INVESTIGATION OF TICK-BORNE PATHOGENS RESISTANCE MARKERS USING NEXT GENERATION SEQUENCING

**RESEARCH DISSERTATION**

**for**

**MSc degree in Life Sciences**

**Submitted to the**

**DEPARTMENT OF LIFE AND CONSUMER SCIENCES**

**UNIVERSITY OF SOUTH AFRICA**

**by**

**AUBREY D CHIGWADA**

**Student No: 61366943**

**SUPERVISOR: DR TM MASEBE**

**CO-SUPERVISOR: DR NO MAPHOLI**

**JULY 2021**

DECLARATION

Name: Aubrey Dickson Chigwada

Student number: 61366943

Degree: Master of Science in Life Sciences

Dissertation title:

INVESTIGATION OF TICK-BORNE PATHOGENS RESISTANCE MARKERS USING NEXT GENERATION SEQUENCING

I, Aubrey D Chigwada, declare that investigation of tick-borne pathogens resistance markers using next-generation sequencing is my work, and sources I have used have been acknowledged by complete references. I submit this thesis for a Master of Science in life science at the college of agriculture and environmental science at the University of South Africa and have not been submitted to any other university.

JULY 2021

---------- ----------

Aubrey Dickson Chigwada Date

ACKNOWLEDGEMENTS

Firstly, I would like to thank God almighty for the strength to carry on and a gift of life.

Words cannot express my deepest gratitude to my supervisor Dr. TM Masebe for her professional guidance, encouragement, support, corrections, instructions, patience, and technical skills thought-out this work. I am forever indebted to your mentorship; you are a role model and an inspiration to me. I would like to extend my sincere appreciation to my Co-supervisor Dr. NO Mapholi for her insightful comments and suggestions. To the woman in research funds used for this project, I am grateful. Much gratitude to Dr. Ramganesh Selvarajan for his mentorship and training from the basics to the actual Miseq sequencing, which made it possible to sequence for this work. Would like to thank Dr. Ogola for taking his time to run data and teaching me how to analyze, use algorithms, and interpret the results. Your input shaped my laboratory techniques and data analysis skills. Thank you for the time and sacrifice you made, for this, I will forever be grateful. Additionally, I would like to thank our research team members 'TICK GROUP' Dr. Mbizeni Sikhumbuzo, Dr. Tambani Tshifhiwa. and Ms. Mohololа Nthabiseng for their helpful contributions. Thank you to Kgomotso Chokoe, Itumeleng Setshedi, and Sharon Lerooibaaki laboratory technicians for their warm accommodation and support in the laboratory. My gratitude also goes out to my family and friends for their encouragement and support all through my studies.

TABLE OF CONTENTS

# TABLE OF FIGURES

LIST OF TABLES

LIST OF ACRONYMS

| | | |
|---|---|---|
| PICRUSt | : | Phylogenetic investigation of communities by reconstruction of unobserved states |
| OTUs | : | Operational taxonomic units |
| NGS | : | Next-generation sequencing |
| KEGG | : | Kyoto Encyclopedia of Genes and Genomes |
| KO | : | KEGG orthologs |
| COG | : | Cluster of Orthologous Genes |
| PCR | : | Polymerase chain reaction |
| DNA | : | Deoxyribonucleic acid |
| rRNA | : | Ribosomal ribonucleic acid |
| ATP | : | Adenosine triphosphate |
| ABC | : | ATP binding cassettes |
| MFS | : | Major facilitator multiple drug efflux pumps |
| MATE | : | Multiple drugs and toxic efflux pumps |
| RND | : | Resistance nodulation |
| CCHFV | : | Crimean-Congo hemorrhagic fever virus |
| TBEV | : | Tick-borne encephalitis virus |
| DHFR/DHPS | : | Dihydrofolate reductase/Dihydropteroate synthase |
| FDR | : | Fault discovery rate |
| PCoA | : | Principal coordinate analysis |

# CHAPTER 1

## 1.1 GENERAL INTRODUCTION

Cattle in South Africa have a general population of about 14 million, indigenous Nguni cattle representing a sizable percentage, surveys have indicated Nguni cattle as well adapted to diseases and the environment, however they are partially resistant to tick infestation (Mapiye et al. 2009). Ticks are obligate blood-feeders that harbor pathogens infecting ruminants and humans worldwide (Narasimhan and Fikrig 2015; Sánchez-Montes et al. 2019; Moses et al. 2017). In arthropods, ticks of veterinary and medical importance, are the leading disease vectors after mosquitoes, with an impressive cause of an estimated annual global loss of US$22-30 billion (Lew-Tabor and Rodriguez Valle, 2016; Nyangiwe et al., 2018). These losses are due to infestations and tick-borne disease resulting in morbidity and mortality, abortions, low production of milk and low weight meat production in livestock, the continent with the highest losses recorded is African (Mapholi *et al.*, 2014; Nyangiwe *et al*., 2018). Several studies confirmed tick-microbiomes to be composed of pathogenic, endosymbiotic, and symbiotic organisms of communities of viruses, bacteria, and eukaryotes (Narasimhan and Fikrig, 2015; Eyer *et al.*, 2017; Keskin *et al.*, 2017; Greay *et al.*, 2018; Portillo *et al.*, 2019; Seo *et al.*, 2020). Worldwide numerous bacterial strains of tick-borne microorganisms that are different in genotype, antigenic characteristics and transmission mode have been identified (Theiler, 1909; Seshadri *et al.*, 2003; Chen *et al.*, 2014; Kumsa *et al.*, 2015; Quiroz-Castañeda *et al*., 2016; Galay *et al.*, 2018; Guo *et al.*, 2019).

In South Africa, ticks of veterinary and medical importance include species of *Ixodes, Hyalomma, Amblyomma,* and *Rhipicephalus* infesting cattle. These ticks transmit bacterial species (spp.) of *Anaplasma, Borrelia, Coxiella, Ehrlichia, Francisella,* and *Rickettsia* (Guo et al. 2019; Halajian et al. 2016; Iweriebor et al. 2017; Mtshali et al. 2017; Ringo et al. 2018a). Tick-borne bacteria

cause diseases such as the relapsing fever (*Borrelia* spp.*)* and spotted fever (*Rickettsia* spp.), ovine and bovine anaplasmosis (*Anaplasma* spp.), Query-fever (*Coxiella* spp.), ehrlichiosis (*Ehrlichia* spp.) (Allsopp, 2009; Yabsley *et al.*, 2012; Kuley, 2017; Halajian *et al.*, 2018). Tick-borne viruses of medical and veterinary importance in South Africa transmitted by *Rhipicephalus* tick species are in the family *Flaviviridae*. The commonly transmitted viruses include the Crimean-Congo hemorrhagic fever virus (CCHFV) and tick-borne encephalitis virus (TBEV) (Mohd Shukri et al. 2015; Palanisamy et al. 2018).

Control of ticks and tick-borne diseases is very important in animal health, meat production, and for enhancing food security (de Castro, 1997; Mapholi *et al.*, 2014; Kirkan *et al.*, 2017; Boulanger *et al.*, 2019). Antibiotics such as tetracyclines, beta-lactams, and macrolides are used by farmers and veterinarians for the treatment of tick-borne bacterial diseases, while antiviral agents like ribavirin and benzavir-2 are used in the treatment of viral fever infections caused by tick-borne viruses (Kersh, 2015; Aslam *et al.*, 2016). However, antimicrobial resistance is rising to dangerously high levels in all parts of the world with devastating effects. Antimicrobial resistance has become a serious threat worldwide medically and in veterinary medicine causing outbreaks unexpectedly (Karkman et al. 2016; Zuñiga-Navarrete et al. 2019). Tick-borne *Rickettsia* species are known to be resistant to the following drug groups rifampin, aminoglycosides, and beta-lactams, while *Ehrlichia* and *Anaplasma* species are also shown to be resistant to groups of macrolides, chloramphenicol, and quinolones (Rolain *et al.*, 1998; Maurin *et al.*, 2001; Branger *et al.*, 2004; Biswas *et al.*, 2008).

Mechanisms by which resistance can occur in bacteria involve firstly, the production of enzymes that deactivate and detoxify antimicrobial agents. Secondly, mutations of the sites in which antibiotics target, as well as post-transcriptional and translational modification of the antimicrobial

action site. Finally, through under-expression of porins, reducing drug permeability and overexpression of drug efflux pumps (Lingzhi et al. 2018; Martinez et al. 2014; Wright and Serpersu 2005). Antiviral drugs such as 6-azauridine, Ribavirin, 2'-C-methylcytidine, and interferon-alpha 2 have shown inhibition capabilities to tick-borne viruses (Te *et al*., 2007; Palanisamy *et al.*, 2018). However, antiviral drug resistance is triggered by random point mutations in the viral genome (Strasfeld and Chou 2010), tick-borne RNA viruses have been shown to have very high rates of mutations in comparison with DNA viruses hence their resistance profiles need to be constantly evaluated (Sanjuán and Domingo-Calap 2016).

Application of Next-generation sequencing (NGS) technology with speedy and high throughput techniques allows in-depth characterization of microbial communities. A bioinformatics tool phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) provides a cheaper accurate alternative to complex functional genome analyses. The PICRUSt algorithm was coded to predict the functional potential of the microbial community from 16S rRNA OTUs (Operational taxonomic units) data. The PICRUSt functional predictions can be used to predict resistance biomarkers with accuracies ranging between 85-90% (Gavin *et al*., 1991; de Scally *et al.*, 2016; Park *et al.*, 2020). The NGS technology accompanied by bioinformatic tools proves to be very powerful tools in the surveillance and screening of drug-resistant strains (Ramanathan et al. 2017).

The present dissertation aims to give new insights into the composition of bacterial communities in ticks and to characterize their functional antimicrobial resistance biomarkers by employing next-generation sequencing and the PICRUSt algorithm. Specifically, by looking into bacterial community composition and resistance biomarkers from *Amblyomma*, *Hyalomma*, and *Rhipicephalus* tick species collected from cattle in Roodeplaat ARC research farm.

## 1.2 RATIONALE OF THE STUDY

Ticks are multiple host vectors of communities of intracellular bacteria, viruses, and protozoans infecting cattle (Portillo et al. 2019; Greay et al. 2018; Onyiche et al. 2019; Bente et al. 2013). In-depth information about the genetic diversity of tick-borne pathogens is important for understanding disease occurrence, virulence, antibiotic resistance profiles, and tick control strategies (Quiroz-Castañeda *et al*., 2016). Branger and co-workers reviewed the resistance patterns of tick-borne bacterial pathogens to antimicrobial agents and concluded that resistance continued to evolve, and susceptibility has remained to fewer antibiotics (Branger *et al*., 2004). Continued active surveillance of the ticks, tick-borne pathogens, and communities of bacteria associated with ticks, is important to provide information significant in tick and tick-borne disease control (Crowder et al. 2011).

In South Africa, the economic losses in cattle production due to tick and tick-borne diseases are a result of reduced weight in cattle, fertility, damaged cattle skin, anemia, and produced milk, as well as in abortions and mortality. These direct and indirect losses amount to billions of Rands annually (Mapholi *et al.,* 2014; Nyangiwe *et al*., 2018). To overcome the losses, dairy and beef cattle farmers employ the dipping method to control ticks, nevertheless, chemical treatment is expensive, hence tick infestations remain problematic. Some farmers vaccinate their livestock as a defensive measure and infected livestock are administered with antibiotics. Groups of prophylactic antibiotics used are tetracyclines, beta-lactams, macrolides, aminoglycosides, rifampin, chloramphenicol, and quinolones (Aubry and Geale 2011; Khumalo 2018; Allsopp 2009; Lim et al. 2018). These antibiotics are added to animal feeds at a subtherapeutic level as growth promoters, however, the extensive use may lead to the proliferation of antimicrobial-resistant strains.

The mechanism by which tick-borne microorganisms develop resistance is highly diverse (Speer *et al.*, 1992), as such, it is important to constantly investigate and monitor, using modern sensitive technologies, to come up with well-informed approaches to counter their resistance. Studies looking at molecular characterization of tick-borne microorganisms are ongoing in South Africa (Mtshali *et al.*, 2017; Halajian *et al.*, 2018; Ringo *et al.*, 2018), however very few address their antimicrobial resistance profiles. Therefore, more studies looking at antimicrobial-resistant profiles, in bacteria transmitted by ticks using modern, and sensitive molecular techniques in South Africa are crucial. Thus, this study sought to identify microbial communities of bacteria in ticks and to investigate resistance markers using next-generation sequencing.

## 1.3 HYPOTHESIS

This study is based on the hypothesis that tick-borne pathogens are highly resistant to antimicrobial agents.

## 1.4 RESEARCH QUESTIONS

1. What is the composition of bacterial communities in ticks from cattle?
2. Which pathogenic, endosymbiotic, and symbiotic bacteria are present in ticks?
3. Which antimicrobial genes are present in tick-borne bacterial communities?

## 1.5 AIMS AND OBJECTIVE

The study aims to determine the genetic composition of tick-borne bacterial communities and investigating their resistance biomarkers using next-generation sequencing.

### 1.5.1 THE SPECIFIC OBJECTIVES:

- To identify and determine tick-borne bacterial communities associated with ticks parasitizing Nguni cattle breeds from Roodeplaat ARC-research farm.
- To apply 16S rRNA metagenomics approach to characterize bacterial communities associated with *Amblyomma* and *Hyalomma* tick species.
- To use 16S rRNA metagenomic sequencing to assess and characterize bacterial communities in *Rhipicephalus evertsi evertsi*, *Rhipicephalus simus*, and *Rhipicephalus decoloratus* tick species.
- To investigate functional antimicrobial resistance biomarkers using bioinformatics (tool: PICRUSt).

CHAPTER 2 LITERATURE REVIEW

Ticks are acarine, ubiquitous, obligate hematophagous arthropods with no segmented body but circular (Andreotti et al. 2011; Junquera 2018). They are a group of arthropod vectors that transmit pathogens from infected cattle to healthy ones (Boulanger et al. 2019; Espinaze et al. 2018). These ectoparasites can be divided into three families i.e., Ixodidae, Argasidae, and the Nuttalliellidae. Currently, there are about 692 species identified in Ixodidae ticks worldwide (Figure 2.1) they are alternatively referred to as the hard ticks because they possess a hard dorsal scutum (de Castro, 1997; Bell-Sakyi *et al.*, 2007; Marufu, 2008; Barker and Walker, 2014; Mastropaolo *et al.*, 2014; Kumsa *et al.*, 2015).

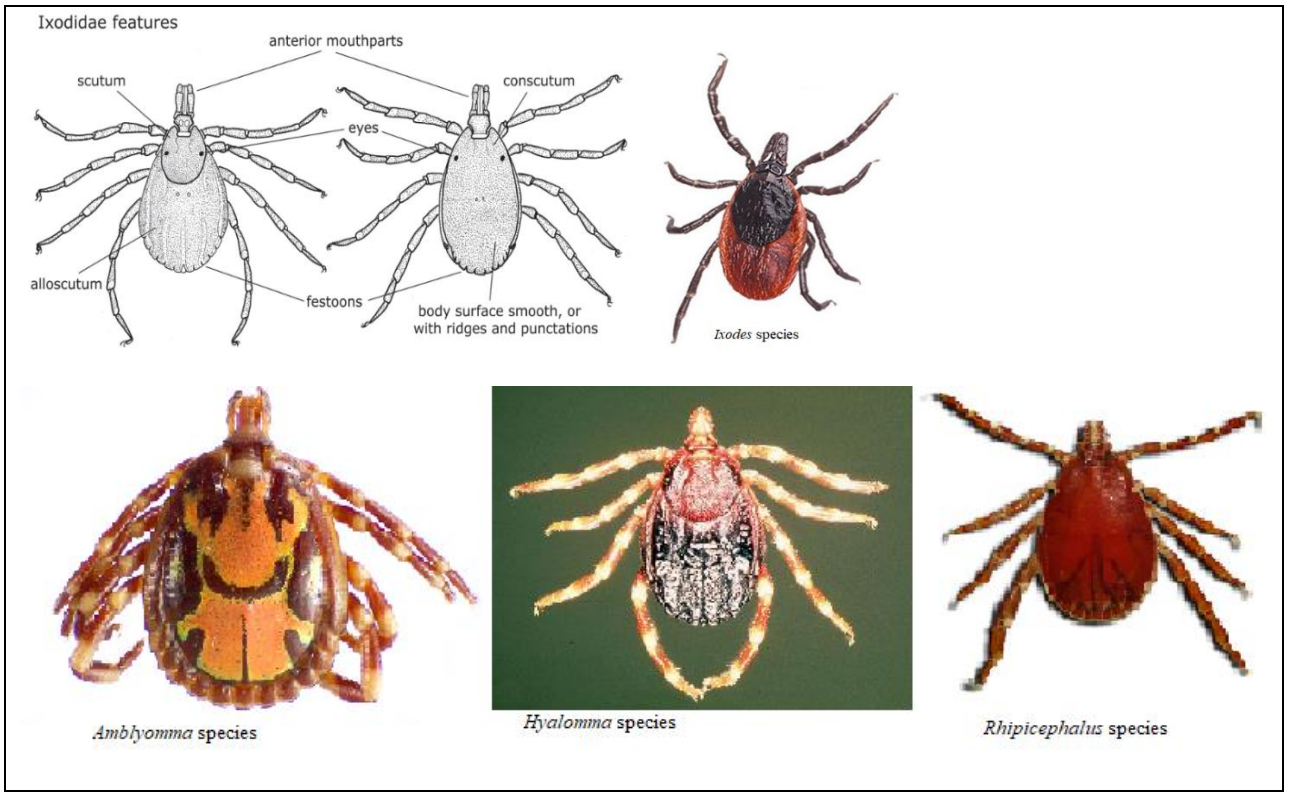



Figure 2.1 Features of ticks in the Ixodidae family (Barker and Walker 2014)

The Argasidae ticks (Figure 2.2a) are the soft ticks with 186 known species that lack a scutum and are also distributed worldwide. The Nuttalliellidae is the third tick family sharing several features

like the Ixodidae and the Argasidae. It is only found in Southern African countries with only one tick species the *Nuttalliella namaqua* (Figure 2.2b)(Latif et al. 2012).

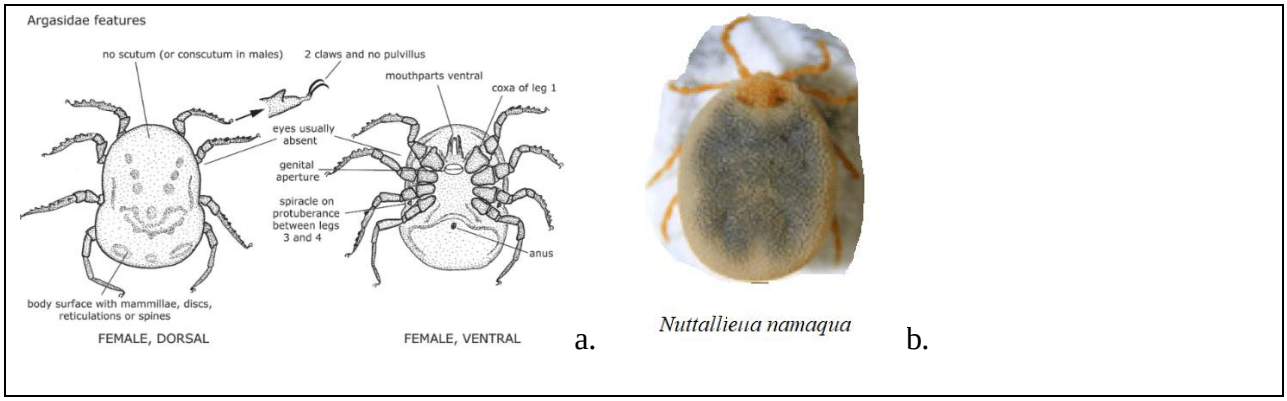



Figure 2.2 Argasidae tick body structure (a.) and Nuttallienna species (b.) (Barker and Walker 2014)

## 2.1 TICKS OF VETERINARY AND MEDICAL IMPORTANCE

The Ixodidae family is composed of about seven genera with about 692 known species. The genera of economic importance are *Amblyomma, Dermacentor, Haemaphysalis, Hyalomma, Rhipicephalus,* and *Ixodes* and within these genera, species of veterinary and medical importance are found (Boulanger et al. 2019).

### 2.1.1 *Amblyomma* species

*Amblyomma* genus comprises of 135 known species known worldwide, with *A. hebraeum* the most important veterinary species. *A. hebraeum* is identified (Figure 2.1) by long mouthparts and banded legs. In males, the conscutum is highly decorated (ornate) with discrete lateral patches of yellow color. *Amblyomma* species transmit the pathogens causing Ehrlichiosis and Rocky Mountain Spotted Fever. In South Africa, *A. hebraeum* has been identified carrying tick-borne pathogens, the *Rickettsia africae, Ehrlichia canis,* and *E. chaffeensis* (Iweriebor et al. 2017; Mtshali et al. 2017).

### 2.1.2 *Argasidae* species

The family *Argasidae* known as soft ticks' family is composed of 193 species (Figure 2.2). They are multiple host tick vectors of relapsing fevers caused by *Borrelia* species in humans. They also transmit Crimean-Congo hemorrhagic fever virus and are considered to be of medical importance (Aslam et al. 2016). *Argasidae* is identified by their lack of scutum and their large size (6-7mm) which makes them soft ticks. These species have short mouthparts located ventral to their body and they do not have pulvilli (Barker and Walker 2014).

### 2.1.3 *Hyalomma* species

*Hyalomma* genera have 25 known ticks species worldwide, these include ticks of veterinary importance, that is, *H. dromedarii, H. marginatum,* and *H. rufipes*. They are identified by their large body size (5- 6mm) with the integument texture that has striations and with mouthparts that are anterior to their body (Barker and Walker 2014). *H. rufipes* can transmit *Anaplasma marginale* to cattle, causing bovine anaplasmosis and gall sickness as reported by a molecular study by Spengler et al., (2018). Also, they showed that ticks of the genus *H. rufipes* can also spread *Crimean-Congo hemorrhagic fever* virus and *Rickettsia conori* to humans (Spengler and Estrada-Peña 2018).

