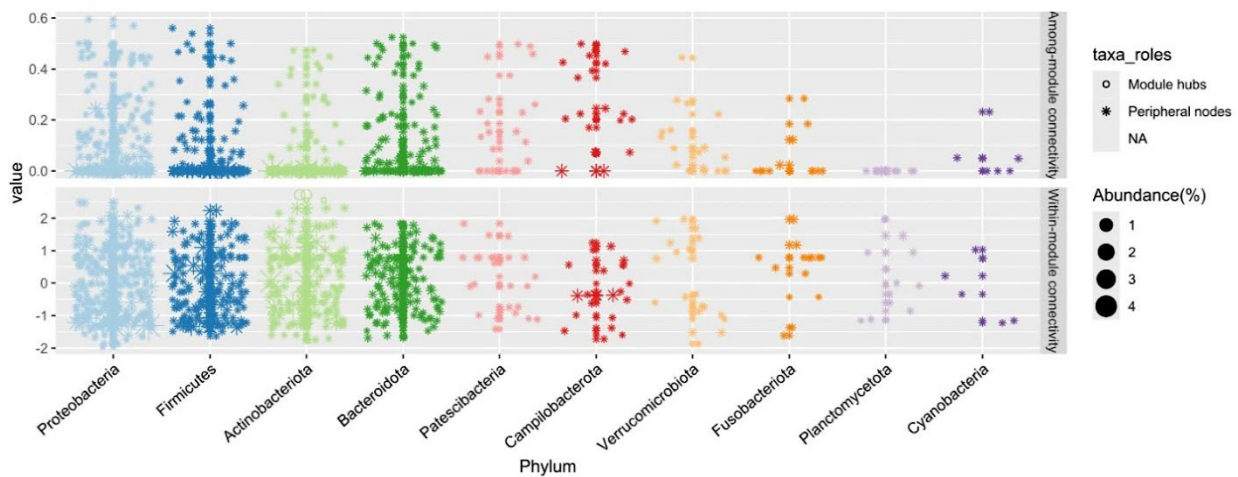


Figure 6.9: Network taxa roles within the tick microbiome of *Rhipicephalus evertsi evertsi* and *Amblyomma hebraeum* ticks.

6.3.9 Functional potential of the bacterial communities in *Rhipicephalus evertsi evertsi* and *Amblyomma hebraeum* ticks

The combined functional analysis of the microbiome from *A. hebraeum* and *R. evertsi evertsi* ticks, organized into co-occurrence network modules (M1–M16), highlights a wide range of metabolic potentials. Modules such as M1, M10, M13, and M14 were associated with various carbon-related processes, including photoheterotrophy, different forms of chemoheterotrophy, fermentation, and the breakdown of methanol and hydrocarbons, with M13 and M14 showing the highest activity. Nitrogen cycle-related functions like nitrogen fixation, nitrate and nitrite reduction, ammonia oxidation, and denitrification were also prominent in M13 and M14, indicating their role in nitrogen processing. Though less frequent, sulfur-related pathways were observed in modules like M5 and M13, particularly involving sulfate respiration and sulfur compound degradation. In contrast, modules M6 through M9 were dominated by genes linked to host interaction traits such as pathogenicity (e.g., septicemia, gastroenteritis), parasitism, and the breakdown of complex organic compounds. Overall, M13 emerged as the most functionally diverse module across nutrient cycles, while M6 to M9 were more specialized in host-associated and environmental survival traits.



Figure 6.10: Functional potential of the tick microbiome *Amblyomma hebraeum* and *Rhipicephalus evertsi evertsi* ticks. M1-M16 are the modules in the co-occurrence network.

6.4 Discussion

This study compared the bacterial communities of *A. hebraeum* and *R. evertsi evertsi* ticks infesting sheep and goats in the North West Province of South Africa. Previous studies have confirmed the presence of *A. hebraeum* and *R. evertsi evertsi* tick species infesting sheep and goats in the North West, South Africa (Spickett *et al.*, 2011; Mtshali, 2012; Mnisi *et al.*, 2022). However, research in South Africa has primarily focused on the identification, epidemiology and conventional control of livestock and zoonotic TBPs, leaving a knowledge gap concerning the bacterial communities harboured by *A. hebraeum* and *R. e. evertsi* and their potential significance, particularly in the North West Province (Spickett *et al.*, 2011; Mtshali, 2012; Jongejan *et al.*, 2020; Mnisi *et al.*, 2022; Thekiso *et al.*, 2023; Makwarela *et al.*, 2024). Despite the significance, studies on the greater tick microbiota of South African small stock are scarce. The study revealed a significant divergence in the bacterial communities associated with *A. hebraeum* and *R. e. evertsi* ticks from sheep and goats, highlighting a species-dependent microbial diversity.

In this study, at the phylum level, *Proteobacteria* (54.32%) was the dominant phylum in *A. hebraeum* and *R. e. evertsi* ticks from both sheep and goats, particularly *A. hebraeum* ticks from sheep exhibited a distinct dominance of *Proteobacteria* (82.81%). These findings are consistent with previous studies

on the *Amblyomma* and *Rhipicephalus* tick genera, particularly concerning the distribution of bacterial phyla (Ackermann, 2019; Kisten *et al.*, 2021). The finding that *A. hebraeum* ticks collected from sheep exhibited a high proportion of *Proteobacteria* aligns with prior research, where *Proteobacteria* were identified as the predominant phylum in various tick species, including the *Amblyomma* genus (Mutai *et al.*, 2022). The dominance of *Proteobacteria* in *A. hebraeum* and *R. e. evertsi* ticks may be attributed to their various functions that are advantageous to tick survival and development. Many members of this phylum are known for their role in nutrient acquisition and metabolic processes crucial for tick survival amidst challenging environments (Narasimhan and Fikrig, 2015). Furthermore, the presence of *Proteobacteria* correlates with potential transmission dynamics of TBPs, as these bacteria may interact with pathogens, influencing the tick vector competence (Gall *et al.*, 2016).

At the genus-level the most dominant genus across all groups is *Rickettsia* (27.98%) in the current study, with significantly higher abundance ($p < 0.05$) in *A. hebraeum* from sheep (61.42%) and goats (61.29%) than in *R. e. evertsi* from goats (0.21%) and sheep (0.1%). This highlights critical ecological and epidemiological implications rickettsial agents. The dominance of *Rickettsia*, particularly in *A. hebraeum*, aligns with previous findings emphasizing this tick genus as a principal vector of *Rickettsia* spp., particularly *Rickettsia africae*, an important pathogen responsible for African Tick-Bite Fever (Magaia *et al.*, 2020; Kisten *et al.*, 2021; Thekisoe *et al.*, 2023). The high prevalence of *Rickettsia* in *Amblyomma* species corroborates findings from sub-Saharan Africa, where the prevalence of *Rickettsia* spp. in various tick species frequently exceeds 80%, reflecting their role as significant reservoirs (Eneku *et al.*, 2023). The important difference in abundance between *Rickettsia* in *A. hebraeum* and *R. e. evertsi* suggests complex ecological interactions and host-tick-pathogen dynamics. Higher infection rates in *A. hebraeum* may result from its feeding habits on specific host types that facilitate *Rickettsia* transmission, as suggested in other geographic regions (Magaia *et al.*, 2020; Kisten *et al.*, 2021). The findings observed in this study are of public health importance as *Rickettsia* species can be transmitted from infected ticks to livestock and subsequently to humans through zoonotic spillover, necessitating critical attention (Ghafar *et al.*, 2020; Shehla *et al.*, 2023).