### 2.1.4 *Rhipicephalus* species

*Rhipicephalus* genus is composed of 75 known species of ticks, most but not all are multiple host ticks (Figure 2.3) that infest mainly cattle and other large ruminants and mostly endemic to Africa. They are identified by small to medium size (2–5 mm) body structure with mouthparts short, anterior, and protruding from their body (Figure 2.1). They also have eyes and an anal groove that is posterior to the anus (Barker and Walker 2014). *Rhipicephalus* species include subgenus like *Boophilus* like *R. B. microplus, R. B. decoloratus.* Several researchers have reported *R. B.*

*decoloratus* transmitting *Anaplasma marginale* causing bovine anaplasmosis and *Borrelia theileri* causing spirochaetosis to cattle, horses, goats, and sheep (Andreotti et al. 2011; Davidson et al. 1999; Fyumagwa et al. 2011). Different pathogen species transmitted by these ticks have been identified (Andreotti et al. 2011; Dantas-Torres 2010; Latrofa et al. 2014; Junquera 2018). In a study by Latrofa et al., (2014) *Anaplasma platys* and *Ehrlichia canis* transmitted by *R. sanguineus sensulato* and *R. turanicus* were identified through next-generation sequencing of the 16S rRNA region (Latrofa et al. 2014). The *R. sanguineus* is a one-host tick that has dogs as preferential hosts. It can also feed on livestock and many other mammals and humans included. *R. bursa* is another two-host tick that is common in the Mediterranean Basin down to the Middle East (Junquera 2018). It thrives in mild climates, (neither too cold, nor too hot) and feeds on cattle, sheep, goats, and horses. *R. bursa* transmits bacterial pathogens such as various species of *Anaplasma*, *Ehrlichia ruminantium,* and the *Crimean-Congo hemorrhagic fever* virus to livestock (Junquera 2018).

2.1.5 *Ixodes* species

Ticks of *Ixodes* genus are of veterinary and medical importance with a wide range of distribution worldwide (Barker and Walker, 2014; Chen *et al.*, 2014; Mansfield *et al.*, 2017; Nyangiwe *et al.*, 2018; Boulanger *et al.*, 2019; Klitgaard *et al.*, 2019). They are identified by their small to medium body size (2–5 mm) (Figure 2.1), with their mouthparts seen anterior to the body. In females, mouthparts appear longer as compared to male mouthparts. Other features include the anal groove loops which are anterior to the anus, the eyes are absent; the legs are typically plain and dark but may vary; males have flat sclerotized plates ventrally that is not visible in females (Barker and Walker 2014). Ixodes are multiple host ticks during their life cycle (Figure 2.3), alternating between hosts in their life stages. All phases of growth of *Ixodes pilosus* infest livestock and small ruminants. *Ixodes rubicundus* is a strictly South African tick and has been identified in the Cape

regions (Iweriebor et al. 2017). In a study conducted in birds by Klitgaard and colleagues (2019) in Denmark, bacterial *Borrelia* species such as *B. spielmanii*, *B. valaisiana*, *B. garinii*, *B. burgdorferis*, *B. miyamotoi*, and *B. afzelii* were identified from *Ixodes* tick species, infecting birds (Klitgaard et al. 2019). *Rickettsia* species were also detected in the same study, confirming Ixodes ticks as multiple vectors of different pathogens to their hosts.

## 2.2 THE LIFE CYCLE OF TICKS

There are four developmental stages in the lifecycle of ticks, that is the egg, larva, nymph, and adult, in which ticks undergo metamorphosis. One host ticks feed on the same host and after each blood meal detach and molt to the next life stage but do not leave the host. While multiple host ticks fall off to the ground to molt transforming to the next life stage and thereafter attaching to a new host after metamorphosis (Figure. 2.3) (Barker and Walker 2014; Junquera 2018).

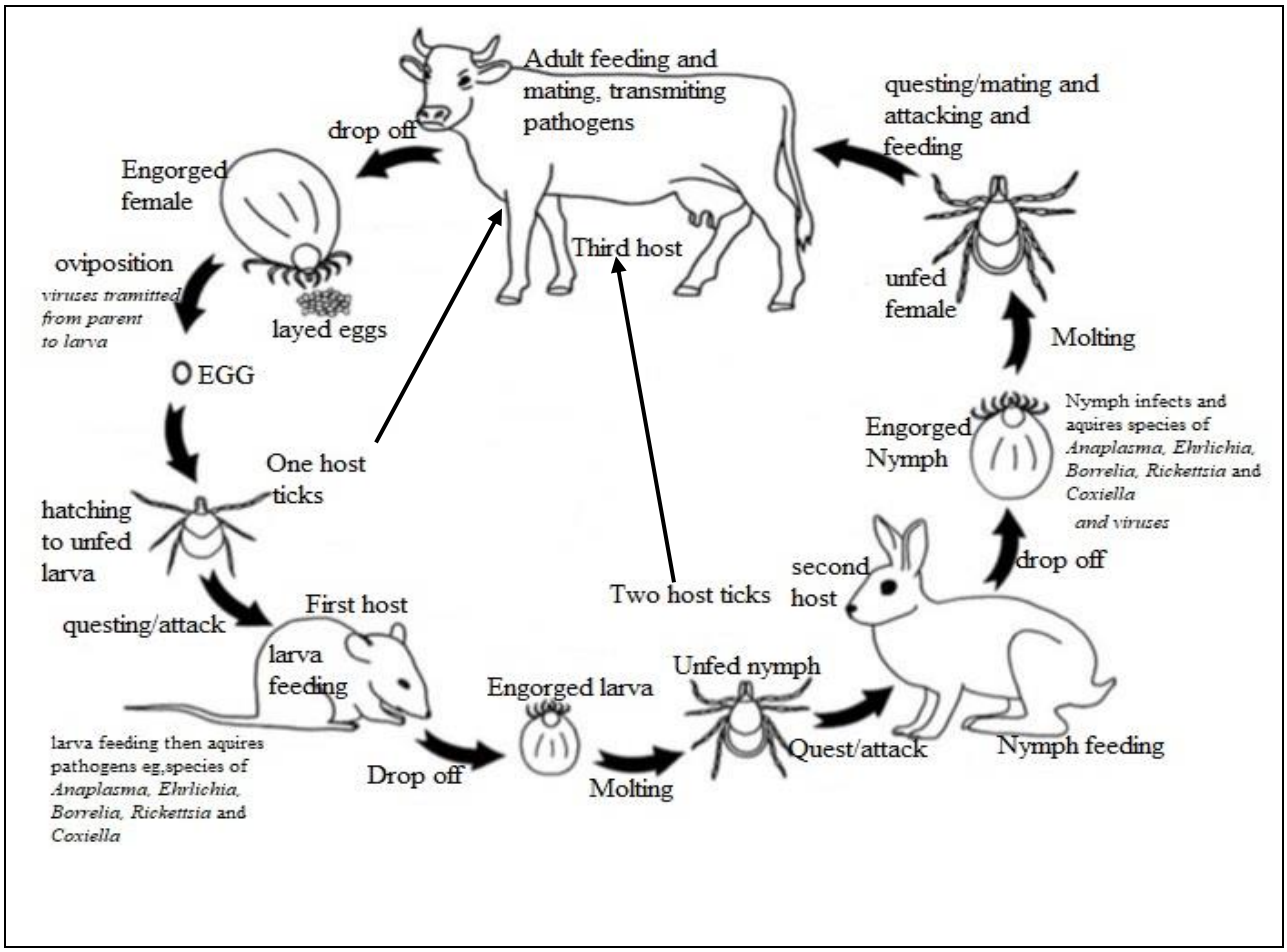



Figure 2.3 Life cycle of ticks and possible hosts

Tick-borne pathogens are transmitted to hosts during feeding. After a blood meal, pathogens replicate in several tissues within the ticks including the salivary glands. When ticks feed on a new host, the transmitted pathogens infect the endothelial cell linings of the small blood vessels by parasite-induced phagocytosis (Scherler *et al*., 2018). Once inside the cells, pathogens lyse the phagosome membrane with a phospholipase and get into the cytoplasm where they replicate. They exit by exocytosis or by lysis of the host cell. Since ticks can be multiple host vectors parasitizing small wild animals and cattle, this enables them to spread a wide variety of tick-borne bacteria (Iweriebor et al. 2017).

## 2.3 TICK-BORNE DISEASES OF CATTLE

Cattle and dairy farming are a huge contribution financially to small communal farmers, commercial dairy, and meat farmers. About 80% of cattle worldwide are at risk of tick-borne diseases, causing global annual losses of 22–30 billion US$ as estimated by De Castro in 1996 (Mapholi *et al.*, 2014; Lew-Tabor and Rodriguez Valle, 2016; Nyangiwe *et al*., 2018). This has led to losses of meat, milk, skin, and cash from sales, which has impacted the economy negatively (Mapholi et al. 2014). Viral tick-borne pathogens implicated in cattle diseases include Crimean-Congo hemorrhagic fever virus, Encephalitis virus, whereas the bacterial pathogens are species of *Rickettsia*, *Borrelia*, *Francisella*, *Anaplasma*, *Ehrlichia,* and *Coxiella* (Greay et al. 2018). The most common diseases from these pathogens in South Africa are bovine anaplasmosis (gall sickness), relapsing or spotted fever, hemorrhagic fever, and heartwater (Choopa 2015; Theiler 1912; Halajian et al. 2016; Khumalo 2018).

### 2.3.1 Bacterial tick-borne pathogens

Tick-borne bacteria have an endosymbiotic relationship with ticks. This relationship is important for tick survival, molting, reproductive capabilities, and also provides for cofactors and other nutrients that are not present in the tick blood meal (Weinert *et al*., 2009; Machado-Ferreira *et al*., 2016; Greay *et al*., 2018).

#### 2.3.1.1 *Anaplasma* species

Sir Arnold Theiler's research in 1909 led to the discovery of the first bacteria species of the *rickettsial* order, *Anaplasma marginale* (Theiler 1910). Over the years more species have been documented, species such as *A. phagocytophilum*, *A. bovis*, and *A. Ovis;* these are intracellular tick-borne pathogens causing anaplasmosis in sheep, cattle, and birds respectively (Khumalo 2018; Kocan et al. 2003; Theiler 1910; J. Yang et al. 2015). In cattle, *A. marginale* causes bovine

anaplasmosis a communicable, noncontagious haemotropic disease. Characterized by symptoms of fever; anemia; animal weakness and reduced appetite; constipation and dehydration; yellowing of the mucous membran**es** as well as difficulties in breathing (J. Yang et al. 2015). Acute infections result in the slow recovery of diseased cattle, hence low milk and meat production as cattle shed a lot of weight and this might also result in the death of cattle (Fedorina et al. 2019). *A. phagocytophilum, A. marginale, A. bovis,* and *A. Ovis* have been identified from cattle ticks countrywide in South Africa, constant surveillance of these pathogens is important as they pose a threat to food production (Iweriebor et al. 2017; Halajian et al. 2018; Ringo et al. 2018b; Guo et al. 2019).

2.3.1.2 *Borrelia* species

The *Borrelia* species are tick-borne bacterial pathogens classified in the order *Spirochaetales* and family *Spirochaetaceae*. They are the main disease agents of Lyme borreliosis and relapsing fever worldwide (Davidson et al. 1999; Yabsley et al. 2012; Livanova et al. 2018; Boulanger et al. 2019; Brinkmann et al. 2019). *B. theileri* is transmitted by *Rhipicephalus* tick species causing bovine borreliosis. Yabsley and colleagues (2012) collected and studied tick from penguins in the South African western cape coast and detected *Borrelia* species (Yabsley et al. 2012). There is limited data on the prevalence of *Borrelia* species in South African cattle, as such, there is a need for additional molecular research on this pathogen.

2.3.1.3 *Ehrlichia* species

*Ehrlichia* genus is composed of Rickettsiales transmitted by ticks. Ehrlichiosis is a communicable disease caused by *Ehrlichia* bacterial species, that are Gram-negative, obligately intracellular classified under the phylum *Proteobacteria*. *Ehrlichia* species of veterinary and medical

importance include *E. canis*, *E. chaffeensis*, *E. ruminantium*, *E. muris*, and *E. ewingii* these are mostly transmitted by *Amblyomma* tick species (Sreekumar *et al.*, 1996; Mansueto *et al.*, 2012; Chitanga *et al*., 2014; Ringo *et al.*, 2018a). *E. equi* causes equine granulocytic ehrlichiosis in cattle and dogs (Branger *et al*., 2004). Heartwater is caused by the *E. ruminantium*, an important disease in South Africa and worldwide (Brouqui and Raoult, 1990; Branger *et al*., 2004; Iweriebor *et al.*, 2017; Mtshali *et al.*, 2017; Halajian *et al.*, 2018; Guo *et al.*, 2019).

2.3.1.4 *Francisella* species

*Francisella* species have been detected in *Hyalomma dromedarii* ticks. Genus *Francisella* is comprised of several bacterial species, *F. tularensis*, *F. philomiragia*, *F. novicida*, *F. noatunensis*, and *F. hispaniensis* (Brinkmann et al. 2019). *F. tularensis* is a Gram-negative facultative intracellular bacteria causing tularemia, a disease that can be deadly to humans and a variety of animal species (Ghoneim *et al*., 2017). *F. tularensis* has been reported in ticks from Egypt, Ethiopia, and the southern hemisphere through PCR based 16S rRNA amplification (Szigeti *et al.*, 2014; Ghoneim *et al*., 2017). Knowledge of the presence of organisms of the genus *Francisella* and their occurrence is limited in Africa, therefore, more studies to determine their prevalence are required.

2.3.1.5 *Rickettsia* species

Veterinary and medical *Rickettsia* species of importance cause diseases in cattle and humans. These are obligate intracellular bacteria causing diseases that progress to fever, headache, rash, and vasculitis (Noh et al. 2017). The species *R. conorii* and *R. rickettsii*, the causative agents of spotted fever in cattle are transmitted by *Ixodid* ticks (Noh et al. 2017). African tick bite fever is

caused by the bacteria *R. africae* transmitted by *Amblyomma* tick species to cattle and has been observed in rural sub-Saharan Africa (Mtshali et al. 2017). African tick-borne fever caused by *R. africae* is endemic in Southern Africa and the disease has been reported in travelers, mainly tourists and game hunters departing Southern Africa (J. M. Rolain et al. 1998; J.-M. Rolain et al. 2002; Mtshali et al. 2017; Ringo et al. 2018b; Halajian et al. 2018). Rickettsia is of paramount importance as it affects cattle health and directly affects tourism, therefore, in-depth knowledge of their occurrence and distribution is important.

#### 2.3.1.6 *Coxiella burnetii*

*Coxiella burnetii* is an intracellular bacterium classified under the subdivision *Proteobacteria* with *Rickettsiella* and *Franciscella* genera as closest relatives upon sequence analysis of the16S rRNA. *C. burnetii* is a causative agent of Q fever (Greay et al. 2018) and has been detected in cattle in all South African provinces (Halajian et al. 2016; 2018; Iweriebor et al. 2017; Mtshali et al. 2017; Ringo et al. 2018b; Guo et al. 2019).

## 2.4 CONTROL AND PREVENTION

In South Africa, prevention and control of tick-borne diseases involve immunization of livestock and the use of chemicals for tick-vector control. Immunization involves the use of vaccines, classified into inactivated, live attenuated, and recombinant vaccines (Allsopp 2009). Dairy and cattle farmers use vaccines for prevention against heartwater disease and gall sickness. However, this is not recommended for use in pregnant animals as abortion may occur (Allsopp 2009). Tick control is done by the dipping method or spraying insecticides such as acaricides. Acaricides used by cattle farmers are synthetic pyrethroids, formamidines, and organophosphates (Onyiche et al. 2019). However, tick infestations are still a major challenge.

## 2.5 TREATMENT OF TICK-BORNE BACTERIAL DISEASES

The most commonly used drugs for the treatment of infections caused by bacterial tick-borne pathogens in cattle are antibiotics like tetracyclines or doxycycline, these are very effective in the treatment of fever, heartwater, and gall sickness (Hedayatianfard *et al.*, 2014; Espinaze *et al.*, 2018). Sulfamethazine can also be used for the treatment of heartwater during its early stages of infection (Biswas *et al.*, 2008).

## 2.6 RESISTANCE DEVELOPMENT OF BACTERIA TO ANTIMICROBIAL AGENTS

Drug resistance to antibiotics is due to mutations resulting in over-expression of multidrug efflux pumps, under-expression of porins, mutations in the target sites of the drugs, and the production of drug-modifying enzymes (Ramanathan et al. 2017). Mobile extrachromosomal elements called plasmids are also involved in horizontal gene transfer within bacteria of different species. The environmental microorganisms harbor resistance genes from their evolution, making it a ready supplier of new and emerging genes of resistance to drugs (Perry and Wright 2014).

Tick-borne pathogens have obligated intracellular lifestyle, therefore, susceptibility to antimicrobial agents cannot be assessed with conventional microbiological tests. The use of mammalian cell culture, animal Guiney pigs, models of embryonated eggs, and molecular detection techniques have been considered for susceptibility studies (Kimberlin and Whitley, 1996; Xu *et al.*, 1997; Biswas *et al.*, 2008; Brinkmann *et al.*, 2019; Guo *et al.*, 2019)

### 2.6.1 Mechanisms of action of antibiotics and resistance development.

Macrolides such as clarithromycin and erythromycin have been used in the treatment of tick-borne diseases. Their mode of action involves inhibiting translation by acting on the 50S ribosomes. They bind on the peptidyl transferase center of the 50S subunit, and they are involved in domains II and V of the 23S rRNA (Biswas *et al.*, 2008). Resistance mechanisms occur because of post-

transcriptional mutation of the 23S rRNA region and changes occurring on the ribosome target site. The gene encoding the methylases is the *er*m (erythromycin ribosome methylation) (Table 2.1), and the drug efflux pumps are encoded by *mef* (A) gene and are in the membrane of these pathogens. *E. chaffeensis, E. canis, A. phagocytophilum,* and *F. tularensis* have been confirmed to have *mef* (A) genes and mutations on 23S rRNA which code for resistance to macrolides (Branger *et al.*, 2004; Biswas *et al.*, 2008; Gestin *et al.*, 2010; Makarov and Makarova, 2018). Some strains of *R. typhi* and *R. prowazekii* are susceptible while some have become resistant to the macrolide erythromycin, and this is due to a mutation observed in the 23S rRNA ribosomal region (Biswas *et al*., 2008).

Table 2.1 Antimicrobial agents, resistance genes, and bacterial strains associated with resistance.

| Antimicrobial agent | Resistance genes | Mode of resistance | Resistant Microorganism | reference |
|---|---|---|---|---|
| Macrolides | *Erm* in 23S rRNA<br>*mef* (A) | Site mutation<br>Efflux pumps | *Ehrlichia chaffeensis, Francisella tularensis, Rickettsia prowazekii* | (J. M. Rolain and Raoult 2005) |
| Chloramphenicol | *cat,* 23S rRNA | Mutation in target 23S ribosome subunit | *Ehrlichia* | (Brennan and Samuel 2003) |
| Aminoglycosides | 16S rRNA, *rps*, aminoglycoside-modifying enzymes | Producing protein aminoglycoside 3′-phosphotransferase | *Rickettsia conorii* and *Rickettsia felis* | (J. M. Rolain 2005) |
| Tetracycline | 16SrRNA, tet gene, | Ribosomal protection and efflux pumps<br>Inactivating enzymes | *Francisella tularensis* | (Biswas *et al*., 2008) |
| Beta-lactam | *bla*A<br>AmpG protein | Beta-lactamases inactivating enzyme and increased permeability proteins | *Rickettsia* typhi and R. prowazekii *Rickettsia felis and R. conorii* | (Rolain, 2005; Biswas *at el.*, 2008) |
| Quinolones | *gyr*A, *gyr*B, *par*C, *par*E | Mutation in these target genes | *Ehrlichia* | (Rolain, 2005; Biswas at el., 2008) |
| Rifampicin | *Rpo* (B-D) | The mutation in these target gens | *Rickettsia conorii* | (Rolain, 2005; Biswas *at el.*, 2008) |
| Trimethoprim Sulfamethoxazole | *fol*A<br>*folP* | The mutation on the target gene and overproduction of *fol*P | *Rickettsia* | (Rolain, 2005; Biswas *at el.*, 2008) |

Chloramphenicol is a bacteriostatic antimicrobial agent, that binds to the 50S ribosomal subunit thus preventing the peptidyl transferase step during protein synthesis (Makarov and Makarova 2018). Resistance is due to the reduction in drug permeability in bacterial membranes, mutations in the ribosomes, and amplification of chloramphenicol acetyltransferase. The *cat* gene codes for chloramphenicol acetyltransferase which deactivates chloramphenicol (Table 2.1). Mutations in ribosomes have previously been reported in *Ehrlichia* species, hence the strains are found to be resistant to chloramphenicol (Makarov and Makarova 2018).

Aminoglycosides are antibiotics that inhibit cell wall protein synthesis by binding to ribosomal site, this leading to the bacteria with no or weak cell wall and consequently rapture of the pathogens (Rolain, 2005; Pachori *et al*., 2019). Resistance to these drugs is by aminoglycoside-modifying enzymes such as nucleotidyl transferases, overexpressed efflux pumps, and the presence of an *rpLs* gene that codes for ribosomal protection protein (Wright and Serpersu, 2005; Biswas *et al*., 2008). Rolein et al., (2002) molecular studies showed that *Rickettsia conorii* and *Rickettsia felis* had sequences that encoded an aminoglycoside 3′-phosphotransferase enzyme, leading to streptomycin resistance (J.-M. Rolain et al. 2002).

Tetracyclines are a broad-spectrum antibiotic used in the treatment of infections in humans and animals. They inhibit protein synthesis by preventing aminoacyl-tRNA contact to the ribosomal acceptor site (Hedayatianfard *et al*., 2014). Tetracyclines at subtherapeutic levels can be used as animal feeds to act as growth promoters. Over 40 different tetracycline resistance genes (tet genes) have been characterized (Roberts, 2005; Hedayatianfard *et al*., 2014; Jurado-Rabadán *et al*., 2014), that codes for active efflux pumps [tet (A-H)], ribosomal protection [tet (M-P)], and drug modifying enzymes [tet (X)]. Tetracycline resistance in most bacteria is due to the presence of these genes and are often located in mobile elements, plasmids, or transposons, and are always

ready to be transferred to other bacteria conjugatively (Aminov et al. 2002). The resistance of *Francisella tularensis* to tetracyclines has been reported and is due to the presence of [tet (A)], [tet (B)], and [tet (C)] genes (Biswas *et al*., 2008).

Beta-Lactam antibiotics act by interfering with cell wall synthesis by inhibiting the transpeptidases and carboxypeptidases that catalyze peptidoglycan synthesis (Lingzhi et al. 2018). Inhibited peptidoglycan will lead to bacteriolysis because bacteria will not be able to withstand osmotic forces due to the lack of a cell wall. Resistance to beta-lactams is due to the production of beta-lactam inactivating enzymes known as the beta-lactamases, which act by degrading the drugs before they reach the target site (Choi and Lee, 2019; Laws *et al*., 2019). Beta-lactamases are coded for by *bla*A genes located in the main bacterial chromosomes or plasmids. Resistance can also be due to the modification of the target site, efflux pumps increased activity, and as well as an increase in membrane permeability to the drug (Lingzhi et al. 2018). In a study conducted by Biswa *et al*., (2008), whole-genome analysis of *Francisella* strains showed the possession of *bla*A gene, AmpG protein, and Metallo-*B*-lactamase proteins that conferred resistance to beta-lactam. In another study, *Rickettsia felis* and *R. conorii* strains had a class C β-lactamase enzyme, while *Rickettsia typhi* and *R. prowazekii* possessed AmpG genes that conferred increased permeability to export Beta-lactams. *Francisella tularensis* demonstrated Erythromycin resistance in a study by Tarnvik et al., (2007)(Renesto *et al*., 2005; Biswas *et al.*, 2008). This emphasizes the need for more resistance studies to determine the resistance profiles of tick-borne bacteria to the commonly used drugs.

Quinolones or fluoroquinolones are antibacterial drugs used in human and veterinary medicine. They act by inhibiting DNA gyrase and topoisomerase IV in bacteria. DNA gyrase is encoded by the genes *gyr*A and *gyr*B (Table 2.1), while DNA topoisomerase IV is coded for by genes *par*C,

*parE*. Resistance to quinolones is attributed to the mutations in these genes (Aldred *et al*., 2014; Siebert *et al*., 2019). Maurin and colleagues conducted a bioinformatics analysis of *Ehrlichia* whole-genome sequence and found *gyr*A gene sequence was substituted by alanine(A) sequences in quinolone-resistant strains, meaning the *gyr*A gene was absent, as a result, DNA gyrase inhibition was not possible (Maurin *et al*., 2014).

The rifampicin antibiotics mode of action is by inhibiting DNA dependent RNA polymerase enzyme encoded for by genes *rpo*A, *rpo*B, *rpo*C, and *rpo*D (Kim et al. 2005; Troyer et al. 1998; Brennan and Samuel 2003). Bacterial resistance to this drug is mainly caused by mutations in the *rpo*B gene. Rolain et al., (1998) reported that tick-borne *Rickettsia* species were resistant to rifampin antibiotics by growing them in vitro cell monolayer cultures. They concluded that this had occurred because of mutations in the *rpo*B gene, this mutation prevented rifampin from reaching the target site (J.-M. Rolain et al. 2002).

Trimethoprim inhibits the dihydrofolate reductase (DHFR) enzyme. Resistance is by the production of more DHFR by the host, presence of mutations in the physical gene for DHFR (i.e. the *fol*A gene), and attainment of *dfr* gene that encodes the production of resistant DHFR enzyme (Brolund et al. 2010). Sulphonamide targets dihydropteroate synthase (DHPS) enzyme with structural gene *fol*P (Table 2.1). Sulphonamide resistance is facilitated by the mutations and the presence of a drug-resistant DHPS enzyme encoded by *fol*P gene. In *Rickettsia* species it has been reported that the absence of *fol*P and *fol*A genes enables them to be resistant to Sulphonamide. Similarly, *Francisella* strains do not possess genes coding for *fol*P dihydropteroate synthase and *fol*A dihydrofolate reductase making them resistant to co-trimoxazole (Biswas *et al*., 2008). Cotrimoxazole resistance was also detected against strains of *Rickettsia conorii* in the study conducted in France in 1998 (J. M. Rolain et al. 1998). Thus, tick-borne bacteria are highly

resistant microorganisms only susceptible to a few selected antibiotics, their resistance profiles need to be continuously updated. Furthermore, there are few recent papers published on the resistance of tick-borne microorganisms hence there is a need for more recent surveillance studies of tick-borne microorganisms using recent molecular technologies. A Summary of genes associated with antibiotic resistance, to commonly used antibiotic agents, is presented in Table 2.1.

## 2.7 DIAGNOSTIC TECHNIQUES FOR TICK-BORNE DISEASES

Diagnostic identification of tick-borne microorganisms can be done using serology, cell culture, immunohistochemistry, and molecular biology (Bielawska-Drózd et al. 2013; Baldridge et al. 2008). In serology, tests use surface protein antigens and are considered the most hands-on way to screen tick-borne pathogens, however, serological tests may detect antibodies from previous infections. Serological methods used include enzyme-linked immunosorbent assay (ELISA), the card agglutination test, indirect fluorescent antibody test (IFAT), and complement fixation test (Al-Adhami et al. 2011). Microscopy identification involves the preparation, staining, and examination under a microscope of blood smears, this has proved effective in the diagnosis of tick-borne pathogens. The blood smears are stained with Romanowsky type stain, and tick-borne pathogens are identified by their morphological characteristics (Kirkan *et al.*, 2017). However, recently sensitive, and highly accurate molecular techniques for diagnosis have proven to be more effective tools. Molecular methods allow analyses of microbial communities avoiding the need for extensive culture-based techniques. Allowing identification of species that are not possible to culture, several studies prove their effectiveness (X. J. Yang *et al.*, 2015; Greay *et al.*, 2018; Van Camp *et al.*, 2020). Bioinformatics drug resistance genetic profiling using whole-genome and metagenomics has proved to be sensitive, reliable, and cost-effective in recent functional profiling

studies (Z. J. Chen et al. 2020; Mukherjee et al. 2017). It is, therefore, important to use high throughput techniques to generate information that is accurate to describe communities of bacteria in ticks.

## 2.8 MOLECULAR DIVERSITY AND FUNCTIONAL RESISTANCE BIOMARKERS IN BACTERIAL COMMUNITIES

Andreotti et al., (2011) reported the first-ever nonculture molecular approach to study bacterial diversity in ticks through next-generation sequencing. In their study, the 16S ribosomal RNA variable regions V3-V4 were amplified. The V3-V4 hypervariable regions of the bacterial 16S ribosomal RNA genes were used to profile and identify bacterial communities (Andreotti et al. 2011). The study of nine variable regions in 16S RNA allows improved understanding of microbial ecology in diverse niches (Douglas *et al*., 1991). The taxonomic assignment of generated OTUs is achieved by comparing attained reads against databases such as Silver 33, Green-genes, and HOMD to identify bacterial communities (Johnson 2017).

Tyson *et al* (2015) reported whole-genome sequencing precisely predicting antibiotic resistance genes, detected resistance-related mutations, and predicted resistance genotypes. Resistance gene databases which include, ResFinder, DTU, the Center for Genomic Epidemiology, and as well as GenBank have been used to identify resistant genes (Tyson et al. 2015).

The 16S rRNA metagenomics biomarker discovery is a very important tool that can be used to characterize bacterial communities by translating amplicon sequence data into clinical practice. Biomarker discovery coupled with linear discriminant analysis effect size (LEfSe) provides a high dimensional class comparison in metagenomics genes and functions using statistical significance tests. LEfSe allows predictions of biomarkers that are responsible for expressed phenotypes (Segata et al. 2011).

Bioinformatic pipelines such as Muthor, QIIME2, and DADA can employ PICRUSt to predict functional pathways and genes using 16S rRNA data in combination with LEfSe that predicts biomarkers a comprehensive biomarkers analysis is achievable. PICRUSt predictions for functional composition using KEGG (Kyoto Encyclopedia of Genes and Genomes) orthologs (KO) and KEGG modules for pathways, databases for inference can predict biomarkers (Van Camp *et al*., 2020). The inference is done according to sequenced organism's ancestors for gene predictions and it has been proven that these predictions are 85-90% accurate in microorganisms (Park et al. 2020). Several studies have used PICRUSt for predicting functional genes, pathways, and proteins as well as LEfSe for biomarker identification, and characterization, or both (Park et al. 2020; Dube et al. 2019; Mukherjee et al. 2017; Z. J. Chen et al. 2020; Langille et al. 2013a; Segata et al. 2011).

## 2.9 SUMMARY

Ticks and tick-borne microorganisms are a significant constraint in cattle production. Information regarding bacterial communities in ticks and their antibiotic resistance is very limited and scarce in South African publications. Therefore, a gap exists in bacterial communities and their resistance markers in ticks infecting cattle, the need to determine this is important in veterinary and medical understanding of the treatment required from infection. The main objective of the study was to determine the bacterial communities in ticks, infesting Nguni cattle, and to determine their functional resistance biomarkers for improved treatment strategies.

# CHAPTER 3 BACTERIAL COMMUNITIES AND THEIR FUNCTIONAL ANTIBIOTIC RESISTANT MARKERS IN *AMBLYOMMA* AND *HYALOMMA* TICKS.

## 3.1 ABSTRACT

Ticks are ectoparasites of vertebrates that are vectors of numerous disease-causing pathogens to cattle and other vertebrates. The present study examined microbial communities associated with *Hyalomma* and *Amblyomma* ticks using 16S rRNA metagenomics sequencing, and characterizing PICRUSt predicted antibiotic resistance biomarkers. A total of 19 ticks *Hyalomma* (n=4) and *Amblyomma* (n=15) were collected from Nguni cattle. About 1671347 sequence reads were generated, and 16374 bacterial genera were identified in all the examined tick samples. Bacterial communities detected showed significant differences in the number of OTUs (Operational taxonomic units) in the beta-diversity analysis. Among the communities previously described pathogenic, endosymbionts, and tick normal flora bacteria detected included genera of *Rickettsia, Corynebacterium, Escherichia, Porphyromonas, Anaerococcus, Arthrobacte, Aerococcus, Trueperella, Coxiellaceae_uc, Acinetobacter, Helcococcus, Peptoniphilus, Enhydrobacter, Porphyromonadaceae_uc, Coxiella, Sporobacter, Brachybacterium,* and *Phycicoccus*. Moreover, genera of *Rickettsia, Corynebacterium, Escherichia,* and *Porphyromonas* were found to be the most dominant. There was a clear separation in clusters between communities of bacteria from both tick species after PCoA (Principal coordinate analysis) beta-diversity analysis, suggesting that bacterial communities might be influenced by cattle skin microflora, feeding site, and host blood. Comparative metagenomics showed significant differences ($P<0.051$) in core microflora between tick species. KEGG Level 2 predicted PICRUSt functional analysis suggested that functions related to genetic, environmental information processing, and metabolism were highly enriched. Further KO analysis revealed functional antibiotic-resistant biomarkers in both communities, composed of efflux pumps (ABC, MFS, and MDR), drug degrading and modifying enzymes, ribosomal protection proteins pathways as well as secretion systems. Data from this

study revealed how cattle are exposed to multiple tick-borne bacterial communities with biomarkers, conferring resistance to antibiotics such as Beta-lactam, tetracycline, and Macrolides. Tick-borne microbial communities are of medical and veterinary importance, characterizing and providing knowledge of their antibiotic resistance markers, may lead to novel control strategies in disease outbreaks.

## 3.2 INTRODUCTION

Ticks are very important arthropods that act as vectors of various bacterial communities infecting cattle. The enormous annual global loss of about 22US$ to 30US$ billion has been recorded due to tick-borne pathogens, therefore, ticks and tick-borne disease control is very important in animal health, and meat production (de Castro, 1997; Falvey, 2015; Lew-Tabor and Rodriguez Valle, 2016; Nyangiwe *et al*., 2018). Several studies in South Africa have identified tick genera such as *Ixodes, Hyalomma, Amblyomma,* and *Rhipicephalus* in Nguni cattle (Mapholi *et al*. 2016). *Hyalomma* and *Amblyomma* ticks microbial communities transmitted include bacterial species of *Anaplasma, Borrelia, Coxiella, Ehrlichia, Francisella,* and *Rickettsia* (Guo *et al*. 2019; Halajian *et al.* 2016; Iweriebor *et al*. 2017; Mtshali *et al*. 2017; Ringo *et al*. 2018a; Berggoetz *et al*. 2014).

Diseases associated with tick-borne bacteria include relapsing fever (*Borrelia* agent*)* and spotted fever (*Rickettsia* agent); ovine and bovine anaplasmosis (*Anaplasma* agent); Q fever (*Coxiella* agent); ehrlichiosis and heartwater (*Ehrlichia* agent) (Allsopp, 2009; Yabsley *et al.*, 2012; Kuley, 2017; Halajian *et al.*, 2018). Antibiotics such as tetracyclines, macrolides, beta-lactams, aminoglycosides, and fluoroquinolones are used against tick-borne pathogens. However, several studies have shown antibiotic resistance of tick-borne bacteria to antibiotics, and studies looking at their resistance biomarkers are limited (Brouqui and Raoult, 1990; Rolain *et al.*, 1998; Branger *et al.*, 2004; Siebert *et al.*, 2019). Bacterial antibiotic resistance is achieved through mechanisms employing over-expression of efflux pumps, iron transport proteins, enzymes that modify or degrade antibiotics, mutations resulting in under-expression of porins, and mutations in drug target sites (Venter *et al*., 2015; Wang *et al.*, 2018; Van Camp *et al*., 2020). Algorithms such as PICRUSt enable the prediction of resistance genes from 16S rRNA sequences (Langille *et al*., 2013b). Several studies have proven the effectiveness of the PICRUSt algorithm in the characterization of

functional and resistance biomarkers of intracellular bacteria, this may not be possible using cultural techniques (Mukherjee *et al*., 2017; Langille *et al*., 2013b).

Most studies reported microbial communities in whole intact ticks (Lejal et al. 2019). However, this approach does not consider organ-specific community distribution. Organ-specific studies are important in characterizing microbes transmitted, acquired, and maintained within the salivary glands and mouthparts (Lejal et al. 2019). Salivary glands and mouthparts of ticks serve as barriers for efficient pathogen transmission and maintenance of endosymbionts (Rikihisa 2011; Lejal et al. 2019). Furthermore, characterizing microbial community resistance biomarkers sheds a light on the antibiotic-resistant, and allows us to elucidate possible recommendations for effective tick-borne disease treatment.

This study aimed to examine bacterial communities associated with *Amblyomma* and *Hyalomma* tick species collected from Nguni cattle, by performing 16S rRNA metagenomics and characterize functional antibiotic resistance biomarkers using a bioinformatics tool (PICRUSt). The flow chat illustrating the methods used is shown in figure 3.2.1.

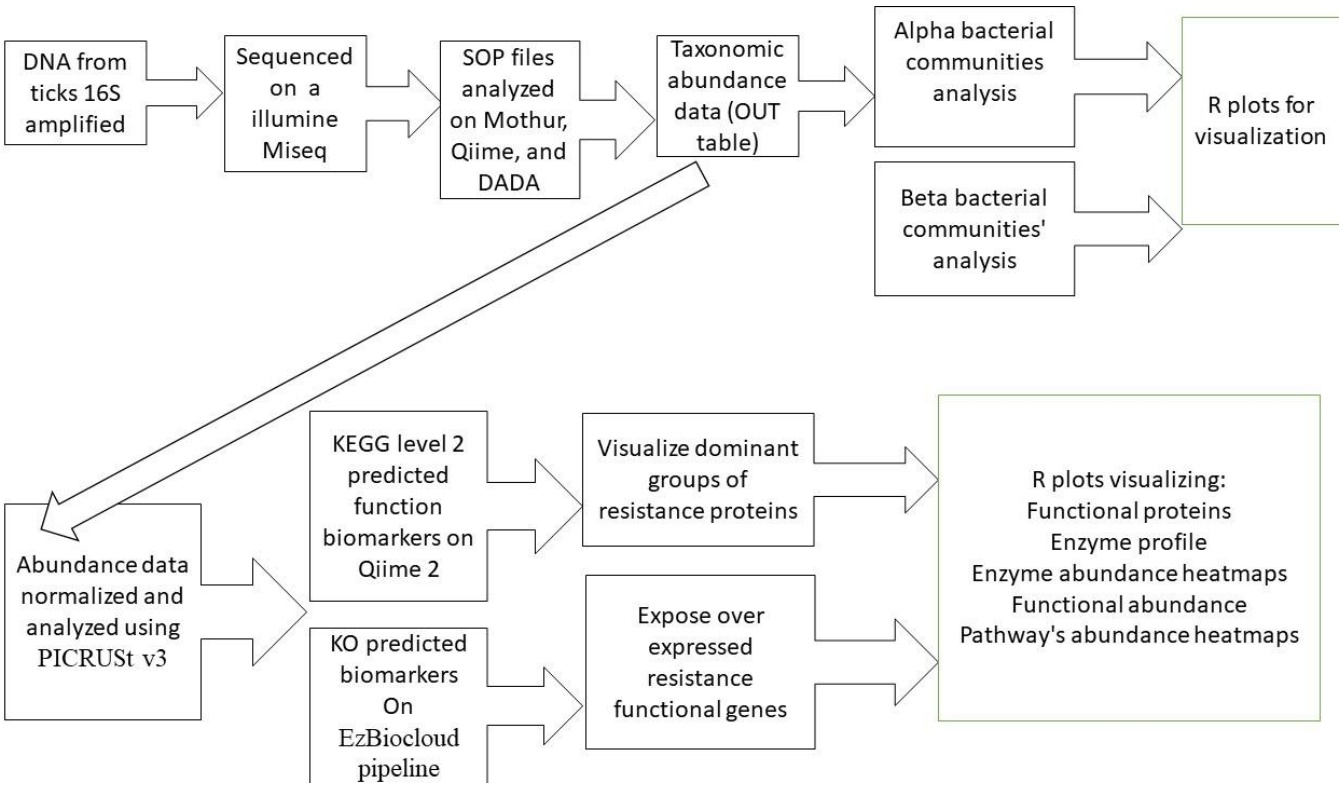



Figure 3.2.1 Methodology workflow chat used in the current study.

## 3.3 METHODOLOGY

### 3.3.1 Ethics statement

Ethical clearance to conduct the study was sought from the University of South Africa, College of Agriculture and Environmental Sciences - Animal Ethics Review Committee before the study commenced (2019/CAES_AREC/152) (Appendix 1). Recommendations made by the Research Committee were strictly adhered to. The objectives of the study were part of an ongoing study, and authorization to use animals and collection of ticks was attained from Department of Agriculture, Land Reform and Rural Development *(*DALRRD*)* and The Agriculture and Research Council-Animal Production Institute. Ethical clearance to conduct the study at the Agricultural Council (ARC) was granted.

### 3.3.2 Sampling site

Ticks used in this study were collected between September 2018 and February 2019 from the Roodeplaat ARC-research farm (29º 59” S, 28º 35” E) (Figure 3.3.1) as part of an ongoing collaborative study between ARC and different research farms.

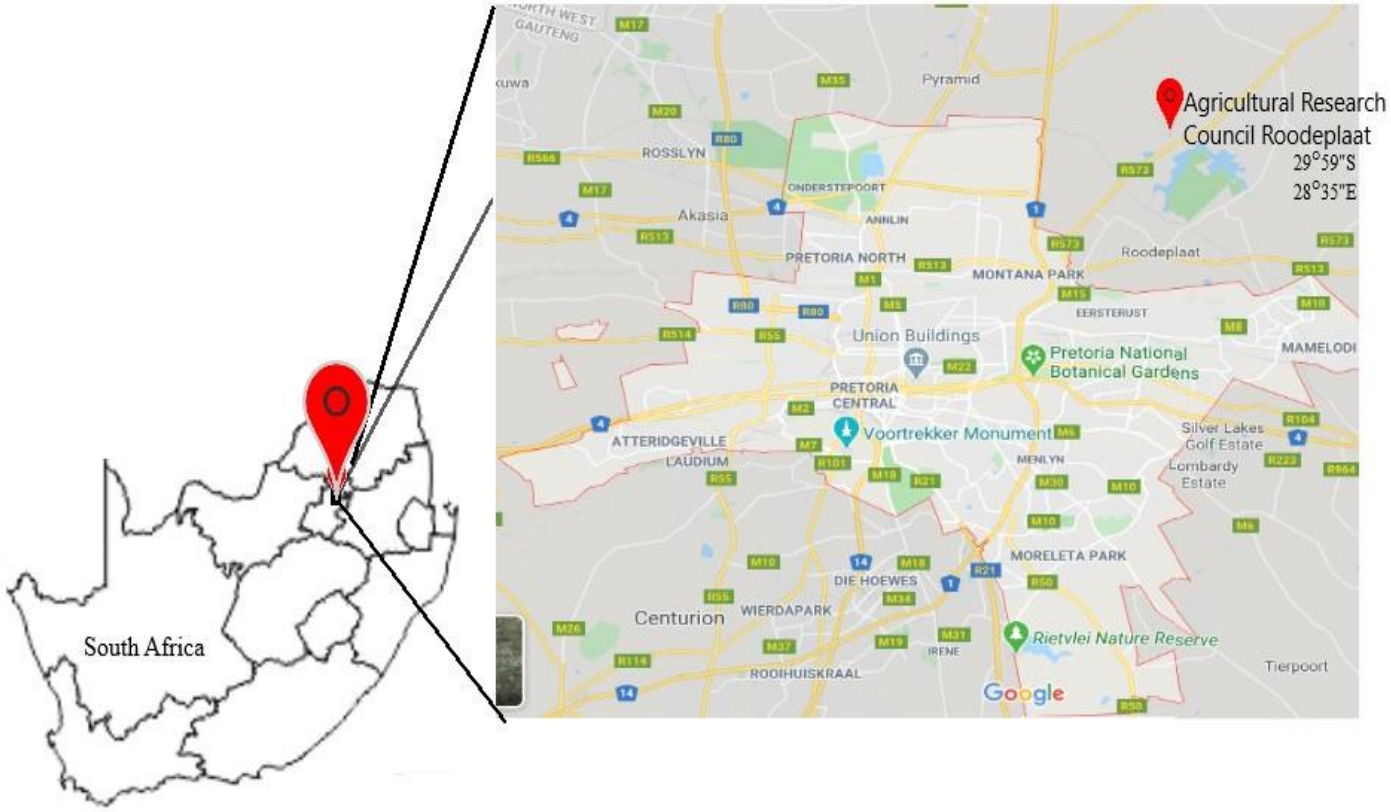



Figure 3.3.1 Sampling site for ticks, Agriculture Research farm Roodeplaat, Pretoria, South Africa

### 3.3.3 Tick samples collection and processing

To collect the ticks, tweezers were used to remove ticks from cattle ensuring the mouthparts remained intact. Ticks were then placed into Eppendorf test tubes containing 70% ethanol for preservation. The cattle bite site was carefully cleaned with 70% ethanol. Collected ticks were then stored at -80C° at UNISA Eureka Life science laboratory for further DNA extraction. A total of 110 ticks were collected and morphologically identified to species level using standard taxonomic identification keys (Barker and Walker 2014; Estrada-peña et al. 2003) and a stereomicroscope with the help of a Veterinarian Dr. Skhumbuzo Mbizeni (UNISA) and Goodwill Makwarela (Ph.D. student). For the purpose of this chapter, nineteen ticks were identified as *Amblyomma* (n=15) and *Hyalomma* (n=4) and the remaining were *Rhipicephalus* tick species which were considered for analysis for the subsequent chapter (Chapter 4).