This study validated the presence of *Anaplasma/Ehrlichia* species in *A. hebraeum* and *R. e. evertsi* ticks through a group-specific quantitative PCR (qPCR) assay targeting the *16S rRNA* gene. The results indicated a higher infection rate of *Anaplasma/Ehrlichia* species in *R. e. evertsi* ticks, recorded at 37.1% (13 out of 35), compared to 25.7% (9 out of 35) in *A. hebraeum* ticks collected from goats. These findings are consistent with the detection of *Ehrlichia* species at low abundance using the 16S rRNA metabarcoding sequencing approach. This raises significant concerns regarding animal health

and the potential for zoonotic transmission to humans, as other *Anaplasma* spp. known to occur in South Africa are of zoonotic importance. Previous research in South Africa, including the work by Iweriebor *et al.* (2017), has documented the presence of *Anaplasma* and *Ehrlichia* pathogens in various tick species, particularly in the Eastern Cape Province. However, their distribution is not restricted to this region; several studies have also reported these genera in ticks from KwaZulu-Natal, Limpopo, and other provinces, highlighting their widespread occurrence across the country (Mtshali *et al.*, 2015; Thekiso *et al.*, 2007; Allsopp *et al.*, 2007).

In addition, the core microbiome identified across all tick species from host animals revealed dominant bacterial genera such as *Bacillus*, *Staphylococcus*, *Corynebacterium*, *Acinetobacter* and *Pseudomonas* many of which are commonly associated with host skin, mucosal surfaces, or environmental sources (Loftis *et al.*, 2008; Andreotti *et al.*, 2011; Qiu *et al.*, 2014; Adegoke *et al.*, 2020). Their presence may therefore reflect both potential symbiotic or opportunistic roles within the tick microbiome, such as aiding in metabolic functions or pathogen interactions, while also raising the possibility that some of these taxa could represent environmental or external contaminants introduced during host contact or from the tick's immediate habitat (Ross *et al.*, 2018; Van Treuren *et al.*, 2015). However, the bacterial genera distribution varies depending on the tick species and the animal host. This observation is consistent with previous research indicating that the bacterial composition may be influenced by the feeding habits of ticks, their host specificity, and environmental factors (Bonnet *et al.*, 2017). For example, host-switching in tick species that adapt to different host environments can lead to significant changes in the structure of the microbiome. These changes may impact the ticks' behaviour, survival, and ability to act as vectors for pathogens (Du *et al.*, 2023).

The Principal Coordinates Analysis (PCoA) of tick microbiomes indicate significant beta-diversity differences between the tick species, *A. hebraeum* and *R. e. evertsi*, collected from sheep and goats. The observed species-specific differences in microbiome composition highlight how the microbial communities associated with ticks are influenced by both the tick species and their host-related variability. This suggests that while the tick species plays a crucial role in shaping the resident microbial communities, host factors also contribute to microbiome structuring, with evidence leaning towards tick species as the primary driver of microbial diversity (Rynkiewicz *et al.*, 2015; Chicana *et al.*, 2019; Namiņa *et al.*, 2023). Studies that align with these findings demonstrate that the intrinsic characteristics of ticks, such as their immune responses and specific ecological niches, significantly influence the composition of their microbiomes (Rynkiewicz *et al.*, 2015; Bruley *et al.*, 2025). The presence of overlapping microbiome profiles among samples collected from different hosts primarily

indicates shared environmental conditions and host interactions, where the tick species itself remains a dominant factor in microbiome specificity (Bonnet and Pollet, 2020; Mapholi *et al.*, 2022). Consistent with this, studies have shown that distinct tick species can harbour unique microbial populations despite existing in the same geographical area. This supports the notion of host specialization influencing microbial communities (Chicana *et al.*, 2019; Hussain *et al.*, 2022).

Moreover, the bacterial composition based on genus-level OTUs provided insights into shared and unique microbial communities between *R. e. evertsi* and *A. hebraeum* ticks collected from sheep and goats. Identifying a core microbiome comprising 1,534 bacterial taxa, which constitute 63.1% of the total microbiome, indicates a significant core microbiome shared between both tick species. *R. e. evertsi* harbours 8,425 unique taxa (27.8%), while *A. hebraeum* possesses 4,402 unique taxa (9%). These findings highlight the presence of a shared core microbiome among ectoparasites, which indicates evolutionary adaptations that may enhance the survival and reproductive success of the ticks by providing essential metabolic functionalities or pathogen resistance (Adegoke *et al.*, 2020). Interestingly, in the current study, *R. e. evertsi* harbours a considerably larger proportion of unique bacterial taxa than *A. hebraeum*. Previous studies have shown that distinct host interactions and environmental factors can lead to varied microbiome compositions in ticks. Blood meals can influence the diversity and composition of the microbiome, which is facilitated by the nutritional composition and host immune responses in ticks (Zolnik *et al.*, 2016; Munjita *et al.*, 2024). Unique taxa may play crucial roles in tick biology, impacting digestion, immunity, and interactions with pathogens, thereby influencing the overall health and fitness of the ticks (Andreotti *et al.*, 2011).

The network connectivity of bacterial taxa within the tick microbiome of *R. e. evertsi* and *A. hebraeum* ticks at the phylum level demonstrated their ecological roles in microbial community structure. *Proteobacteria* and *Firmicutes* exhibited the highest number of highly connected taxa, indicating their potential role in core microbiome functions. Studies suggest that specific bacteria, particularly within the *Proteobacteria* and *Firmicutes* phyla, may contribute to tick survival, reproduction, and the modulation of vector efficacy in disease transmission (Varela-Stokes *et al.*, 2018; Chicana *et al.*, 2019). Moreover, the significance of these findings extends beyond basic ecological understanding into practical applications in vector control strategies. For instance, understanding how these dominant phyla interact and the overarching structural features of the tick microbiome can reveal new strategies to manipulate tick-associated bacteria to disrupt pathogen transmission mechanisms. Studies have shown that altering the microbiome can decrease tick fitness and affect their ability to harbour pathogens (Clayton *et al.*, 2015; Estrada-Peña *et al.*, 2019). These insights are highly relevant given the global rise in tick-borne diseases, thereby encouraging

innovative approaches manipulating microbial community knowledge to mitigate health risks posed by these vectors.