### 3.3.4 Tick lysis and DNA extraction

After tick identification, about 19 ticks were washed with nuclease-free water until ethanol was washed off, then air-dried. Ticks were then cut from the second leg going up the capitulum to target salivary glands under a light microscope (Berggoetz et al. 2014). The upper sections of ticks were cut into pieces and added to 0.5 ml screw-cap tubes. The omega TL lysis buffer and 25µl of Proteinase K were added to each tube for lysis to occur over a 24-hour incubation at 56℃. DNA extraction was performed using the tissue DNA extraction kit, E.Z.N.A. (Omega Bio-Tek), according to the manufacturer's instructions.

### 3.3.5 Library sample preparation for 16S rRNA metagenomic sequencing

Library preparation for the 16S rRNA library for 19 DNA samples, was carried out using the 16S rRNA metagenomic sequencing library preparation protocol (Illumina, Inc., San Diego, CA 2013). To characterize the bacterial communities, DNA samples were amplified targeting the V3-V4 hypervariable region of the 16S rRNA. The target variable region is used to identify bacteria when aligned against a Silver bacterial database. Amplification was performed using 27 reverse and 518 forward primers with overhang adapters listed in Table 3.1. Briefly, PCR reaction mixture was prepared in a total volume of 25µl and comprised of 2.5µl of DNA, 12,5µl of 2x KAPA HiFi Hot Start Ready Mix (Kapa Biosystems, Boston, MA, USA), and 5µl of each of the primers. The thermocycling conditions used are indicated in Table 3.2.

Table 3.1. 16S rRNA reverse and forward primers with overhang adapters

| **Target** | **Primer sequence** | **Target region** | **Size of amplicon** | **Reference** |
|---|---|---|---|---|
| Bacteria | 16S rRNA Forward 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3’<br>16S rRNA Reverse 5’-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3’ | 16S rRNA gene | 518 | (Illumina, Inc., San Diego, CA 2013) |

Table 3.2. Thermocycling conditions for Amplicon PCR.

| Cycling conditions | Temperature | Time | |
|---|---|---|---|
| Initial Denaturation | 94°C | 5 minutes | |
| Denaturation | 94°C | 30 seconds | |
| Annealing | 58°C | 30 seconds | 36x cycles |
| Elongation | 72°C | 40 seconds | |
| Final Elongation | 72°C | 10 minutes | |
| Hold | 4°C | ∞ | |

The resulting amplified products were visualized in ethidium bromide-stained, 1 % agarose gel. The DNA pools which yielded amplified products with fragments of approximately 560bp were selected. The DNA was quantified with a Qubit fluorometer (Invitrogen life Technologies) using a dsDNA assay kit (Invitrogen life technologies).

Subsequently, the amplified products were cleaned using AMpure XP beads (Beckman Coulter), 80% EtOH, and magnetic beads following manufacture instructions. Purified products were attached to dual indices using Nextera index PCR using the Nextera XT v2 Index Kit (Illumina, Inc., San Diego, CA 2013). Briefly, a total reaction mixture of 25 µl comprising of 5 µl DNA, 2.5 µl each of Nextera index primers forward (S5XX) and reverse (N7XX), 12.5 µl of 2x KAPA HiFi Hot Start Ready Mix (Kapa Biosystems, Boston, MA, USA), and 2.5 µl of PCR grade water was prepared. The reaction mixture was amplified under thermocycling conditions in Table 3.3.

Table 3.3 The dual index PCR cycling conditions.

| Cycling conditions | Temperature | Time | |
|---|---|---|---|
| Initial Denaturation | 95°C | 3 minutes | |
| Denaturation<br>Annealing<br>Elongation<br>Final Elongation | 95°C<br>55°C<br>72°C<br>72°C | 30 seconds<br>30 seconds<br>40 seconds<br>5 minutes | 8x cycles |
| Hold | 4°C | ∞ | |

The resulting products were purified using Ampure XP beads, 80% EtOH, and magnetic beads following manufacture instructions. Quantification of the final product was performed using Qubit. The concentrated final library samples were diluted to 4nM using 10mM Tris at PH 8.5. A volume of 5µl of each sample was pooled into a multiplexed library and a negative control sample was included.

### 3.3.6 Library sequencing

The 6 pM of the pooled libraries and the PhiX control library were denature using diluted 0.2 N NaOH, to achieve cluster generation during sequencing. The final library was sequenced on an Illumina MiSeq next-generation sequencer using a MiSeq Reagent Kit v3 (Illumina, Inc., San Diego, CA 2013).

### 3.3.7 Sequences analysis of 16S rRNA bacterial data

The 16S rRNA amplicons were sequenced using Illumina Miseq 2x 300 base pairs, with primers 27F and 518R using Nextera dual indexes targeting the V3-V4 hypervariable region of bacterial

16S rRNA. Sequence processing was performed using Mothur (version 14) software as per Miseq SOP (Kozich et al. 2013). The SILVA-based reference sequences were used to classify unique sequences, executing a Bayesian classifier on Mothur against Silva's (v133) reference taxonomy. UCHIME was used with the Silva SEED database to identify chimeras and removing them for downstream analysis, while a rare function was used to remove singletons (Weber et al. 2018). Using the average-neighbor algorithm classified 16S rRNA were assigned operational taxonomic units (OTUs) at 97.0%. Generated OTUs table was then used for downstream analysis, R Studio platform (version 4.0.3) and STAMP (version 2.1.3) software were used for data visualization.

3.3.8 Functional resistance biomarker analysis.

Resistance biomarkers were estimated using PICRUSt v3, algorithm software using 16S rRNA sequence data, and reference databases to infer biomarker gene contents as described by Langille *et al.*, (2013). Using the PICRUSt v3 algorithm, COG (Cluster of Orthologous Genes), and KEGG (Kyoto Encyclopedia of Genes and Genomes) databases, resistance biomarkers were identified to Level 2 Orthology. KEGG Ortholog (KO) genes were predicted using PICRUSt and EzBiocloud (Yoon et al. 2017) and analyzed using LEfSe analysis. In brief, demultiplexed fast Q files were removed adapters, noise reads, chimera, cluster, and dereplicate sequences. The resultant OTUs table was normalized with the lowest read OTU and genes predicted using PICRUSt v3. STAMP (version 2.1.3) and R studio (version 1.3) were used to visualize results as illustrated in APPENDIX 5.

## 3.4 RESULTS

From the 110 ticks' samples collected, a total of 19 ticks were selected 15 of which were *Amblyomma* species and 4 were *Hyalomma* species. After initial amplification positive nymphs, males, and females of *Amblyomma* and *Hyalomma* tick species were selected as individual samples. A total of 15 *Amblyomma* (assigned code A1 to A15), 3 *Hyalomma* (assigned code H1, H2, H3, and H4), were sequenced.

### 3.4.1 Sequencing quality and estimated richness of bacterial communities

To gain insights on the bacterial community genetic composition from *Hyalomma* and *Amblyomma* tick species, paired-end reads of the V3 -V4 region of the 16S rRNA gene amplicons were generated for the metagenomics dataset. From the 18 samples sequenced a total of 1671347 reads were generated providing 11019 OTUs, average trimmed valid reads were 87965.63 per sample after being processed by the Mothur pipeline. The sample (H2) had 0.0003% valid reads and was excluded in the downstream analysis and was considered a control blank. PICRUSt command was executed on the Mothur pipeline for functional antimicrobial resistance biomarkers encoded in the tick-associated bacterial communities.

Average Good's coverage of the library ranged from 99.34% to 99.90% and rarefaction curves reaching a plateau, suggesting enough sequencing depth with all OTUs with good coverage reads as seen in the rarefaction plots in figure 3.4.1a and figure 3.4.1b.

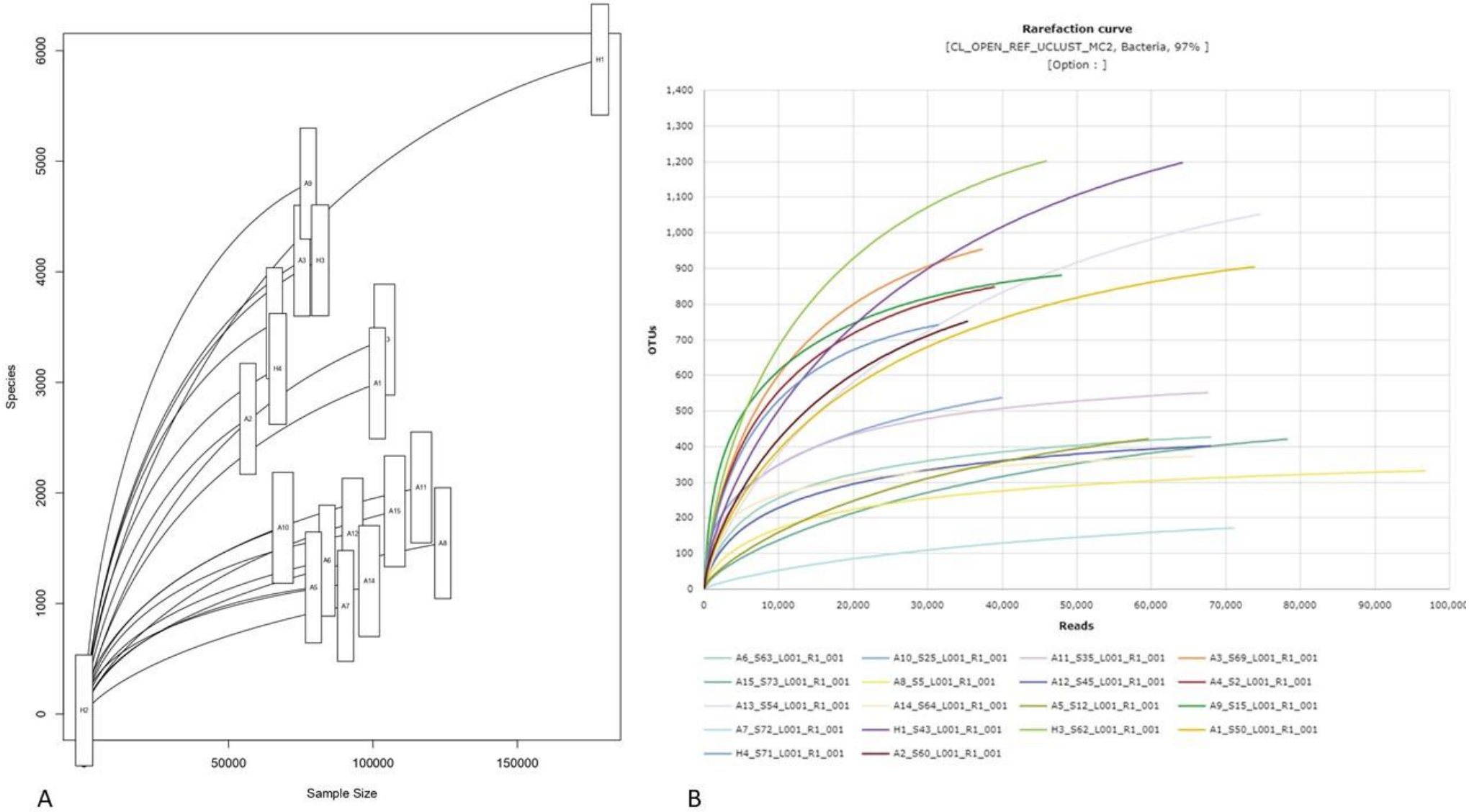



Figure 3.4.1. Operational taxonomic units (OTUs) per sample are shown as rarefaction curves of *Hyalomma* and *Amblyomma* ticks DNA samples. The curves A and B, represent reads observed against OTUs on the y-axis and reads per sample on the x-axis.

Alpha diversity and bacterial communities' species richness were analyzed using diversity indices (Observed OTUs, Shannon, Chao1, Simpson, Inv Simpson, Fisher, and ACE) in figure 3.4.2. There were no significant differences in observed OTUs, Chao1, and ACE indices between communities of bacteria from *Hyalomma* and *Amblyomma* tick species. However, Shannon, Simpson, Inv Simpson, and Fisher diversity indices had high significant differences, observed at (P=0.021) using Wilcoxon rank-sum test, in OTUs indices that were normalized to the lowest read count (31484). This suggested significant differences in species richness and genetic composition of bacterial communities from *Hyalomma* and *Amblyomma* tick species.

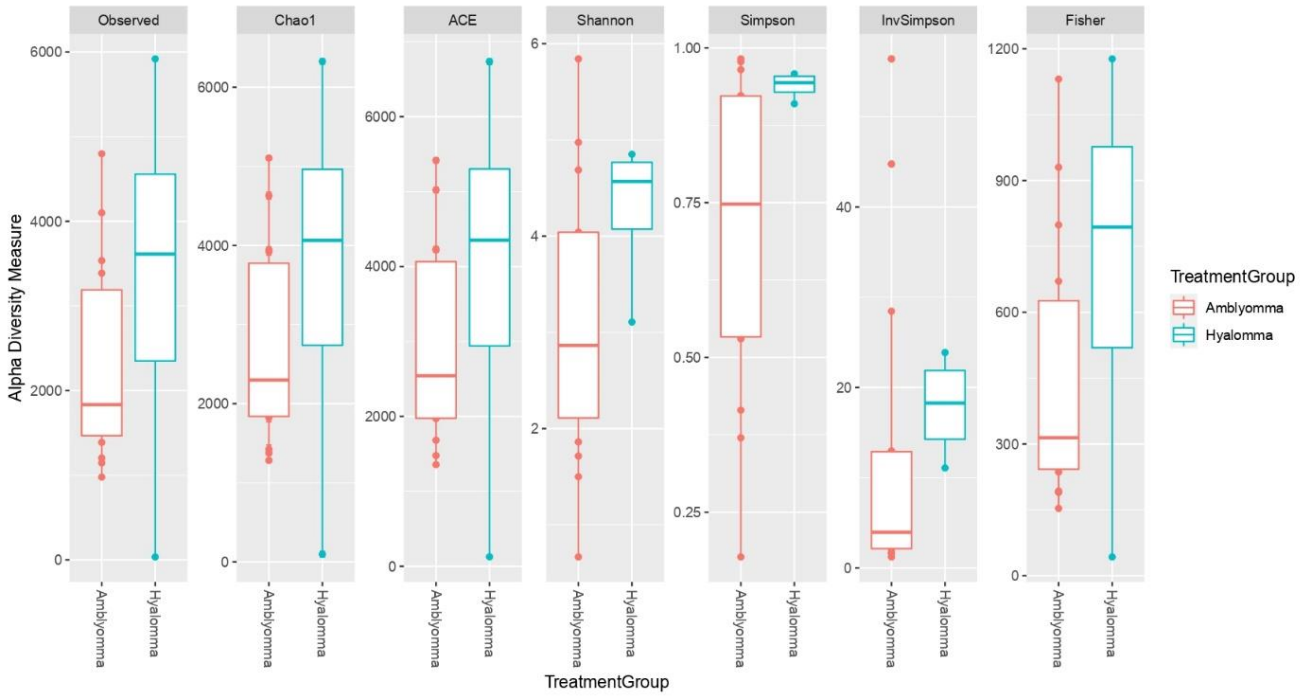



Figure 3.4.2 Diversity indices for Bacteria OTUs from *Amblyomma* and *Hyalomma* tick bacterial communities.

3.4.3 Comparative metagenomics of bacterial communities between *Amblyomma*, and *Hyalomma* tick species

High throughput sequencing and comparative metagenomics of *Amblyomma* and *Hyalomma* tick species revealed significant differences in microbial composition. However, the co-occurrence of some bacteria genera was also identified. A comparative metagenomic analysis performed using PCoA analysis showed selected significant differences in beta-diversity at (P>0.051), from tick samples resulting in tick samples clustering separately according to tick species but same samples overlapping (Figure 3.4.7). The total x-axis variances PCA1 was 36.6% and y-axis PCA2 was 13.1%, with prediction ellipses observed having tick species falling in a different ellipse and some sharing the same ellipse, reflecting similarities and variances in associated bacterial communities in tick samples.

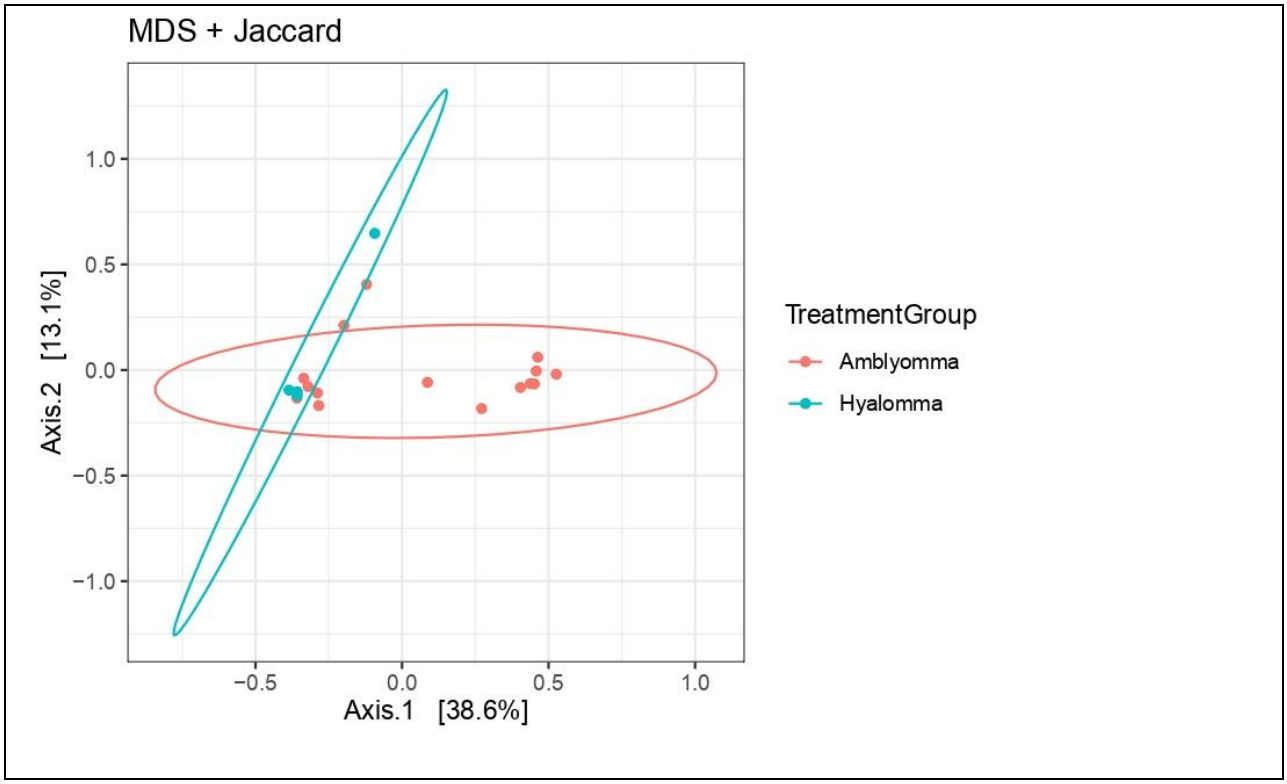



Figure 3.4.7 Principal coordinate analysis (PCoA) plot based on *Amblyomma* and *Hyalomma* ticks bacterial communities' genetic composition at the phylum level. Using MDS and Jaccard distances analysis with their significant differences observed at P>0.051.

### 3.4.4 Comparative metagenomics of bacterial communities from *Amblyomma* and *Hyalomma* tick species

Comparative metagenomics revealed variations in abundance of core microbiota in communities of bacteria in *Hyalomma,* and *Amblyomma* tick species (Figure 3.4.8). At the phylum level the relative abundance of Proteobacteria, Actinobacteria, was relatively high in *Amblyomma,* while Bacteroidetes and Firmicutes were dominant in *Hyalomma* tick species (Figure 3.4.8A). At the genus level, *Amblyomma* tick species had a comparatively high abundance of *Rickettsia* (38.9%), *Escherichia* (7%), *Arthrobacter* (3.6%), and *Coxiella* (2%), while bacterial communities in *Hyalomma* tick species had a high abundance of *Corynebacterium* (35.9%), *Porphyromonas* (14.4%), *Anaerococcus* (11.1%), *Trueperella* (3.7%), and *Helcococcus* (4.7%) shown in figure 3.4.8.B.

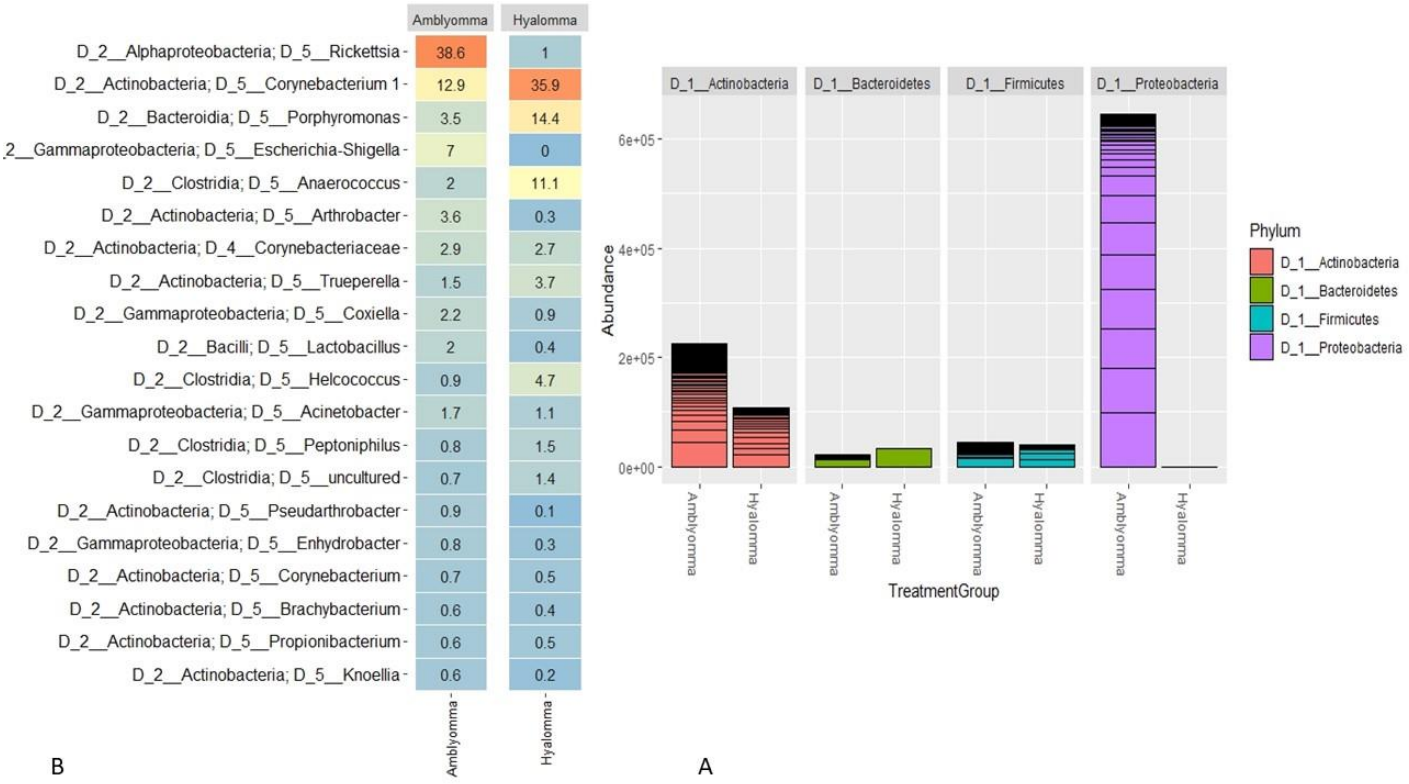



Figure 3.4.8 Comparative distribution and abundance pattern of 16S rRNA OTUs associated with *Hyalomma*, and *Amblyomma* tick species. A) Comparative stacked bar plot of bacterial composition at the Phylum level. B) Comparative heatmap showing percentage grouped abundance of combined samples of ticks at Genus level.

An extended error plot further revealed significant differences in bacterial communities at the phylum level, differences in mean proportions were observed mainly in Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria (Figure 3.4.9). Two-sided t-test statistical analysis and a multiple test correction storey FDR (fault discovery rate) at P<0.95 confidence interval using a bootstrap method. Corrected q-values showed significant differences in mean proportion at 95% confidence interval (Figure 3.4.9) between tick species.

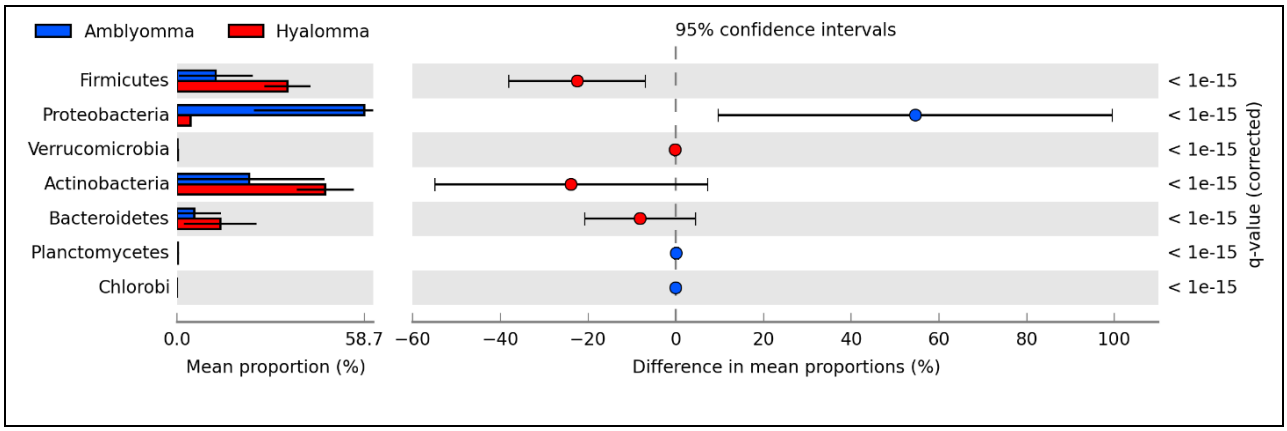



Figure 3.4.9. Extended error plot, illustrating comparative phylum-level bacterial composition in *Hyalomma* and *Amblyomma* tick species, and showing significant differences between mean proportions. This plot used a two-sided t-test statistical analysis and a multiple test correction storey FDR at bootstrap P<0.95. Corrected q-values are shown on the right.

3.4.2 Genetic composition and distribution of microbial communities in *Amblyomma* and *Hyalomma* tick specimens.

*Hyalomma* and *Amblyomma* tick species showed distinct microbial diversity and composition. At the phylum level, classified sequence reads revealed four leading major phyla: Proteobacteria (31.41%), Firmicutes (23,40%), Bacteroidetes (9.37%), Actinobacteria (34.65%), and while the remaining phyla sequence reads accounted for 1.15% in total abundance (Figure 3.4.3).

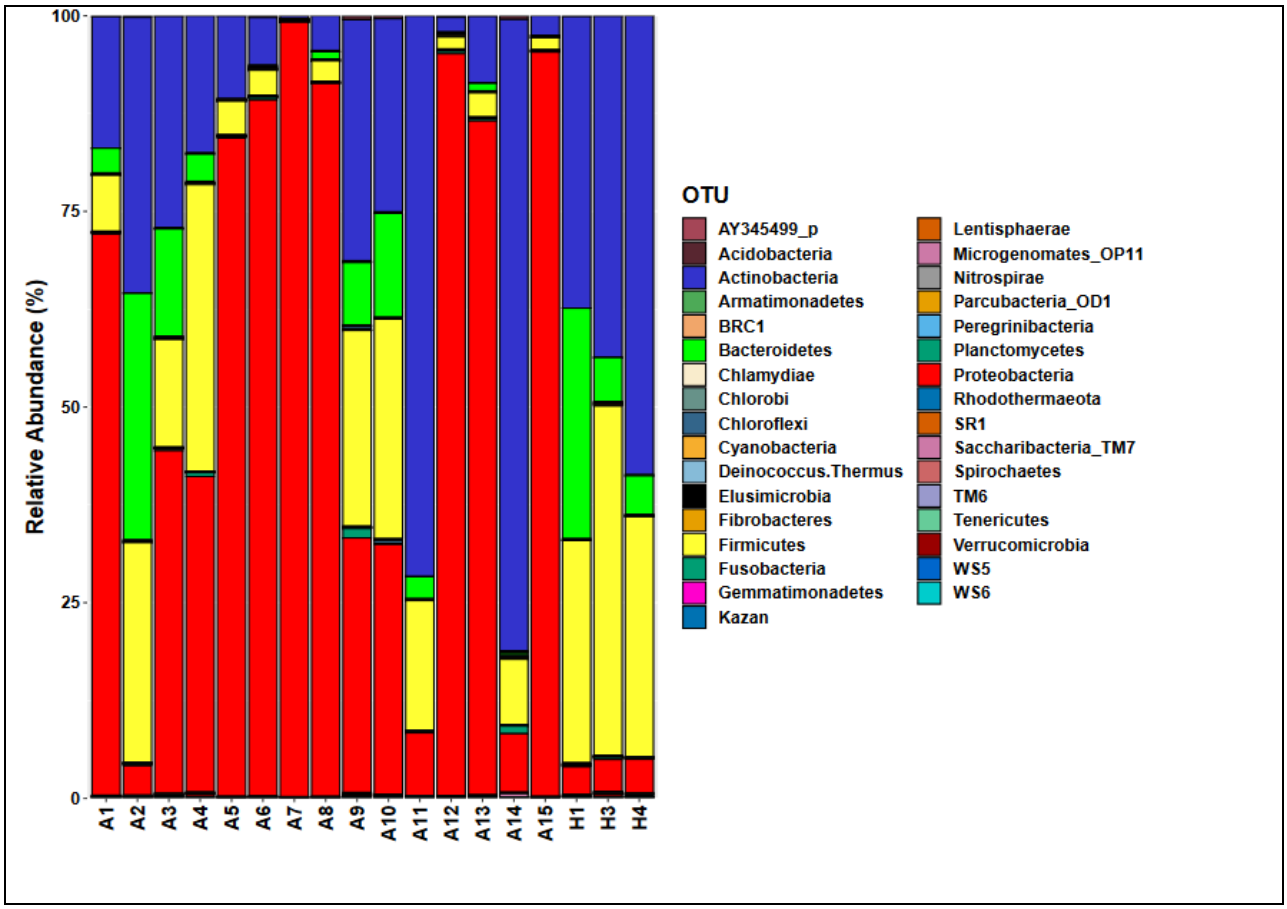



Figure 3.4.3 Stacked bar chart representing the taxonomic bacterial composition at phylum level based on 16S rRNA metagenomics of 18 tick samples. Each phylum is indicated in colour on the right-hand side.

A dendrogram heatmap was plotted to illustrate bacterial communities from each tick sample relatedness at genera level (Figure 3.4.4). *Amblyomma* tick species clustered together, while *Hyalomma* tick species clustered with two *Amblyomma* samples A2 and A11, suggesting shared genera and similarities in bacterial communities in both tick species. The top genera identified in this study consisted of *Rickettsia, Corynebacterium, Escherichia, Arthrobacter, Porphyromonas, Anaerococcus, Aerococcus, Trueperella, Coxiellaceae_uc, Helcococcus, Acinetobacter, Peptoniphilus, Coxiella, Enhydrobacter, Sporobacter, Brachybacterium, Porphyromonadaceae_uc, Bradyrhizobium, Phycicoccus, Brevibacterium, Cutibacterium, Pseudomonas, Staphylococcus, Romboutsia, Streptococcus, Mesorhizobium, Cupriavidus, Bacillus,* and *Ehrlichia* as shown in a heat map and stacked bar plot ( Figure 3.4.5, and Figure 3.4.6).

*Amblyomma* tick sample A5 had a very high abundance in *Ehrlichia* genera as illustrated in a stacked bar blot in figure 3.4.5, while A11 and H3 revealed high abundance in *Coxiella* genera. *Rickettsia* genus was dominant in most of the *Amblyomma* tick samples but was less abundant in *Hyalomma* tick species shown in figure 3.4.6. *Corynebacterium* and *Porphyromonas* were prevalent in both tick species varying in percentage distribution in each tick.

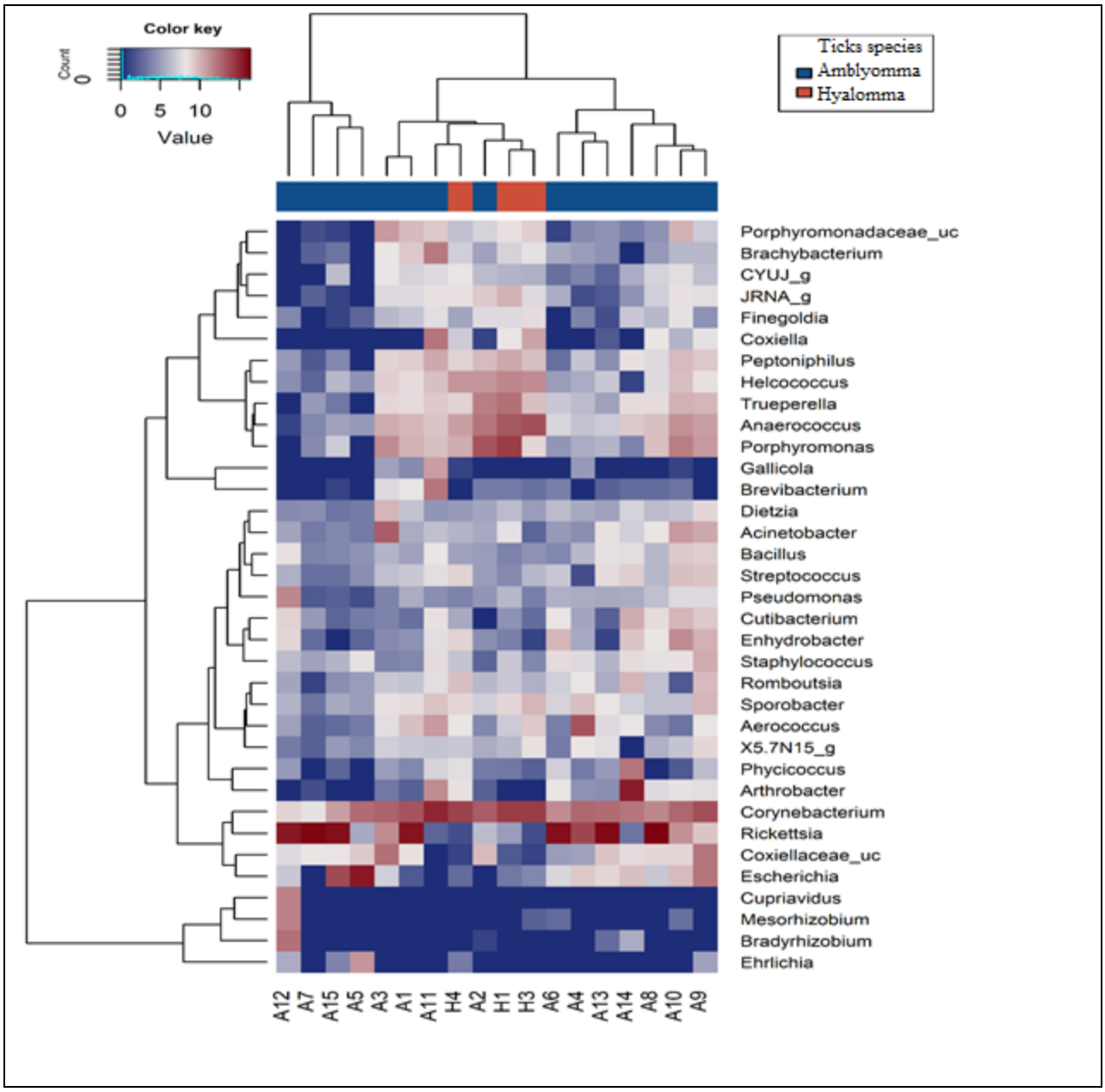



Figure 3.4.4 Dendrogram heatmap based on similarity and distribution of bacteria communities in *Hyalomma*, and *Amblyomma* tick species, at the genus level. Dendrogram linkages and distance of the bacterial genus are not phylogenetic but based upon the relative abundance of genera within individual tick samples. Heatmap showing top 35 genera abundance at y-axis while the x-axis represents the individual tick samples.

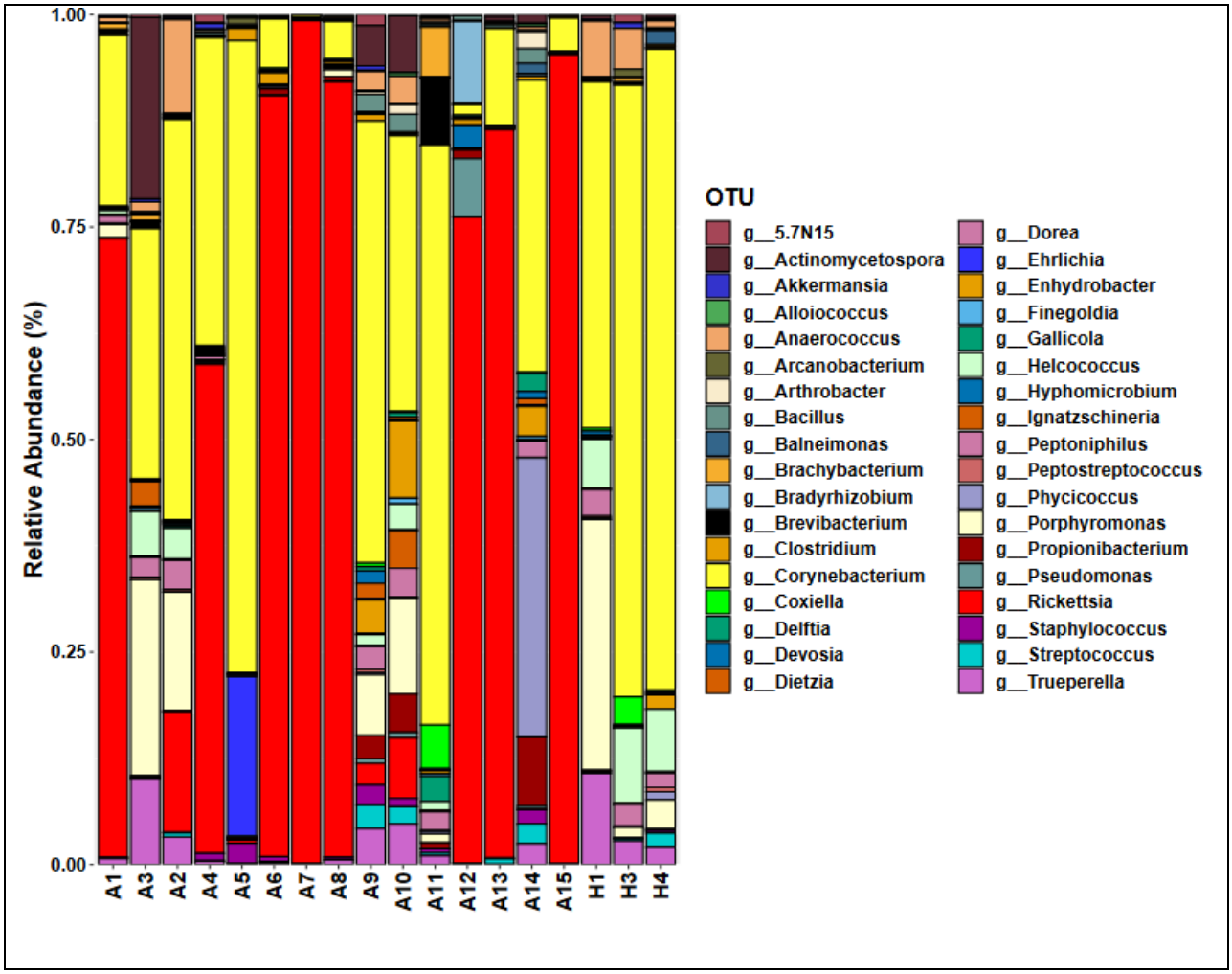



Figure 3.4.5 Stacked bar chart representing the taxonomic bacterial composition at genus level based on 16S rRNA identification. The colour on the right shows the genera and its relative abundance is indicated in the graph.

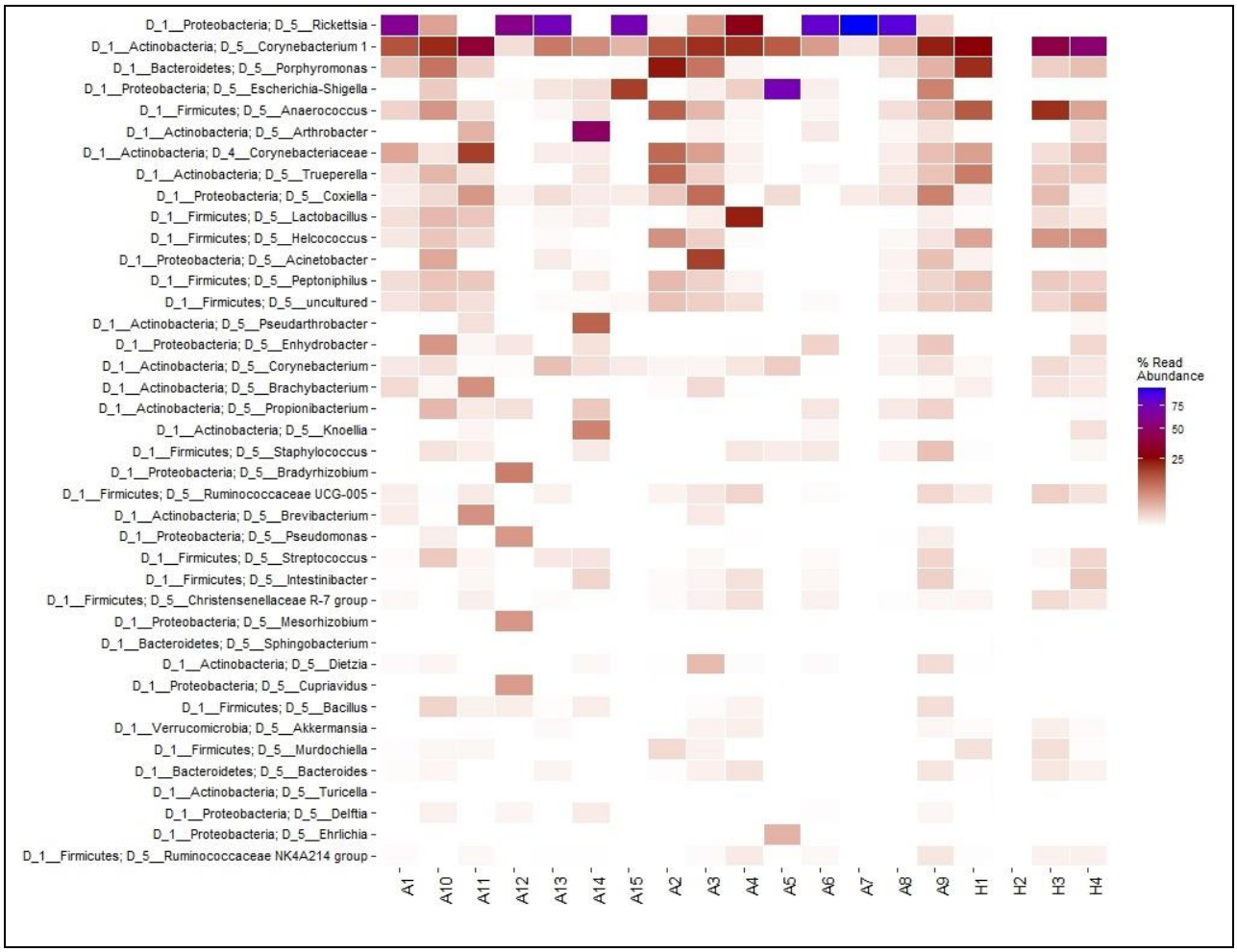



Figure 3.4.6 Heatmap based on percentage abundance reads of bacterial OTUs at class and genus level between tick samples.