Furthermore, the functional analysis of the microbiomes of *A. hebraeum* and *R. e. evertsi* ticks, structured into co-occurrence network modules, revealed significant insights into microbial communities' ecological roles and metabolic potentials. The presence of specific modules associated with carbon cycling processes, including photoheterotrophy, chemoheterotrophy, fermentation, and the breakdown of methanol and hydrocarbons, suggests that these tick-associated microbiomes are not incidental but play crucial roles in nutrient cycles relevant to their ecological niches (Obregón *et al.*, 2019). Specific modules exhibited high metabolic activity, indicating their critical function in sustaining microbial diversity and facilitating nutrient turnover within the tick's environment. Moreover, these modules' pronounced nitrogen cycle-related functions, including nitrogen fixation and denitrification, suggest a significant nitrogen processing capability within the tick microbiome (Obregón *et al.*, 2019). This is especially relevant, as nitrogen cycling is essential for maintaining soil health and plant productivity, which may indirectly influence the habitats where these ticks thrive, highlighting the interconnectedness between tick microbiomes and broader ecological processes (Obregón *et al.*, 2019). The presence of sulfur-related pathways is less frequent, further supporting that these ticks may contribute to sulfur cycling. However, this aspect warrants further investigation to understand its implications fully.

This study provides the first comparative analysis of bacterial communities in *A. hebraeum* and *R. e. evertsi* ticks infesting sheep and goats in the North West Province of South Africa, highlighting significant species and host-associated bacterial diversity. *Proteobacteria* emerged as the dominant phylum, particularly in *A. hebraeum* from sheep, suggesting functional advantages for tick survival. *Rickettsia* species were significantly more abundant in *A. hebraeum*, aligning with its known vector competence for *R. africae*. The use of qPCR detection method of *Anaplasma/Ehrlichia* spp. confirmed the microbiome data, revealing higher infection rates in *R. e. evertsi*, supporting concerns for potential zoonotic spillover. Identifying core and unique microbiota revealed substantial bacterial diversity, with *R. e. evertsi* harbouring more unique taxa, indicating potential ecological and host-driven influences. Functional and network analyses underscored the environmental importance of microbial taxa in nutrient cycling and tick physiology. The findings underscore the significance of tick microbiomes in shaping pathogen dynamics and ecological interactions, providing foundational data for future vector control and zoonotic disease mitigation strategies.

References

1. Ackermann, R., 2019. *Bacterial microbiome of Rhipicephalus sanguineus ticks collected from dogs in the Mnisi Community, South Africa*. MSc thesis. University of Pretoria, South Africa.
2. Adegoke, A., Kumar, D., Bobo, C., Rashid, M.I., Durrani, A.Z., Sajid, M.S. and Karim, S., 2020. Tick-borne pathogens shape the native microbiome within tick vectors. *Microorganisms*, 8(9), 1299.
3. Andreotti, R., Pérez de León, A.A., Dowd, S.E., Guerrero, F.D., Bendele, K.G. and Scoles, G.A., 2011. Assessment of bacterial diversity in the cattle tick *Rhipicephalus (Boophilus) microplus* through tag-encoded pyrosequencing. *BMC Microbiology*, 11, 1–11.
4. Bastian, M., Heymann, S. and Jacomy, M., 2009, March. Gephi: an open source software for exploring and manipulating networks. In *Proceedings of the international AAAI conference on web and social media* (Vol. 3, No. 1, pp. 361-362).
5. Bokulich, N.A., Kaehler, B.D., Rideout, J.R., Dillon, M., Bolyen, E., Knight, R., Huttley, G.A. and Gregory Caporaso, J., 2018. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. *Microbiome*, 6(1), p.90.
6. Bonnet, S.I. and Pollet, T., 2021. Update on the intricate tango between tick microbiomes and tick-borne pathogens. *Parasite Immunology*, 43(5), e12813.
7. Bonnet, S.I., Binetruy, F., Hernández-Jarguín, A.M. and Duron, O., 2017. The tick microbiome: why non-pathogenic microorganisms matter in tick biology and pathogen transmission. *Frontiers in Cellular and Infection Microbiology*, 7, 236.
8. Booysen, S., Horak, I.G., Heyne, H., Smit, W.J. and Halajian, A., 2017. Parasites of domestic and wild animals in South Africa. L. Ixodid ticks infesting horses and donkeys. *Onderstepoort Journal of Veterinary Research*, 84(1), pp.1-6.
9. Bournez, L., Cangi, N., Lancelot, R., Pleydell, D.R.J., Stachurski, F., Bouyer, J., Martinez, D., Lefrancois, T., Neves, L. and Pradel, J., 2015. Parapatric distribution and sexual competition between two tick species, *Amblyomma variegatum* and *A. hebraeum* (Acari, Ixodidae), in Mozambique. *Parasites & Vectors*, 8, 1–14.
10. Bray, J.R. and Curtis, J.T., 1957. An ordination of the upland forest communities of southern Wisconsin. *Ecological monographs*, 27(4), pp.326-349.

11. Bruley, M., Pasternicki, C., Fattar, N., Amoros, J., Duhayon, M., McCoy, K. and Duron, O., 2025. Culturable bacteria and fungi in *Ixodes*, *Dermacentor*, *Amblyomma* and *Ornithodoros* ticks. *Parasite*, 32, 20.
12. Chaisi, M.E., Baxter, J.R., Hove, P., Choopa, C.N., Oosthuizen, M.C., Brayton, K.A., Khumalo, Z.T., Mutshembele, A.M., Mtshali, M.S. and Collins, N.E., 2017. Comparison of three nucleic acid-based tests for detecting *Anaplasma marginale* and *Anaplasma centrale* in cattle. *Onderstepoort Journal of Veterinary Research*, 84(1), 1–9.
13. Chicana, B., Couper, L.I., Kwan, J.Y., Tahiraj, E. and Swei, A., 2019. Comparative microbiome profiles of sympatric tick species from the far-western United States. *Insects*, 10(10), 353.
14. Clayton, K.A., Gall, C.A., Mason, K.L., Scoles, G.A. and Brayton, K.A., 2015. The characterization and manipulation of the bacterial microbiome of the Rocky Mountain wood tick, *Dermacentor andersoni*. *Parasites & Vectors*, 8, 1–5.
15. Couper, L.I., Kwan, J.Y., Ma, J. and Swei, A., 2019. Drivers and patterns of microbial community assembly in a Lyme disease vector. *Ecology and Evolution*, 9(13), 7768–7779.
16. Csardi, G. and Nepusz, T., 2006. The igraph software. *Complex syst*, 1695, pp.1-9.
17. Du, L.F., Zhang, M.Z., Yuan, T.T., Ni, X.B., Wei, W., Cui, X.M., Wang, N., Xiong, T., Zhang, J., Pan, Y.S. and Zhu, D.Y., 2023. New insights into the impact of microbiome on horizontal and vertical transmission of a tick-borne pathogen. *Microbiome*, 11(1), 50.
18. Eneku, W., Erima, B., Byaruhanga, A.M., Atim, G., Tugume, T., Ukuli, Q.A., Kibuuka, H., Mworosi, E., Douglas, C., Koehler, J.W. and Cleary, N.G., 2023. Wide distribution of Mediterranean and African spotted fever agents and the first identification of Israeli spotted fever agent in ticks in Uganda. *Public Library of Science Neglected Tropical Diseases*, 17(7), p.e0011273
19. Estrada-Peña, A., Cabezas-Cruz, A. and Obregón, D., 2020. Behind taxonomic variability: the functional redundancy in the tick microbiome. *Microorganisms*, 8(11), 1829.
20. Estrada-Peña, A., Cabezas-Cruz, A., Pollet, T., Vayssier-Taussat, M. and Cosson, J.F., 2018. High throughput sequencing and network analysis disentangle the microbial communities of ticks and hosts within and between ecosystems. *Frontiers in Cellular and Infection Microbiology*, 8, 236.