Attempts to identify bacteria communities to species level revealed *Rickettsia rickettsii* group as the most abundant group followed by *Corynebacterium* groups such as *C. xerosis, C. falsenii, C. resistens, C. striatum, C. epidermidicanis,* and *C. pseudotuberculosis group.* Species such as *Porphyromonas levii, Trueperella pyogenes, JQ480818_s* (Coxiella endosymbiont), *Ehrlichia ruminantium* group, and *Coxiella_uc* were among the highly expressed species of bacteria (Figure 3.4.6b). The top 60 species identified are shown in Appendix 2 (Supplementary 3.2).

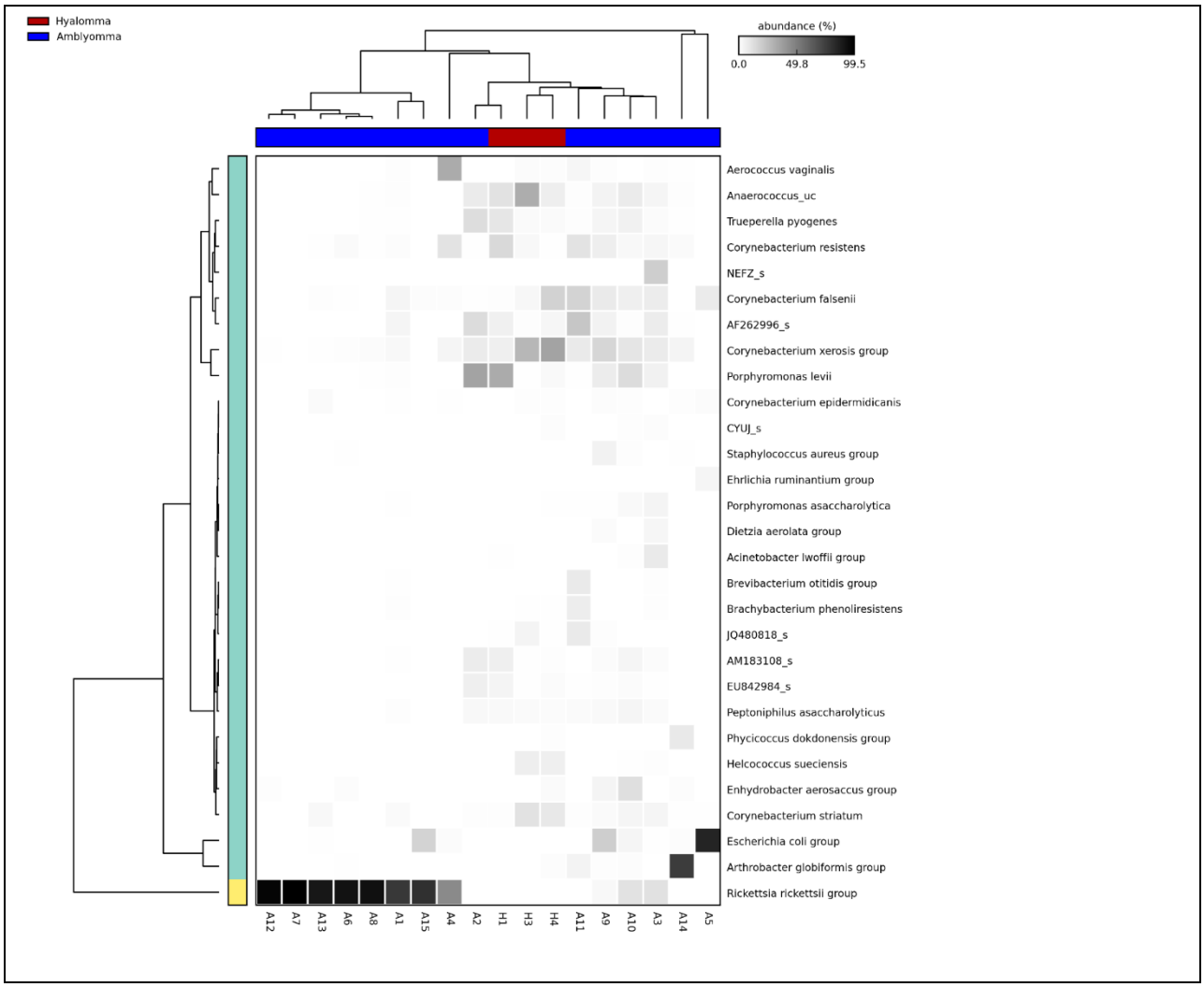



Figure 3.4.6b Dendrogram heatmap based on similarity and distribution of bacteria communities in *Hyalomma*, and *Amblyomma* tick species, at the species level. Dendrogram linkages and distance of the bacterial genus are not phylogenetic but based upon the relative abundance of species within individual tick samples. Heatmap showing top 35 species abundance at y-axis while the x-axis represents the individual tick samples.

3.4.5 Unique and core-occurrence of bacterial genera in communities between *Amblyomma* and *Hyalomma*

A Venn diagram was created using the Amp Venn package of the R program to illustrate unique and shared bacterial genera between communities of bacteria in *Amblyomma* and *Hyalomma* (Figure 3.4.10). Overall, 74.4% OTUs of core microbial bacteria were shared between tick species, while 6.3% OTUs were found to be unique to communities in *Amblyomma* and 8.9% OTUs were unique to communities in *Hyalomma* ticks. About 10.6% of non-core microbial bacteria were identified.

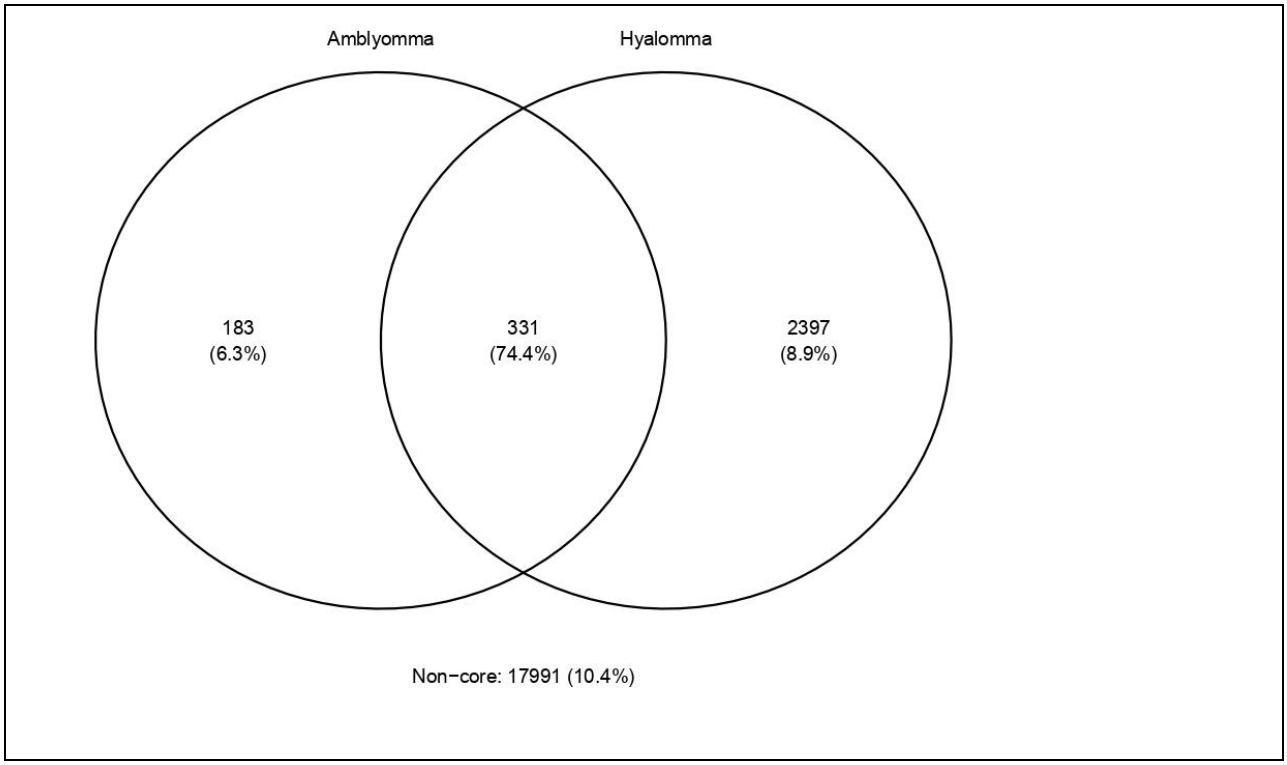



Figure 3.4.10 A Venn diagram illustrating shared and unique core microbial bacterial OTUs in communities of bacteria associated with *Amblyomma,* and *Hyalomma* tick samples. Shared core microbial OTUs were 74.4% and unique OTUs were *Amblyomma* 6.3% OTUs and *Hyalomma* 8.9% OTUs, 10.4% of non-core microbes.

3.4.6 A comparison in pathogenic microorganism distribution in tick samples

Pathogenic microorganisms identified in ticks included bacteria in the genera *Rickettsia, Ehrlichia, Coxiella, Porphyromonas, Trueperella, Corynebacterium,* and *Helcococcus.* An extended error plot revealed significant differences in the means of occurrence of bacterial pathogens in *Amblyomma* and *Hyalomma* ticks (Figure 3.4.11). The extended error plot was constructed using a two-sided t-test statistical analysis and a multiple test correction storey FDR (fault discovery rate) at a 95% confidence interval using a bootstrap method. In Amblyomma tick species, dominant pathogens were identified in the following genera *Rickettsia, Ehrlichia, Coxiellaceae_uc, Bacillus, Escherichia,* and *Porphyromonadaceae_uc* while in *Hyalomma* ticks pathogens highly expressed were in the genera *Porphyromonas, Trueperella, Corynebacterium, Coxiella,* and *Helcococcus.*

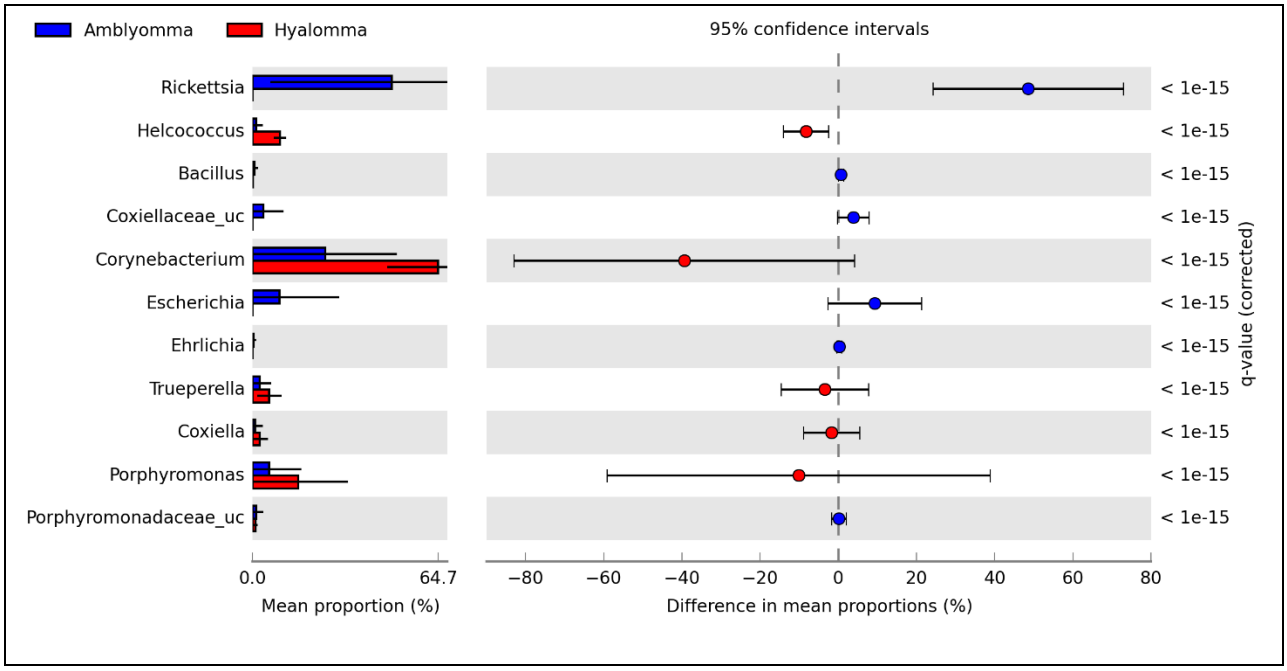



Figure 3.4.11 Extended error plot, illustration comparative pathogenic bacterial composition in *Hyalomma* and *Amblyomma* tick species. Using two-sided t-test statistical analysis and a multiple test correction storey FDR at bootstrap (P>0.95). Pathogens that dominated in *Amblyomma* are in blue while pathogens dominating *Hyalomma* are in red.

3.4.7 Predicting functional profiles through 16S rRNA gene-based metagenomics analysis.

Bacterial communities 16S rRNA metagenomics data from *Hyalomma* and *Amblyomma* tick samples were analyzed for functional biomarkers using the PICRUSt algorithm, inferring from KEGG annotated database level 2 pathways. Pooled predicted data was imported to STAMP version 3 software for statistical analysis and visualization. Differences were considered significant at $P< 0.05$ using White's non-parametric t-test.

Genetic information processing, environmental information processing, and metabolism were the most enriched KEGG level 2 pathways from both microbial communities of *Hyalomma*, and *Amblyomma* tick species. This was closely followed by cellular information processing pathways and finally human disease pathways being the least as shown in the heatmap figure 3.4.12. to compare KEGG pathways between two communities of bacteria in *Hyalomma* and *Amblyomma* ticks an extended error plot was constructed in figure 3.4.13.

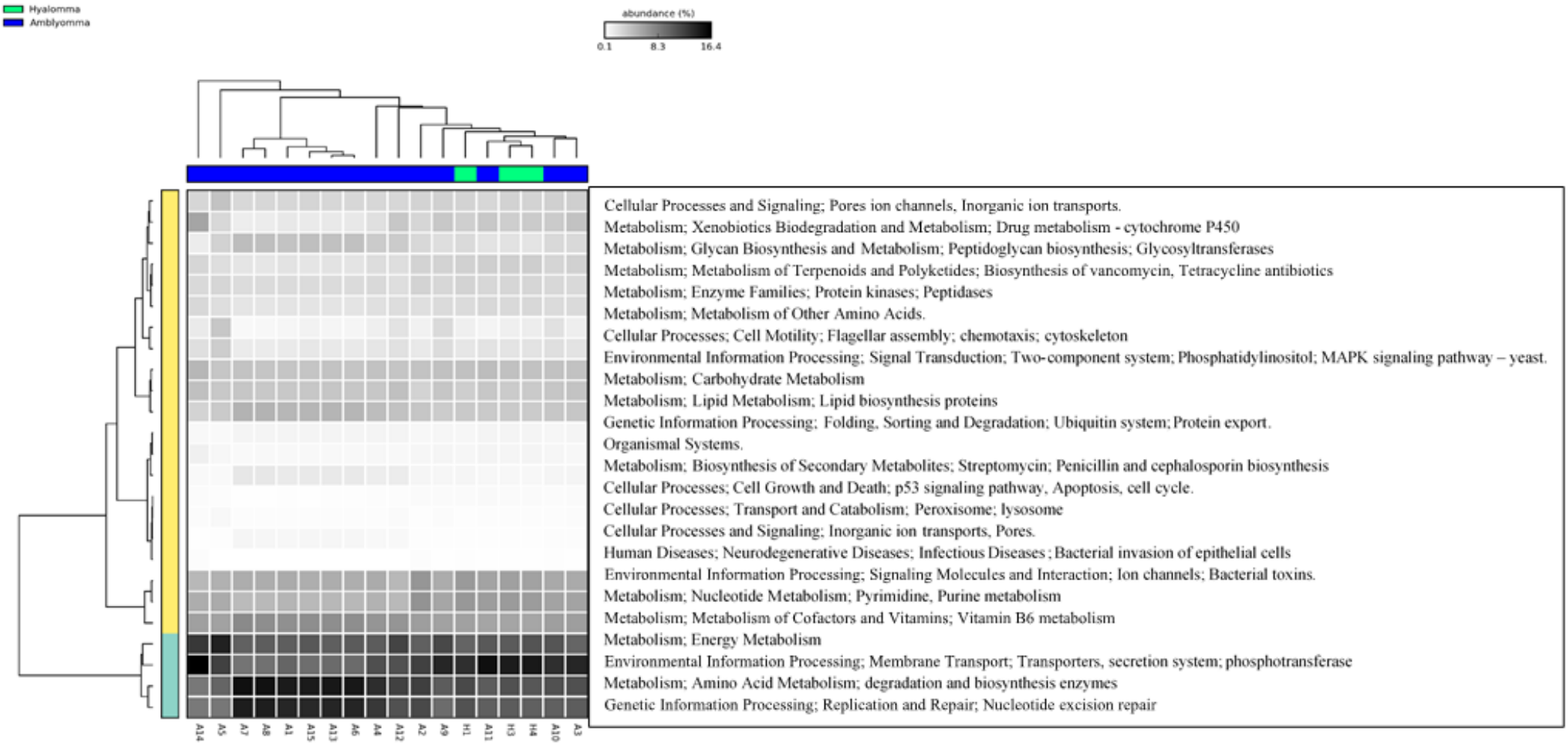



Figure 3.4.12 A heatmap showing detected level 2 of KEGG functional pathways of biomarkers predicted using PICRUSt. Dendrograms demonstrate how each tick sample cluster.

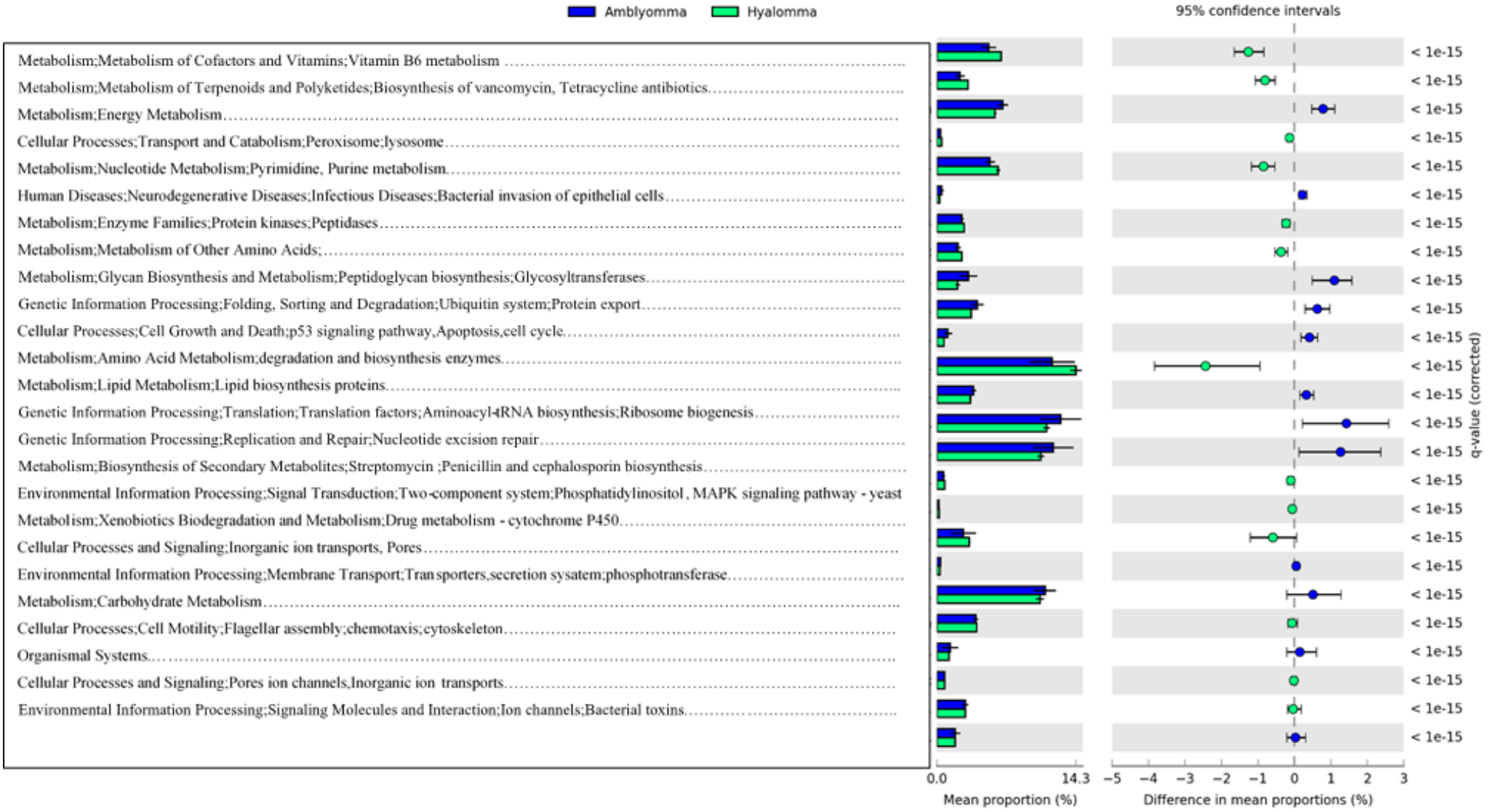



Figure 3.4.13 Extended plot based on storey FDR of predicted functional categories. Plotted using White's non-parametric t-test, using a 95% confidence interval using bootstrap CI method. Comparing pulled functional genes from communities of bacteria in *Amblyomma*, and *Hyalomma* ticks.

The most abundant pathways were the genetic information processing pathways involved in firstly, the biogenesis of ribosomal protection proteins. Secondly protein sorting, protein export, and finally aminoacyl-tRNA biosynthesis from microbial communities of both tick species. Foundationally, these pathways are very important in antimicrobial resistance.

The highly expressed metabolic pathways included the following; amino acids metabolism, degradation, and biosynthesis of enzymes pathways; pathways in biosynthesis of secondary metabolites such as streptomycin, penicillin, and cephalosporin; biosynthesis of glycan, peptidoglycan, and glycosyltransferases pathways; pathways in the metabolism of cofactors, vitamins pyrimidine, and purine; pathways in biodegradation of vancomycin, tetracycline antibiotics, and metabolism of terpenoids and polyketides and lastly, xenobiotics biodegradation by cytochrome P450 pathways. Consequently, these pathways are responsible for enzyme-derived antibiotic resistance from communities of bacteria detected in ticks.

The highly enriched environmental information processing pathways involved; genes responsible for membrane transport proteins and efflux pumps; pathways of secretion systems and phosphotransferases enzymes; and two-component systems, phosphatidylinositol, and MAPK signaling pathways.

Cellular processing pathways were highly enriched involved; porins regulation pathways, inorganic ion transport pathways; and pathways involved in bacterial invasion of the epithelial cells of eukaryotes. Overall, these pathways are significant contributors to bacterial survival against antimicrobial agents.

At KEGG level 2, no significant differences were observed in tick bacterial communities' composition of functional biomarkers, based on a very high correlation R2 value of R2=0.957, at

P<0.05 (Figure 3.4.14). This is demonstrated on a scatter plot in figure 3.4.14 based on storey FDR plotted using White's non-parametric t-test analysis at a 95% confidence interval.

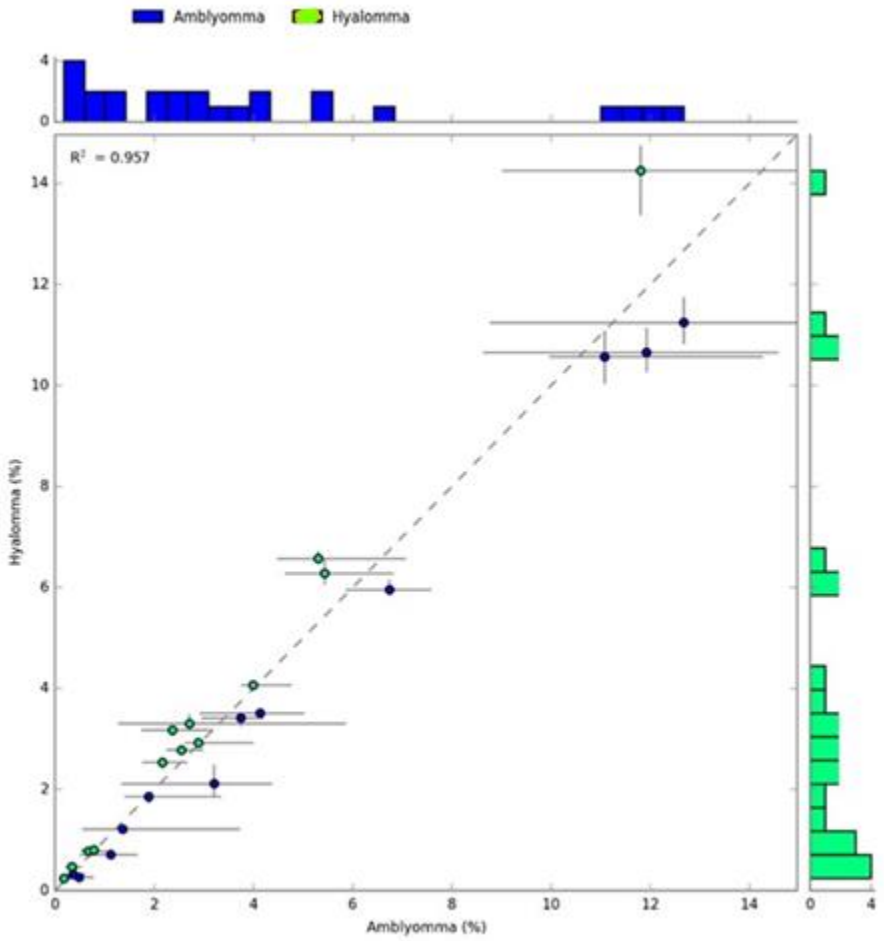



Figure 3.4.14 Scatter plot showing how bacterial communities predicted functional genes scattered in comparison, in *Amblyomma* and *Hyalomma* tick species. The white's non-parametric t-test using bootstrap dissimilarity showed that clusters were significant at (R=0.957, P<0.05).

3.4.8 KEGG Ortholog predicted drug resistance biomarkers in bacterial communities.

To identify tick-borne bacterial resistance biomarkers, we adopted the PICRUSt algorithm. That Inferred from the KEGG Ortholog (KO) database and analyzed using LEfSe. Visualization and statistical analysis were performed using STAMP software (version 2.1.3). PCoA was generated to show the clustering of microbiota-predicted genes, while heatmaps showed gene enrichment in bacterial communities from tick species. The top 50 KO resistance biomarkers are shown in figure 3.4.16 and Appendix 3. These included genes that code for efflux pumps, drug degrading enzymes, drug modifying enzymes, secretion systems proteins, and ribosomal protection proteins, their relative abundance is illustrated in a stacked bar plot (Figure 3.4.15) and a heatmap (Figure 3.4.16).

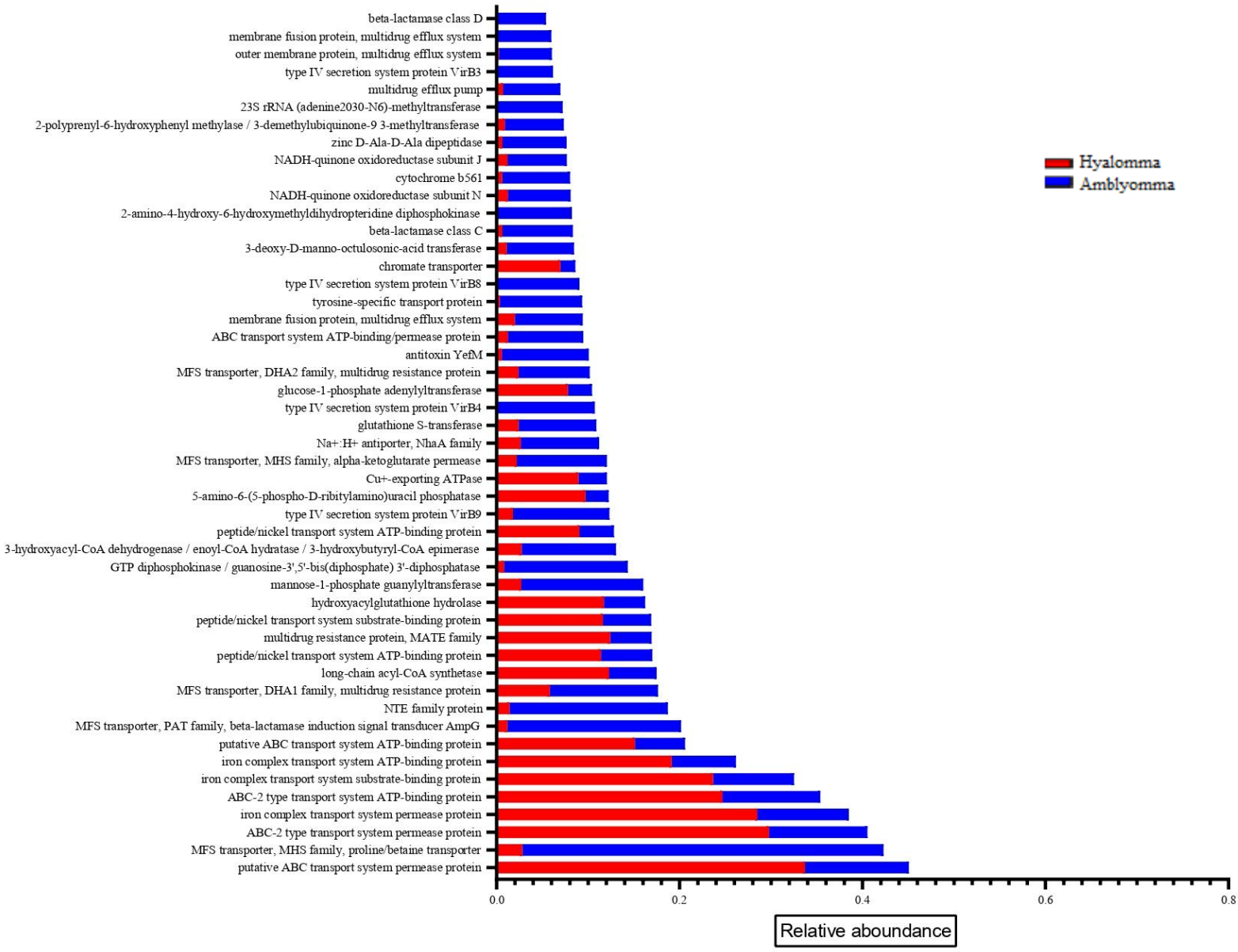



Figure 3.4.15 Stacked bar plot comparing relative abundances in KO functional biomarkers from tick bacterial communities. The red colour represents the relative abundance of genes from bacterial communities in the *Hyalomma* tick species while the blue colour represents the relative abundance of genes from bacterial communities in the *Amblyomma* tick species.


Figure 3.4.16 Heatmap comparing KO functional biomarkers predicted using PICRUSt and LEfSe, associated with bacterial communities in *Hyalomma* and *Amblyomma* tick species.

The resistance markers exceedingly enhanced were drug resistance efflux pumps, transporters, and porins (Figure 3.4.15). The major facilitator superfamily (MFS) transporters were dominant in bacterial communities from *Amblyomma* tick species. While ATP binding and permeases ABC-2 type transport systems were greatly enhanced in communities of bacteria in *Hyalomma* tick species. Outer membrane multiple drug efflux pumps (MDR) and efflux protein of the MATE family co-occurred in both communities.

Drug resistance MFS efflux pumps identified in this study fell under three major families. Firstly, genes of the MHS family encoding alpha-ketoglutarate permeases and the proline/betaine transporters. Secondly, the PAT family genes encoding beta-lactamase induction signal transducer AmpG. Finally, the DHA1 and DHA2 family genes encoding for multidrug resistance proteins as well as MFS transporter family efflux pumps (tetA) were identified.

Antimicrobial resistance multiple drug efflux systems were also revealed, such as membrane fusion proteins, outer membrane proteins, and the MATE family of proteins. Equally, major contributors to fluoroquinolone resistance are drug antiporters in the NhaA family such as Na+: H+ antiporters were also identified. Moreover, metal resistance genes identified in these communities involved ATP-binding protein systems of the iron complex transport, peptide/nickel transport, and Cu+-exporting ATPase.

Most importantly KEGG KO predicted analysis of bacterial communities from *Hyalomma* and *Amblyomma* tick species revealed drug resistance enzymes. Penicillin degrading enzymes such as the beta-lactamases in classes c and d, as well as the penicillin inhibiting, and modification guanylyltransferase (GTase) and methyltransferase enzymes were identified. Furthermore, both communities revealed enzymes conferring ribosomal resistance to macrolides such as 23S rRNA (adenine2030-N6)-methyltransferase and 3-deoxy-D-manno-octulosonic-acid transferase.

Additionally, ribosomal protection protein biosynthesis enzymes such as GTP diphosphokinase or guanosine-3',5'-bis (diphosphate) 3'-diphosphatase were also detected. These enzymes are important in ribosomal protection drug resistance.

Furthermore, drug detoxification enzymes namely the glutathione S-transferases known to inhibit the MAP kinase pathway, and antitoxin YefM proteins involved in modulation of toxins as well as environmental stress were also detected. Genes coding for enzymes and proteins involved in virulence and pathogenesis such as AraC family transcriptional regulators; the transcriptional activator of adiA; and bacterial transpeptidases (Sortase A); as well as versatile type iv secretion system proteins (virB4, virB6, virB8, virB9, and virB11) were also identified (Figure 3.15 and Figure 3.16). These are protein complexes normally powered by ATP to secrete protein toxins essential in pathogenesis. They are imperative in bacterial survival as well as drug resistance. Significant differences in community biomarkers were identified based on a very low correlation value of R2= 0.001 (Figure 3.4.17) in a scatter plot.

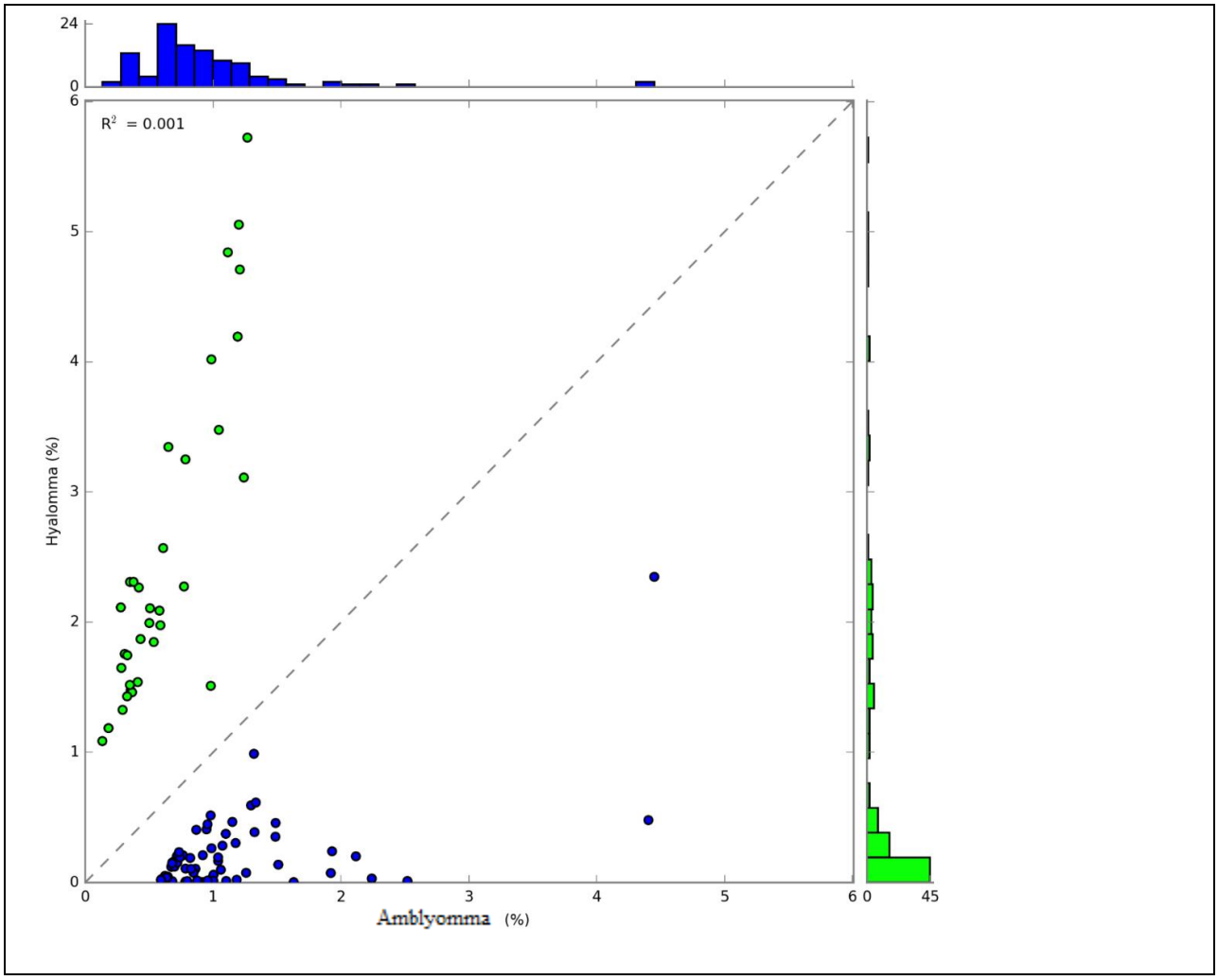



Figure 3.4.17. KO functional biomarkers scatter plot showing clustering of biomarkers from communities of bacteria in *Amblyomma* and *Hyalomma* tick species. The plot used a two-sided, White's parametric t-test at 95% confidence interval with DP bootstrap method and R=0.001. Bacterial communities in *Amblyomma* tick species were plotted in blue colour while bacterial communities in *Hyalomma* tick species were plotted in green colour.

## 3.5 DISCUSSION

In the current study, the multiple host ticks from genera *Amblyomma* and *Hyalomma* tick were collected infesting Nguni cattle in Roodaplate ARC research farm. Previous studies have confirmed the presence of these tick species in South African cattle (Fyumagwa *et al.*, 2011; Spickett *et al*., 2011; Mtshali *et al.*, 2017; Halajian *et al*., 2018). *Amblyomma* and *Hyalomma* tick species are known to harbor pathogenic bacteria such as *Rickettsia*, *Coxiella*, *Ehrlichia*, and *Anaplasma* (Fedorina *et al*., 2019; Guo *et al*., 2019). Hence, in this study, a total of 612 bacterial genera were identified from *Amblyomma* and *Hyalomma* tick's species. These microbial communities are composed of pathogenic, nonpathogenic, and tick commensal flora, as well as endosymbiotic bacteria in *Amblyomma*, and *Hyalomma* tick species. Our analysis revealed dominant bacterial genera to be *Rickettsia*, *Corynebacterium*, *Porphyromonas*, *Trueperella*, *Helcococcus*, and *Actinomycetospora* detected at different abundances within tick samples. While, an attempt to identify detected bacteria to species level figure 3.4.6b revealed species in groups, this is due to a conserved nature of the 16S rRNA gene having a low resolution to identify bacteria to species level (Papa *et al*., 2020). The most dominant species were groups of *Rickettsia rickettsia*, *Corynebacterium* group (*C. xerosis*, *C. falsenii*, *C. resistens*, *C. striatum*, *C. epidermidicanis*, and *C. pseudotuberculosis group*), *Porphyromonas levii*, *Trueperella pyogenes*, *JQ480818_s* (Coxiella endosymbiont), *Ehrlichia ruminantium* group, and *Coxiella_uc* species groups.

The composition of bacterial communities was further characterized using next-generation sequencing bioinformatics tools for alpha and beta diversity comparative analysis. Beta diversity indices measures indicated high diversity of bacterial species in *Hyalomma* tick species in comparison with *Amblyomma* tick species shown in Figure 3.4.2. The alpha diversity PCoA plot (Figure 3.4.7) indicated sample clustering according to tick species. This observation used

generalized UniFrac distances and differences were considered significant at P>0.05, indicating differences in bacterial composition between species. Non-parametric t-test analysis, using an extended error bar chart (Figure 3.4.9) also showed significant differences between communities of bacteria, at P>0.05. Also, metagenomic microbiome comparative analysis between *Amblyomma* and *Hyalomma* tick species found significant differences in diversity, composition, and core microbial genera (Ven diagram Figure 3.4.10). The observed differences in bacterial communities of *Amblyomma* and *Hyalomma* tick species are in conformation with previous studies of microbiome ecological studies in tick species (Baauw *et al*., 2019; Lim *et al*., 2020; Mtshali *et al*., 2017).

Comparative metagenomics findings revealed *Amblyomma* tick species bacterial communities dominated by *Rickettsia* genera, followed by *Corynebacterium*, *Escherichia*, *Porphyromonas*, and *Coxiellaceae_uc* bacterial genus. While, *Hyalomma* tick species were dominated by *Corynebacterium* genera, and other core-genera most likely endosymbionts such as *Porphyromonas, Coxiella, Anaerococcus*, *Aerococcus*, *Helcococcus*, and *Trueperella*. This was also previously reported as bacterial genera associated with *Amblyomma*, and *Hyalomma* tick species (Lim *et al*., 2020; Sánchez-Montes *et al*., 2019; Halajian *et al*., 2016; Lim *et al*., 2018). Bacterial community composition might have been influenced by the presence of cattle host blood engorgements; feeding habits; shape and size of mouthparts, and geographical location of the ticks samples as well as previous tick hosts as previously described (Lim *et al*., 2020).

Endosymbiotic bacteria were also identified, these are important in physiology, adaptation, molting between life stages, reproduction as well as essential nutrient supplies to ticks (Rounds *et al*., 2012; Szigeti *et al*., 2014; Brinkmann *et al*., 2019). *Amblyomma* species were found ununiformly associated with endosymbionts primarily *Rickettsia* and *Ehrlichia* genera, known for

their important contribution in supplement nutrients not found in blood (Rounds *et al*., 2012). Additionally, *Amblyomma* ticks nutritional dependence on bio-symbionts such as *Coxiellaceae_uc* and *Coxiella* genera has been well documented, experimentally proven, and bioinformatically supported (Ramaiah and Dasch 2018), and as a result, their significance is demonstrated by their detection in these communities. Similarly, endosymbionts detected in *Hyalomma* tick species involved genera of *Coxiellaceae_uc* and *Coxiella* as previously detected (Brinkmann et al. 2019). However, *Rickettsia* genera endosymbiotic reads were detected in trace amounts demonstrating their insignificance in *Hyalomma* tick species compared to *Amblyomma* tick species. Several genera identified require further investigation to determine their role as tick endosymbionts, as they may be important in veterinary and public health care, as well as in mitigation of tick-borne pathogen transmissions.