21. Etiang, P., Musoba, A., Nalumenya, D., Ndekezi, C., Bbira, J., Ochwo, S., Tweyongyere, R. and Muhanguzi, D., 2023. Distribution and prevalence of ixodid tick species (Acari: Ixodidae) infesting cattle from North-eastern Uganda [Karamoja]; 2019–2022. *Parasites & Vectors*, 16(1), p.171.
22. Gall, C.A., Reif, K.E., Scoles, G.A., Mason, K.L., Mousel, M., Noh, S.M. and Brayton, K.A., 2016. The bacterial microbiome of *Dermacentor andersoni* ticks influences pathogen susceptibility. *The International Society for Microbial Ecology (ISME) Journal*, 10(8), pp.1846–1855.
23. Ghafar, A., Khan, A., Cabezas-Cruz, A., Gauci, C.G., Niaz, S., Ayaz, S., Mateos-Hernández, L., Galon, C., Nasreen, N., Moutailler, S. and Gasser, R.B., 2020. An assessment of the molecular diversity of ticks and tick-borne microorganisms of small ruminants in Pakistan. *Microorganisms*, 8(9), 1428.
24. Hoffmann, A., Müller, T., Fingerle, V. and Noll, M., 2021. Presence of human pathogens of the *Borrelia burgdorferi* sensu lato complex shifts the sequence read abundances of tick microbiomes in two German locations. *Microorganisms*, 9(9), 1814.
25. Hussain, S., Perveen, N., Hussain, A., Song, B., Aziz, M.U., Zeb, J., Li, J., George, D., Cabezas-Cruz, A. and Sparagano, O., 2022. The symbiotic continuum within ticks: Opportunities for disease control. *Frontiers in Microbiology*, 13, 854803.
26. Jongejan, F., Berger, L., Busser, S., Deetman, I., Jochems, M., Leenders, T., de Sitter, B., van Der Steen, F., Wentzel, J. and Stoltsz, H., 2020. *Amblyomma hebraeum* is the predominant tick species on goats in the Mnisi Community Area of Mpumalanga Province South Africa and is co-infected with *Ehrlichia ruminantium* and *Rickettsia africae*. *Parasites & Vectors*, 13, 1–12.
27. Khleborodova, A., Gamboa-Tuz, S.D., Ramos, M., Segata, N., Waldron, L. and Oh, S., 2024. lefser: implementation of metagenomic biomarker discovery tool, LEfSe, in R. *Bioinformatics*, 40(12), p.btae707.
28. Kisten, D., Brinkerhoff, J., Tshilwane, S.I. and Mukaratirwa, S., 2021. A pilot study on the microbiome of *Amblyomma hebraeum* tick stages infected and non-infected with *Rickettsia africae*. *Pathogens*, 10(8), 941.
29. Ledger, K.J., Beati, L. and Wisely, S.M., 2021. Survey of ticks and tick-borne rickettsial and protozoan pathogens in Eswatini. *Pathogens*, 10(8), 1043.

30. Liu, Z.L., Qiu, Q.G., Cheng, T.Y., Liu, G.H., Liu, L. and Duan, D.Y., 2024. Composition of the Midgut Microbiota Structure of *Haemaphysalis longicornis* Tick Parasitizing Tiger and Deer. *Animals*, 14(11), 1557.
31. Loftis, A.D., Reeves, W.K., Szumlas, D.E., Abbassy, M.M., Helmy, I.M., Moriarity, J.R. and Dasch, G.A., 2006. Rickettsial agents in Egyptian ticks collected from domestic animals. *Experimental & applied acarology*, 40(1), pp.67-81.
32. Louca, S., Parfrey, L.W. and Doebeli, M., 2016. Decoupling function and taxonomy in the global ocean microbiome. *Science*, 353(6305), pp.1272-1277.
33. Magaia, V., Taviani, E., Cang, N. and Neves, L., 2020. Molecular detection of *Rickettsia africae* in *Amblyomma* ticks collected in cattle from Southern and Central Mozambique. *The Journal of Infection in Developing Countries*, 14(06), 614–622.
34. Mahlobo-Shwabede, S.I., Zishiri, O.T., Thekiso, O.M. and Makalo, M.J., 2021. Molecular detection of *Coxiella burnetii*, *Rickettsia africae* and *Anaplasma* species in ticks from domestic animals in Lesotho. *Pathogens*, 10(9), 1186.
35. Maitre, A., Wu-Chuang, A., Mateos-Hernández, L., Foucault-Simonin, A., Moutailler, S., Paoli, J.C., Falchi, A., Díaz-Sánchez, A.A., Banović, P., Obregón, D. and Cabezas-Cruz, A., 2022. *Rickettsia helvetica* infection is associated with microbiome modulation in *Ixodes ricinus* collected from humans in Serbia. *Scientific Reports*, 12(1), 11464.
36. Makgabo, S.M., Brayton, K.A., Oosthuizen, M.C. and Collins, N.E., 2023. Unravelling the diversity of *Anaplasma* species circulating in selected African wildlife hosts by targeted 16S microbiome analysis. *Current Research in Microbial Sciences*, 5, 100198.
37. Makwarela, T.G., Djikeng, A., Masebe, T.M., Nkululeko, N., Nesengani, L.T. and Mapholi, N.O., 2024. Vector abundance and associated abiotic factors that influence the distribution of ticks in six provinces of South Africa. *Veterinary World*, 17(8), 1765.
38. Mapholi, N.O., Banga, C., Dzama, K., Matika, O., Riggio, V., Nyangiwe, N. and Maiwashe, A., 2022. Prevalence and tick loads in Nguni cattle reared in different environmental conditions across four provinces of South Africa. *Veterinary World*, 15(8), 1943.
39. Mateos-Hernández, L., Obregón, D., Maye, J., Borneres, J., Versille, N., de La Fuente, J., Estrada-Peña, A., Hodžić, A., Šimo, L. and Cabezas-Cruz, A., 2020. Anti-tick microbiota vaccine impacts *Ixodes ricinus* performance during feeding. *Vaccines*, 8(4), 702.
40. Matsimbe, A.M., Magaia, V., Sanches, G.S., Neves, L., Noormahomed, E., Antunes, S. and Domingos, A., 2017. Molecular detection of pathogens in ticks infesting cattle in Nampula province, Mozambique. *Experimental and Applied Acarology*, 73, 91–102.

41. McMurdie, P.J. and Holmes, S., 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *Public Library of Science ONE*, 8(4), e61217.
42. Mnisi, S.S., Mphuthi, M.B., Ramatla, T., Mofokeng, L.S., Thekiso, O. and Syakalima, M., 2022. Molecular detection and genetic characterization of *Ehrlichia ruminantium* harbored by *Amblyomma hebraeum* ticks of domestic ruminants in North West Province, South Africa. *Animals*, 12(19), 2511.
43. Mtshali, K., 2012. *Molecular detection of zoonotic tick-borne pathogens in livestock in different provinces of South Africa*.
44. Munjita, S.M., Mubemba, B., Tembo, J., Bates, M. and Munsaka, S., 2024. *Rhipicephalus simus* ticks: new hosts for phleboviruses. *Parasitology*, 151(9), 962–970.
45. Mutai, B., Njaanake, K., Gathii, K., Estambale, B.B. and Waitumbi, J.N., 2022. Bacteriome in ticks collected from domestic livestock in Kenya. *Advances in Microbiology*, 12(2), 67–82.
46. Namina, A., Kazarina, A., Lazovska, M., Akopjana, S., Ulanova, V., Kivrane, A., Freimane, L., Sadovska, D., Kimsis, J., Bormane, A. and Capligina, V., 2023. Comparative microbiome analysis of three epidemiologically important tick species in Latvia. *Microorganisms*, 11(8), 1970.
47. Narasimhan, S. and Fikrig, E., 2015. Tick microbiome: the force within. *Trends in Parasitology*, 31(7), 315–323.
48. Obregón, D., Bard, E., Abrial, D., Estrada-Peña, A. and Cabezas-Cruz, A., 2019. Sex-specific linkages between taxonomic and functional profiles of tick gut microbiomes. *Frontiers in Cellular and Infection Microbiology*, 9, 298.
49. Peña, A.E., Cabezas-Cruz, A. and Obregón, D., 2019. Resistance of tick microbiome to biological disturbance.
50. Perveen, N., Muzaffar, S.B., Vijayan, R. and Al-Deeb, M.A., 2020. Microbial communities associated with the camel tick, *Hyalomma dromedarii*: 16S rRNA gene-based analysis. *Scientific Reports*, 10(1), 17035.
51. Peschel, S., Müller, C.L., Von Mutius, E., Boulesteix, A.L. and Depner, M., 2021. NetCoMi: network construction and comparison for microbiome data in R. *Briefings in bioinformatics*, 22(4), p.bbba290.

52. Qiu, Y., Nakao, R., Ohnuma, A., Kawamori, F. and Sugimoto, C., 2014. Microbial population analysis of the salivary glands of ticks; a possible strategy for the surveillance of bacterial pathogens. *PloS one*, 9(8), p.e103961.
53. Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J. and Glöckner, F.O., 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic acids research*, 41(D1), pp.D590-D596.
54. R Core Team, 2016. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. <http://www.R-project.org/>.
55. Ross, B.D., Hayes, B., Radey, M.C., Lee, X., Josek, T., Bjork, J., Neitzel, D., Paskewitz, S., Chou, S. and Mougous, J.D., 2018. *Ixodes scapularis* does not harbor a stable midgut microbiome. *The International Society for Microbial Ecology (ISME) Journal*, 12(11), pp.2596–2607.
56. Rynkiewicz, E.C., Hemmerich, C., Rusch, D.B., Fuqua, C. and Clay, K., 2015. Concordance of bacterial communities of two tick species and blood of their shared rodent host. *Molecular Ecology*, 24(10), 2566–2579.
57. Shehla, S., Almutairi, M.M., Alouffi, A., Tanaka, T., Chang, S.C., Chen, C.C. and Ali, A., 2023. Molecular survey of *Rickettsia raoultii* in ticks infesting livestock from Pakistan with notes on pathogen distribution in palearctic and oriental regions. *Veterinary Sciences*, 10(11), 636.
58. Spearman, C., 1987. The proof and measurement of association between two things. *The American journal of psychology*, 100(3/4), pp.441-471.
59. Spickett, A.M., Heyne, I.H. and Williams, R., 2011. Survey of the livestock ticks of the North West province, South Africa. *Onderstepoort Journal of Veterinary Research*, 78(1), 1–12.
60. Sungirai, M., Abatih, E.N., Moyo, D.Z., Clercq, P.D. and Madder, M., 2017. Shifts in the distribution of ixodid ticks parasitizing cattle in Zimbabwe. *Medical and Veterinary Entomology*, 31(1), 78–87.
61. Thekiso, O., Ramatla, T., Ringo, A., Mnisi, S., Mphuthi, N., Mofokeng, L., Lekota, K. and Xuan, X., 2023. Molecular detection of *Rickettsia africae* from *Amblyomma hebraeum* ticks in Mafikeng city of North West Province, South Africa. *Research in Veterinary Science*, 164, 105027.
62. Van Treuren, W., Ponnusamy, L., Brinkerhoff, R.J., Gonzalez, A., Parobek, C.M., Juliano, J.J., Andreadis, T.G., Falco, R.C., Ziegler, L.B., Hathaway, N. and Keeler, C., 2015.

- Variation in the microbiota of Ixodes ticks with regard to geography, species, and sex. *Applied and environmental microbiology*, 81(18), pp.6200-6209.
63. Varela-Stokes, A.S., Park, S.H., Stokes, J.V., Gavron, N.A., Lee, S.I., Moraru, G.M. and Ricke, S.C., 2018. Tick microbial communities within enriched extracts of *Amblyomma maculatum*. *Ticks and Tick-borne Diseases*, 9(4), 798–805.
64. Wickham, H., 2016. Data analysis. In *ggplot2: elegant graphics for data analysis* (pp. 189-201). Cham: Springer international publishing.
65. Zolnik, C.P., Prill, R.J., Falco, R.C., Daniels, T.J. and Kolokotronis, S.O., 2016. Microbiome changes through the ontogeny of a tick pathogen vector. *Molecular Ecology*, 25(19), 4963–4977.

7. CHAPTER 7

Conclusion and recommendations

7.1 Tick identification using morphological and molecular techniques

This comprehensive study primarily focuses on the presence, distribution and identification of tick species in South African livestock systems, using combined morphological and molecular approaches. The successful application of 16S rRNA and 18S rRNA gene sequencing not only confirmed the taxonomic identity of *Amblyomma hebraeum* and *Rhipicephalus evertsi evertsi* with high precision (99.23-100% sequence homology) but also established valuable genetic reference data through GenBank deposition (accession numbers PP80989-PP830102). These molecular markers proved particularly effective in resolving phylogenetic relationships, demonstrating apparent monophyletic clustering that supports their continued use in tick identification and evolutionary studies.

7.2. Tick bacterial communities in *Amblyomma hebraeum*

This study unveils critical host-driven shifts in the bacterial diversity of *A. hebraeum* ticks parasitising sheep and goats in Mahikeng, South Africa, offering fresh insights into the complex microbial ecology of ticks in small-stock systems. Ticks from sheep harboured significantly more diverse and evenly balanced bacterial communities than goats, as confirmed by the Shannon ($p = 0.0138$) and Simpson ($p = 0.0233$) diversity indices. Yet, total species richness remained comparable across hosts. This suggests that while host factors strongly shape the tick microbial structure. No significant differences in overall bacterial alpha diversity were found between the ticks from the two host species. Notable compositional differences emerged, highlighting the influence of host species and tick sex on microbial structure. Beta diversity patterns showed that the tick bacterial communities clustered distinctly by host species. Sheep-associated ticks had a uniform bacterial community, while goat-associated ticks displayed higher variability, pointing to host biology and possibly environmental exposure as key microbial influencers. At the phylum level, *Proteobacteria* led the bacterial landscape across both hosts, an expected but critical finding, reinforcing its central role in tick biology. However, at finer taxonomic scales, goat-derived ticks were overwhelmingly dominated by *Rickettsiaceae* and *Rickettsia* spp. (over 50% abundance), raising red flags for potential rickettsial disease transmission to both livestock and humans.

Ticks from both hosts carry an arsenal of pathogens: *Coxiella*, *Bacillus*, *Staphylococcus*, *Corynebacterium*, *Ehrlichia*, *Acinetobacter*, and *Streptococcus*, many of which are implicated in reproductive losses, mastitis, zoonotic infections, and emerging antimicrobial resistance. Their detection in *A. hebraeum* demands urgent attention from veterinary clinicians and public health stakeholders. A resilient core microbiome of 200 genera was detected across all locations, suggesting ecological stability. Distinct location-specific taxa, particularly the rich microbial diversity in Rooigrond, highlight how environmental factors, habitat, and host diversity shape tick microbiota and, consequently, vector competence.

7.3. Tick bacterial communities in *Rhipicephalus evertsi evertsi*

This study provides novel insights into the gut bacterial communities of *R. e. evertsi* ticks parasitizing sheep and goats in Matlwang village, South Africa, and highlights the significant influence of host species on tick microbial composition. The discovery of a host-dependent divergence in microbial communities, where Actinobacteria dominate in tick populations associated with goats, while Proteobacteria prevail in those linked to sheep, underscores the increasing evidence that the tick microbiome is not static. Instead, it is dynamic and responsive to ecological and host-related factors. The prevalence of *Proteobacteria*, known to include significant veterinary and zoonotic pathogens,

aligns with previous research and highlights the essential role of this phylum in tick physiology and pathogen dynamics. Conversely, the surprising dominance of *Actinobacteriota* in goat ticks introduces a new perspective on the microbial ecology associated with tick-borne diseases in small ruminants. This finding invites further functional studies to explore the protective or metabolic roles that this phylum may play in the tick gut. At the genus level, the consistent identification of *Corynebacterium* and *Staphylococcus* in ticks from both host species suggests their potential roles as commensals or symbionts. These genera may enhance tick fitness and influence pathogen transmission, particularly in ecological changes or interactions between vectors and hosts. The observed variation across host species supports the hypothesis that host-specific factors, such as blood composition, immune responses, and skin microbiota, may shape the internal microbial ecosystem of ticks.

The detection of *Ehrlichia* spp., *Anaplasma* spp., *Pseudomonas* spp., and *Bacillus* spp. within the tick microbiome raises important concerns regarding the potential transmission of tick-borne pathogens in livestock populations and possibly humans. These findings have significant implications for veterinary and public health, particularly in under-surveyed regions like Matlwang, where tick surveillance is limited. Notably, the coexistence of both commensal and pathogenic bacteria suggests that complex microbial interactions may impact the vector competence of *R. e. evertsi*.

Although alpha diversity comparisons did not yield statistically significant differences, trends observed in the diversity indices indicate higher richness and diversity in ticks from goats, especially in females. These non-significant but biologically relevant trends hint at potential gender- and host-associated microbiome shifts, warranting larger-scale studies to validate these observations. This study contributes to the underexplored field of livestock-associated tick microbiomes in South Africa, offering baseline data and revealing ecological patterns that merit further investigation. The findings underscore the need for longitudinal studies that integrate environmental, host, and vector data to disentangle the drivers of tick microbiome diversity and their implications for tick-borne disease emergence and control strategies.

7.4. Key differences in bacterial communities of *Amblyomma hebraeum* and *Rhipicephalus evertsi evertsi*

Proteobacteria dominated both tick species, but *A. hebraeum* from sheep showed a significant bacterial community (82.8%), suggesting a more substantial metabolic reliance on this phylum, which is known to support tick survival, nutrient acquisition, and vector capacity. Moreover, genus-level differences were observed in *A. hebraeum*, dominated by a high abundance of *Rickettsia* (61.42% in

sheep-derived ticks) and *R. e. evertsi*, characterised by greater microbial diversity, including *Corynebacterium* and *Staphylococcus* species. This highlights *A. hebraeum* as a more competent vector of *Rickettsia africae*, the agent of African Tick Bite Fever, with significant zoonotic risk implications. These distinct microbial profiles, influenced by tick species and host animals, highlight the complex interplay between vectors, their microbiota, and vertebrate hosts. At substantial levels, the detection of pathogenic genera (*Ehrlichia*, *Anaplasma*, and *Coxiella*) highlights the ongoing threat these ticks pose to livestock health and potential zoonotic transmission. The *R. e. evertsi* ticks contained a more diverse range of bacterial taxa (8,425; 27.8%) compared to *A. hebraeum* (4,402; 9%), suggesting a wider range of bacterial community that may be influenced by factors such as host range, environmental exposure, or variability in blood meals. From an ecological perspective, identifying 1,534 shared bacterial taxa, representing 63.1% of the core microbiome, suggests conserved microbial functions across these two tick species. In contrast, the thousands of unique taxa indicate species-specific adaptations. Both species' microbiomes were enriched in taxa from Proteobacteria and Firmicutes, which exhibited high network connectivity, implying central roles in microbiome stability and pathogen interactions, and potentially influencing tick vector competence. Predicted functional pathways linked to energy metabolism and carbon and nitrogen biogeochemical cycling further emphasized these microbial communities' ecological adaptability and metabolic sophistication.

7.6. Conclusion

This study successfully combined morphological and molecular approaches to identify *Amblyomma hebraeum* and *Rhipicephalus evertsi evertsi* accurately. The study offers one of the most comprehensive characterizations of tick-associated bacterial communities in South African small-stock systems using next-generation sequencing. The study's findings show that host species significantly shape the tick microbiome, while sheep-associated ticks exhibit stable and less variable bacterial communities compared to goats. *Rhipicephalus evertsi evertsi* ticks demonstrated greater bacterial diversity, including more *Actinobacteria* in goat-derived samples, and harboured several potentially pathogenic genera like *Ehrlichia*, *Anaplasma*, *Acinetobacter* and *Pseudomonas* species. Shared and unique bacterial taxa between tick species underscore species-specific microbial adaptation, while a conserved core microbiome suggests ecological stability and essential microbial functions. The predicted metabolic pathways linked to carbon and nitrogen cycling suggest a functionally versatile microbiome, possibly contributing to tick fitness and pathogen carriage. This study confirms the identity of these two important tick species in South Africa, significantly showing how the bacterial communities shape these ticks, with important implications for tick-borne disease

management in South Africa. The demonstrated efficiency of using morphological and molecular tick identification techniques supports their incorporation into surveillance programs. In contrast, next-generation sequencing suggests potential targets for novel control strategies that could manipulate microbial communities to reduce vector competence. The study establishes a foundation for future research into tick-microbe interactions while providing actionable data for developing more effective, targeted approaches to mitigate these significant ectoparasites' economic and health impacts.

7.7. Recommendations

This study has significantly advanced our understanding of the bacterial communities and genetic diversity associated with *Amblyomma hebraeum* and *Rhipicephalus evertsi evertsi* ticks infesting small stock in South Africa's North West Province. We recommend several strategic directions for future research, disease management, and policy development to build on these findings. First, to overcome current limitations in bacterial identification, future studies should employ long-read sequencing technologies such as Oxford Nanopore or the PacBio system, targeting the full-length *16S rRNA* gene for more accurate species-level resolution. Additionally, integrating metagenomics and metatranscriptomics will enhance our understanding of microbial functions, interactions, and ecological roles within the tick microbiome. Expanding the scope of research to include seasonal and regional sampling, along with longitudinal study designs, will provide deeper insights into temporal and spatial shifts in microbial diversity. Investigating the vertical transmission of bacterial taxa from parent ticks to offspring is also crucial, as it may reveal how microbial communities are maintained across generations.

From a disease management and policy perspective, host-specific factors such as immune responses or skin-associated microbiota should be explored to understand their influence on tick-associated bacterial communities, particularly in ticks derived from sheep versus goats. Developing host-specific tick control strategies, including selective acaricide use and microbiome-informed vaccine development, is essential from a disease management perspective. These efforts should be supported by integrated tick management programs that combine environmental, biological, chemical, and host-targeted approaches. Establishing a regional tick surveillance system using traditional and molecular tools will be vital for early detection of emerging threats. Equally important is community education and improved access to veterinary services, especially in rural areas, to empower livestock keepers in managing tick infestations effectively. Policy frameworks should promote cross-sector collaboration, support applied research through systems thinking, and address the implications of climate change, which may alter tick distribution and disease dynamics. Adopting a One Health

approach that integrates human, animal, and environmental health is critical for sustainable and comprehensive control of tick-borne diseases in the North West Province and the broader Southern African region.

Supplementary Material

Supplementary figures

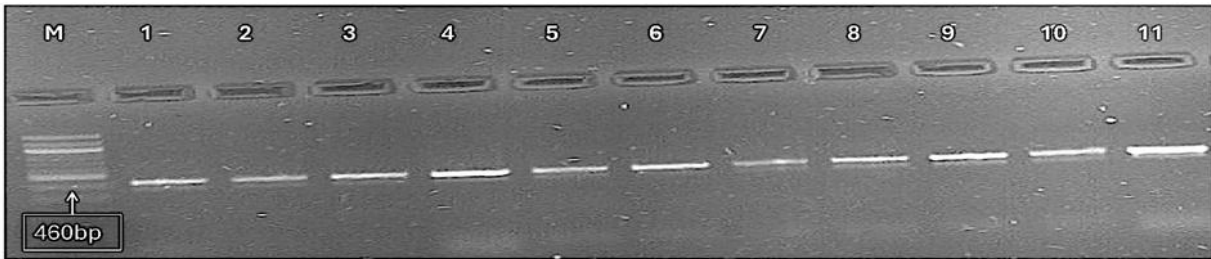


Figure S1: Amplified *16S rRNA* gene DNA of ticks collected from sheep and goats. M indicates DNA ladder, and 1- 11 indicates the amplified gene of the ticks.

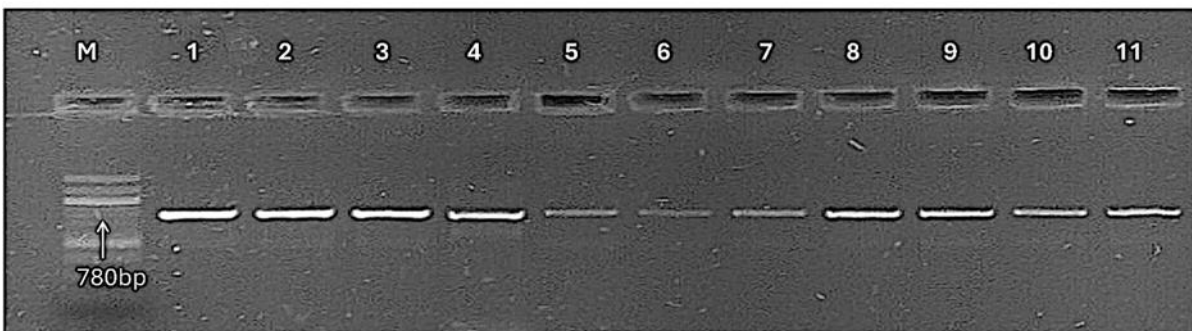


Figure S2: Amplified *18S rRNA* gene DNA of ticks collected from sheep and goats. M indicates DNA ladder, and 1- 11 indicates the amplified gene of the ticks.

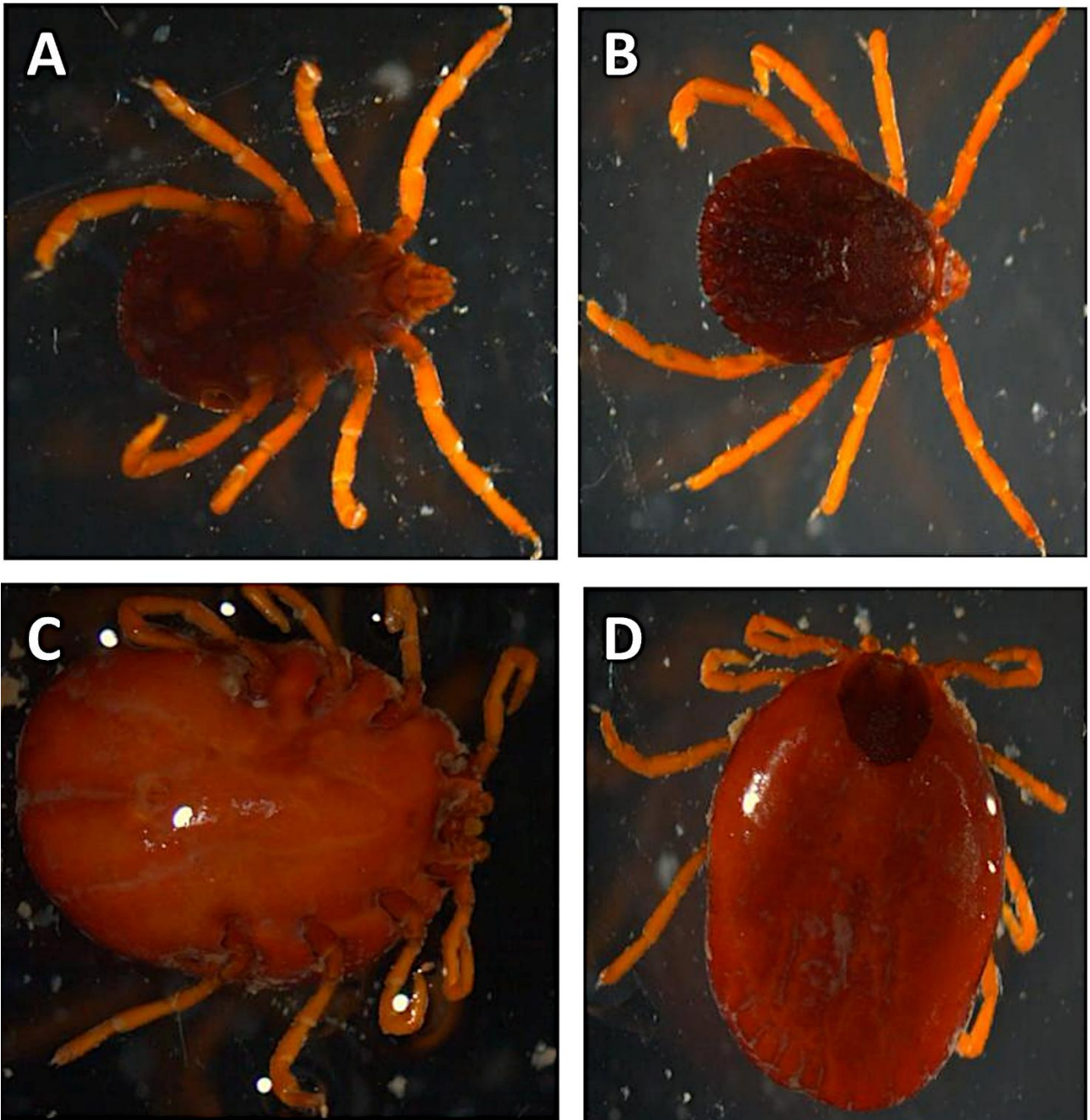


Figure S3: Morphological identification of the tick species *Rhipicephalus evertsi evertsi* collected from sheep ticks in Matlwang. Male ventral view (A), Male dorsal view (B). Female ventral view (C), Female dorsal view (D).

Supplementary Table

Table S1: Detection of *Anaplasma/Ehrlichia* species in *Rhipicephalus evertsi evertsi* and *Amblyomma hebraeum* from sheep and goats using qPCR.

Sample Number	Original sample name	Host	Tick gender	Tick species	Location	<i>Anaplasma/Ehrlichia</i> -group specific qPCR
1	DHG11	Goat	Female	<i>Rhipicephalus evertsi evertsi</i>	Matlwang village	28,42
2	MO2	Sheep	Unknown (pooled samples)	<i>Amblyomma hebraeum</i>	Mafikeng local Municipality	29,52
3	1G	Goat	Male	<i>Rhipicephalus evertsi evertsi</i>	Matlwang village	28,76
4	2G	Goat	Male	<i>Rhipicephalus evertsi evertsi</i>	Matlwang village	28,65
5	3G	Goat	Male	<i>Rhipicephalus evertsi evertsi</i>	Matlwang village	30,21
6	4G	Goat	Female	<i>Rhipicephalus evertsi evertsi</i>	Matlwang village	24,79
7	5G	Goat	Female	<i>Rhipicephalus evertsi evertsi</i>	Matlwang village	29,21
8	6G	Goat	Male	<i>Rhipicephalus evertsi evertsi</i>	Matlwang village	26,72
9	7G	Goat	Male	<i>Rhipicephalus evertsi evertsi</i>	Matlwang village	26,27
10	8G	Goat	Male	<i>Rhipicephalus evertsi evertsi</i>	Matlwang village	19,84
11	10G	Goat	Female	<i>Rhipicephalus evertsi evertsi</i>	Matlwang village	28,16
12	12G	Goat	Female	<i>Rhipicephalus evertsi evertsi</i>	Matlwang village	32,12
13	CDG5	Goat	Female	<i>Rhipicephalus evertsi evertsi</i>	Matlwang village	17,77
14	DHG8	Goat	Female	<i>Rhipicephalus evertsi evertsi</i>	Matlwang village	23,59
15	1S	Sheep	Female	<i>Rhipicephalus evertsi evertsi</i>	Matlwang village	26,77
16	2S	Sheep	Female	<i>Rhipicephalus evertsi evertsi</i>	Matlwang village	26,56
17	3S	Sheep	Female	<i>Rhipicephalus evertsi evertsi</i>	Matlwang village	26,26

18	4S	Sheep	Female	<i>Rhipicephalus evertsi evertsi</i>	Matlwang village	30,48
19	5S	Sheep	Male	<i>Rhipicephalus evertsi evertsi</i>	Matlwang village	24,79
20	6S	Sheep	Male	<i>Rhipicephalus evertsi evertsi</i>	Matlwang village	30,69
21	7S	Sheep	Female	<i>Rhipicephalus evertsi evertsi</i>	Matlwang village	31,36
22	8S	Sheep	Male	<i>Rhipicephalus evertsi evertsi</i>	Matlwang village	30,65
23	9S	Sheep	Female	<i>Rhipicephalus evertsi evertsi</i>	Matlwang village	20,64
24	10S	Sheep	Female	<i>Rhipicephalus evertsi evertsi</i>	Matlwang village	30,88
25	RC3	Goat	Unknown (pooled samples)	<i>Amblyomma hebraeum</i>	Mafikeng local Municipality	30,74
26	RMC4	Goat	Unknown (pooled samples)	<i>Amblyomma hebraeum</i>	Mafikeng local Municipality	31,06
27	MC3	Goat	Unknown (pooled samples)	<i>Amblyomma hebraeum</i>	Mafikeng local Municipality	26,75
28	MC4	Goat	Unknown (pooled samples)	<i>Amblyomma hebraeum</i>	Mafikeng local Municipality	29,26
29	RMC4	Goat	Unknown (pooled samples)	<i>Amblyomma hebraeum</i>	Mafikeng local Municipality	26,99
30	MC5	Goat	Unknown (pooled samples)	<i>Amblyomma hebraeum</i>	Mafikeng local Municipality	23,70
31	RC5	Goat	Unknown (pooled samples)	<i>Amblyomma hebraeum</i>	Mafikeng local Municipality	23,70
32	MC2	Goat	Unknown (pooled samples)	<i>Amblyomma hebraeum</i>	Mafikeng local Municipality	25,95
33	RMC1	Goat	Unknown (pooled samples)	<i>Amblyomma hebraeum</i>	Mafikeng local Municipality	21,91
34	RO3	Sheep	Unknown (pooled samples)	<i>Amblyomma hebraeum</i>	Mafikeng local Municipality	31,30
35	RMO4	Sheep	Unknown (pooled samples)	<i>Amblyomma hebraeum</i>	Mafikeng local Municipality	30,16