*Amblyomma* tick species were dominated by *Rickettsia* pathogenic genus that causes zoonotic diseases, composed of spotted fever and typhus groups (Branger *et al*., 2004; Klindworth *et al*., 2013). In an attempt to classify *Rickettsia* genus to species level, the data revealed *Rickettsia rickettsia* group of species that are agents of rocky mountain spotted fever, and they were detected across all *Amblyomma* tick samples confirming their presence in South Africa (Mtshali *et al*., 2017; Ringo *et al*., 2018). Previous studies have also shown the presence of *Coxiella burnetii* in all tick species identified in South Africa (Halajian *et al*., 2016; Eldin *et al*., 2017; Kuley 2017), and in the current study, organisms of the *Coxiella* genus were also detected. The findings are in agreement with other reports where high serological indices of *Coxiella* were detected all over Africa mainly in tick species of *Amblyomma, Hyalomma,* and *Rhipicephalus* (Mtshali *et al*., 2017; Guo *et al*., 2019; Halajian *et al*.. 2018). Additionally, pathogenic *Ehrlichia ruminantium* group of species that are agents of heartwater (ehrlichiosis) were detected in *Amblyomma* and *Hyalomma*

tick species. Previous studies in South Africa have also identified these pathogens in ticks (Allsopp, 2009; Ringo *et al.*, 2018; Guo *et al.*, 2019), however, sequence reads mostly corresponds to uncultured strains, therefore, isolation, genetic and bioinformatic characterization of detected pathogens are still required.

Apart from known tick-associated pathogenic genera, many other genera were detected, that might have originated from ruminant blood, ticks, and other sources. These include *Corynebacterium*, *Escherichia*, *Arthrobacter*, *Porphyromonas*, *Anaerococcus*, *Aerococcus,* and *Trueperella* genera and their OTU reads were detected in high abundance, and this observation was evident in studies by other researchers (Andreotti *et al.*, 2011; Yang *et al.*, 2015; Lim *et al.*, 2020). There is limited information on the involvement of these genera in tick biology, however, the observed high abundance suggests their importance, and as such more molecular studies are required to ascertain their role.

Bacterial species of the genus *Corynebacterium* are commonly identified on animal skin as the innocuous microbiome, some species have been identified as opportunistic pathogens causing zoonotic diseases. Ticks have not been studied extensively as vectors of microbial pathogens of this genus, however, Lim et al (2020) made this observation and concluded that ticks might have acquired them during feeding (Lim *et al*., 2020; 2018). Zoonotic opportunistic species from the *Corynebacterium* genus such as *Corynebacterium pseudotuberculosis* has been identified associated with secondary meningitis, caseous lymphadenitis, and Otitis media-interna in cattle; while, *C. xerosis*, *C. falsenii*, *C.bovis*, *C. resistans*, and *C. striatum* are associated with an abscess in the brain, mastitis, osteomyelitis, abortions, and arthritis; the mouth of an eagle; mastitis in cattle; bronchial aspirates and blood culture; abscess respectively (Leask, Blignaut, and Grobler 2013; Bernard 2012; Lim *et al*., 2020; 2018; Watts *et al*., 2000). In the current study, the

*Corynebacterium* genus was identified as highly expressed in all tick samples, the findings affirmed the presence of *Corynebacterium* species such as *C. xerosis, C. falsenii, C. resistens, C. striatum, C. epidermidicanis,* and *C. pseudotuberculosis* in *Hyalomma* and *Amblyomma* ticks.

*Trueperella pyogenes* an opportunistic pyogenic infections agent that causes otitis externa, abortions, metritis, infertility, and mastitis in cattle are mainly found in the mucus of livestock (Leask *et al*., 2013; Rezanejad *et al*., 2019). The 16S rRNA metagenomics analysis of ticks in this study implicated *Hyalomma* and *Amblyomma* tick species as reservoirs and possible vectors of *T. pyogenes* pathogens. Data of ticks as vectors of *T. pyogenes* is lacking but their contribution in transmission has been observed (Rzewuska *et al*., 2019).

Species of *Porphyromonas* genera are emerging animal and human pathogens with species such as *Porphyromonas levii* associated with bovine necrotic vulvovaginitis in cattle (Elad *et al*., 2004). In the current study, *Porphyromonas levii* species was the most dominant species in bacterial communities of both *Hyalomma* and *Amblyomma* ticks. This implicates ticks as reservoirs and potential vectors of *Porphyromonas levii,* however, there is a need for future studies, to determine *Amblyomma* and *Hyalomma* tick species as vectors to *P. levii* and the role of *P. levii* as endosymbionts to ticks.

To understand biomarkers related to antimicrobial resistance in communities of bacteria detected in the present study, a PICRUSt 16S-rRNA based functional biomarkers analysis was performed. The use of the PICRUSt algorithm to analyze 16S rRNA data has been proven accurate in predicting functional and resistance genes in microbial communities (Z. J. Chen *et al*., 2020; Langille *et al*., 2013b; Mukherjee *et al*., 2017). In this study, from the KEGG functional biomarkers identified (section 3.4.11), the most dominant KEGG level 2 pathways detected involved in resistance mechanisms of bacteria included metabolism, information processing,

environmental information processing, and cellular information processing (Figure 3.4.12). Antimicrobial genes under these dominant pathways involved those that code for the biosynthesis of ribosomal protection proteins; secondary metabolites; antimicrobial degradation enzymes; excretion proteins; coenzymes, and transport proteins; secretion systems; and phosphotransferases enzymes. This observation supports previous *in silico* findings analyzing resistance genes in tick-borne bacteria (Rolain and Raoult, 2005; Biswas *et al.*, 2008; Santos *et al.*, 2012; Martinez *et al.*, 2014).

Gene composition among bacterial communities was analyzed using an extended bar plot (Figure 3.4.13) based on storey FDR. The plots were made using white's non-parametric t-test, and bootstrap CI method at 95% confidence interval, significant differences were observed from differences in mean values of e-values. Furthermore, a correlation test was performed using a scatter plot (Figure 3.4.14), and samples clustered differently according to tick species demonstrating how bacterial communities influenced resistance gene composition.

PICRUSt algorithm further used KEGG Orthology (KO) database to annotate KO genes that were analyzed using LEfSe and relative abundance of genes calculated according to e-value. The main classes of KO genes associated with drug resistance that were highly expressed involved genes coding for drug efflux pumps; drug degrading and modifying enzymes; secretion systems proteins; and ribosomal protection proteins.

The most expressed efflux pumps were in the family of MFS, ABC-2, and MDR (Figure 3.4.15), their role in bacterial antibiotic resistance has been widely described (Blanco et al. 2016; Spyridaki 2002; J. M. Rolain 2005; Nikaido and Pagès 2012). The findings are in line with a previous study conducted by Rolain, (2005) who showed that *Rickettsia* genera were resistant to macrolides and beta-lactam antibiotics as a result of ABC-2 multiple drug transport systems. Tetracycline

resistance mechanisms have been reported to occur via tetracycline efflux pumps that are integral membrane transporters belonging to the family of MFS efflux pumps (Vranakis et al. 2012). Furthermore, the nomenclature has classified tetracycline efflux pumps (tetA) in the MFS transporter family, which have been shown to infer tetracycline specific resistance (Li *et al*, 2015). Vranakis and colleagues (2010) identified protein channels of MFS efflux pumps responsible for tetracycline resistance in *Coxiella burnetii*. Furthermore, MFS efflux pumps are also described in quinolone resistance in *Coxiella burnetii* (Vranakis et al. 2010). In this study, MFS multiple drug antiporters of NhaA proteins family such as Na+: H+ antiporters genes known for fluoroquinolone resistance were also present in both communities, these were previously identified in the *Coxiella* genus and found to confer resistance to fluoroquinolone (Vranakis et al. 2010). Bacterial communities in *Hyalomma* and *Amblyomma* had a very high relative abundance of MFS efflux pump genes suggesting their importance in tetracycline, quinolone, and fluoroquinolone resistance.

Numerous researchers have emphasized the contribution of MDR efflux pumps to the bacterial acquisition of antibiotic resistance (Vranakis *et al.*, 2010; Li *et al.*, 2015; Blanco *et al.*, 2016; Hwang and Yoon, 2019). Overexpression of efflux pumps in bacterial communities requires the development and addition of efflux pump inhibitors as part of the treatment. This is important in helping improve veterinary and medical treatment for tick-borne bacterial infections. Peptidomimetic compounds that are efflux pump competitive inhibitors such as phenylalanine arginyl beta-naphthylamide are effective in the treatment of infections caused by *Pseudomonas aeruginosa* (Askoura et al. 2011). However, pharmacodynamics and pharmacokinetics studies are required to determine efflux pump inhibitors that can be combined with antibiotics used in tick-borne bacterial communities.

Metal resistance is a co-selection factor that leads to the proliferation of resistance to heavy metals and antibiotics through co-resistance and cross-resistance mechanisms (Nguyen et al. 2019). Consequently, metal resistance genes identified in this study confirmed co-resistance, and cross-resistance mechanisms in both metal and antibiotic resistance among these communities, conferred by ATP-binding protein systems of the iron complex transport; peptide/nickel transport; and Cu+-exporting ATPase. Also, proteins involved in virulence and pathogenies identified in this study were mainly protein complexes normally powered by ATP to secrete protein toxins essential in pathogenesis. The coexistence of virulence and drug resistance has been extensively studied and is very important in bacterial survival and drug resistance (Schroeder *et al.*, 2017; Pan *et al.*, 2020).

Communities of bacteria in both tick species presented enzymatic antibiotic resistance through penicillin-degrading enzymes such as the beta-lactamases in classes C and D; inhibitors and penicillin modification guanylyltransferase (GTase); and methyltransferase enzymes. The presence of these genes is consistent with previous studies showing resistance to penicillin from tick-borne *Ehrlichia* genera (P Brouqui and Raoult 1990; Philippe Brouqui and Raoult 1992), *Rickettsia* genera (J. M. Rolain et al. 1998), and *Corynebacterium* genus (Lim et al. 2018).

Macrolide target site altering enzymes such as 23S rRNA (adenine2030-N6)-methyltransferase and 3-deoxy-D-manno-octulosonic-acid transferase were identified in both communities of bacteria. Additionally, macrolide ribosomal protection protein biosynthesis enzymes such as GTP diphosphokinase or guanosine-3',5'-bis (diphosphate) 3'-diphosphatase were also detected. Macrolide resistance is due to alteration or mutation of 23S ribosomal RNA and methylation of the domain V of 23S rRNA by methyltransferase enzymes (Fyfe et al. 2016). Previous studies have also detected resistance in *Rickettsia, Ehrlichia chaffeensis, Ehrlichia canis, Anaplasma phagocytophilum,* and *Francisella tularensis* tick-borne pathogens (Branger *et al.*, 2004; Biswas

*et al.*, 2008). Our study confirms the identified communities to have genes that confer macrolide resistance.

Enzymes involved in drug detoxification were also detected in both communities. The highly expressed involved glutathione s-transferases and antitoxin YefM. These might be important in the tick-bacterial community endosymbiotic relationship as these enzymes are involved in antibiotics and insecticide biodegradation (Seshadri et al. 2003; Machado-Ferreira et al. 2016).

## 3.6 CONCLUSION

In conclusion, next-generation sequencing-based study is a valuable approach in our understanding of intercellular bacterial community composition, their endosymbionts, and pathogens in ticks affecting cattle. In summary, this study shows *Hyalomma* and *Amblyomma* ticks species collected from Nguni cattle bacterial communities revealed *Rickettsia*, *Corynebacterium*, *Porphyromonas*, *Trueperella*, *Helcococcus*, and *Actinomycetospora* bacterial genera in high abundance. Furthermore, the PICRUSt predicted resistance biomarker analysis genetic tools, provide a good assessment of genes associated with antibiotic resistance, and have the advantage of overcoming several limitations of traditional approaches. The bacterial communities had a high prevalence of resistance biomarker genes that confer resistance to drug groups such as tetracyclines, fluoroquinolones, quinolones, beta-lactam, and macrolides. Combating antibiotic resistance is of great importance worldwide, understanding underlying molecular elements conferring resistance is an important approach towards finding solutions to antibiotic resistance. This type of study can assist veterinary and clinicians with effective therapeutic decisions for treatment, assist ecologists understanding microbial community ecology, as well as insights into the epidemiology of tick-borne diseases, and finally drug discovery. A valuable insight into tick endosymbionts is crucial in understanding their function, enabling us to harness this relationship to control ticks and tick-borne diseases.

# CHAPTER 4 *RHIPICEPHALUS EVERTSI EVERTSI*, *RHIPICEPHALUS SIMUS*, AND *RHIPICEPHALUS DECOLORATUS* TICK SPECIES 16S rRNA AMPLICON ASSESSMENT OF BACTERIAL COMMUNITIES AND THEIR FUNCTIONAL ANTIBIOTIC RESISTANT BIOMARKERS.

## 4.1 ABSTRACT

Ticks have a worldwide distribution and play an important role as vectors of diverse bacterial microorganisms. Tick-borne bacterial communities are composed of endosymbiotic and symbiotic bacteria essential for tick reproduction fitness, providing nutrients not found in blood meals, and biological importance to ticks. However, some of the tick-borne bacteria are pathogens of veterinary and medical important invertebrates. This study characterizes the genetic composition of tick-borne microbial communities using the Miseq Illumina platform to sequence the V3 and V4 variable regions of 16S rRNA and using bioinformatics tool (PICRUSt) to predict functional biomarkers associated with antimicrobial resistance. A total of 28 *Rhipicephalus evertsi evertsi* (n=11/29), *Rhipicephalus simus* (n=9/29), and *Rhipicephalus decoloratus* (n=8/29) tick samples were used. Upon sequencing, about 1326605 sequence reads were generated and assigned to 33424 operational taxonomic units (OTUs) with each tick sample having an average of 45745 reads. The dominant phyla detected in tick samples included Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, and Proteobacteria. Among these phyla, beta diversity indices analysis showed no significant difference ($P<0.05$) in the bacterial communities identified. The top ten predominant genera were *Corynebacterium, Porphyromonas, Anaerococcus, Trueperella, Enhydrobacteria, Rickettsia, Helcoccoccus, Coxiella, Arthrobacter*, and *Lactobacillus*, varying in percentage distribution. Among these genera, pathogenic, endosymbiotic, and symbiotic bacteria were identified. Comparative metagenomics of these genera showed a significant difference in observed OTUs between tick samples, however, all ticks shared 70.8% core microbiome. PICRUSt KEGG Level 2 predicted functional analysis suggested that functions related to genetic, environmental information processing, and metabolism were highly enriched. Furthermore, PICRUSt KEGG Orthology (KO) analysis revealed functional resistant biomarkers conferring resistance to groups

of antibiotics such as beta-lactam, tetracycline, and macrolides. The most dominantly overexpressed KO biomarker genes detected were for efflux pumps such as ABC, ABC-2, MATE, RND, and MFS; followed by metal transporters systems such as iron complexes, energy coupling, multiple sugars, Cu+- exporters, peptide/nickel, and divalent union/Na+; as well as drug degrading and modifying enzymes. These results reveal communities of tick-borne bacterial genetic composition and associated antibiotic resistance biomarkers. The study findings contribute remarkably to our understanding of pathogenic, endosymbionts, and symbiotic tick-borne bacterial functions in tick physiology, pathogen transmission as well as antimicrobial resistance profiles.

## 4.2 INTRODUCTION

Ticks are hematophagous arthropods classified in the order Ixodida and class Arachnida. They are very important vectors of numerous pathogenic, symbiotic, and commensal bacterial communities (Andreotti et al. 2011; Chicana et al. 2019; Brinkmann et al. 2019). There is limited information on bacterial communities of several tick species, this is despite their importance in vector competence and pathogen transmission dynamics (Vila et al. 2019; Chicana et al. 2019). Tick-borne bacterial pathogens of significance to human and animal health include species of *Anaplasma, Borrelia, Coxiella, Ehrlichia, Francisella,* and *Rickettsia* (Kirkan *et al*., 2017; Ringo *et al.*, 2018a; Lim *et al.*, 2020). While, some species of *Rickettsia, Coxiella,* and *Francisella* are endosymbiotic to ticks playing an important role in supplying essential nutrients not found in blood meals, and also provide a reproductive and survival fitness advantage (Machado-Ferreira *et al.*, 2016; Seo *et al*., 2020). Bacterial diseases transmitted by ticks include anaplasmosis caused by (*Anaplasma* sp), Query-fever caused by (*Coxiella* sp), rickettsioses or rocky mountain fever caused by (*Rickettsia*) and heat water caused by (*Ehrlichia* sp) that are of veterinary and medical importance (Santos *et al.*, 2013; Kirkan *et al*., 2017; Nyangiwe *et al.*, 2018).

Multiple hosts *Rhipicephalus* tick species parasitize mainly cattle and large ruminants, several researchers have identified different bacterial communities transmitted by *Rhipicephalus* ticks (Papa et al. 2020; Guo et al. 2019; Halajian et al. 2016; Junquera 2018). However, information on the distribution, diversity, prevalence, and composition of microbial communities needs consistent updates with modern and highly sensitive techniques such as next-generation sequencing (Guo et al. 2019). Next-generation sequencing targeting of 16S rRNA amplicon for variable regions (V1-V9) allows the identification and characterization of microbial communities (Papa et al. 2020).

Furthermore, attaining this data allows the use of bioinformatics tools such as PICRUSt algorithms that can be used to further investigate functional annotations in bacterial communities.

The present study aimed to investigate the composition of microbial communities associated with *Rhipicephalus evertsi evertsi*, *Rhipicephalus simus,* and *Rhipicephalus decoloratus* tick species from Nguni cattle breeds in Roodeplaat ARC-research farm, employing 16S rRNA metagenomics. Furthermore, to the characterization of antimicrobial functional biomarkers using a bioinformatics tool (PICRUSt).

## 4.3 METHODOLOGY

### 4.3.1 Tick samples collection and processing

Ticks were collected from the Roodeplaat ARC research farm (Figure 3.1). To collect the ticks, tweezers were used to remove ticks from cattle ensuring the mouthparts remained intact. Ticks were then placed into Eppendorf test tubes containing 70% ethanol for preservation. The cattle bite site was carefully cleaned with 70% ethanol. Collected ticks were then stored at -80°C at UNISA Eureka Life science laboratory for further DNA extraction. A total of 112 ticks were identified to species level using standard taxonomic identification keys with the help of a Veterinarian Dr. Skhumbuzo Mbizeni (UNISA) and Goodwill Makwarela (Ph.D. student) and 28 *Rhipicephalus* tick species were identified and were given the following identifies *R. evertsi evertsi* (RE1 to 11), *R. simus* (RS1 to 10), and *R. decoloratus* (RD1 to 8) tick species.

### 4.3.2 Tick lysis and DNA extraction

After identification, a total of 28 *R. evertsi evertsi* (n=11), *R. simus* (n=10), and *R. decoloratus* (n=8) ticks were washed with nuclease-free water until ethanol was washed off, then air-dried. Ticks were then cut from the second legs going up the capitulum to target the upper section of the tick containing salivary glands under a light microscope. The upper sections of ticks were cut into pieces and added to 0.5 ml screw-cap tubes. Omega TL lysis buffer and 25µl of Proteinase K were added to each tube for lysis to occurs over 24-hours, then incubated at 56°C. DNA extraction was performed using the E.Z.N.A. tissue DNA kit (Omega Bio-Tek), according to the manufacturer's instructions. Extracted DNA was stored at -20 C for further analysis. Biodrop µLite spectrophotometer (Biochrom Ltd, Cambridge, UK) was used to quantify the DNA concentration and agarose gel was used to check the quality. DNA used for downstream analysis had an $A_{260}$:$A_{280}$ ratio of between 1.79–2.0 and a concentration that ranged from 30-285 ng/µl.

4.3.4 Library preparation for 16S rRNA metagenomics

The first PCR involved amplification of 16S rRNA variable region V3-V4 using Miseq adapters fused, 27F forward, and 518R reverse primers compatible with Illumina indexes (Table 3.1). Briefly, PCR reagents with a total volume of 25µl that consisted of 2.5µl of DNA, 12,5µl of 2x KAPA HiFi Hot Start Ready Mix (Kapa Biosystems, Boston, MA, USA), and 5µl of each of the primers. The PCR was performed with the following thermocycling conditions (denaturation 95°C for 3 minutes; 25 cycles of 95°C for 30, 55°C for 30 seconds and 72°C for 30 seconds; annealing at 72°C for 5 minutes and on hold at 4°C, ∞) and 1% agarose gel was used to check the PCR amplicon. The DNA was quantified with a Qubit fluorometer (Invitrogen life Technologies) using a dsDNA assay kit (Invitrogen life technologies).

The PCR amplicon was cleaned, indexed, pooled, and sequenced on an Illumina Miseq 250®, as described by Selvarajan et al (2019). Briefly, the PCR product was cleaned using AMPure XP magnetic beads (Beckman Coulter, Massachusetts, USA) according to the manufacturer's instructions. Clean amplification products were attached to dual indices using Nextera index PCR that used the Nextera XT v2 Index Kit (Illumina, Inc., San Diego, CA 2013). Briefly, a total reaction mixture of 25 µl comprising of 5 µl DNA, 2.5 µl each of Nextera index primers forward (S5XX) and reverse (N7XX), 12.5 µl of 2x KAPA HiFi Hot Start Ready Mix, and 2.5 µl of PCR grade water. PCR was performed using the following thermocycling conditions (denaturation 95°C for 3 minutes; 8 cycles of 95°C for 30, 55°C for 30 seconds and 72°C for 40 seconds; annealing at 72°C for 5 minutes and on hold at 4°C, for ∞). The resultant PCR product was cleaned again using AMPure XP beads and quantified using the Qubit™ dsDNA HS Assay kit (Thermo Fisher Scientific, Waltham, MA, USA). The concentrated final library samples were diluted to 4nM using

10mM Tris at PH 8.5. A volume of 5µl of each sample was pooled into a multiplexed library including the negative control sample into a 1.5ml tube (Selvarajan et al. 2019).

### 4.3.5 Library sequencing

Libraries pooled at 6 pM and 10% PhiX control library was denatured using diluted 0.2 N NaOH to achieve cluster generation during sequencing. The final library was sequenced on a MiSeq Illumina using a MiSeq Reagent Kit v3 (Illumina, Inc., San Diego, CA 2013).

### 4.3.6 Sequence analysis

The 16S rRNA amplicons were sequenced using Miseq Illumina 2x 300 base pairs, with primers targeting the V3-V4 hypervariable region of bacterial 16S rRNA. Sequences were processed using Qiime2 in a Nephele pipeline (Weber et al. 2018). SILVA-based reference sequences were used to classify unique sequences, a Naïve Bayesian classifier algorithm was executed on Qiime2 against Silva (v133) reference taxonomy. Using the average-neighbor algorithm, classified 16S rRNA sequences were assigned operational taxonomic units (OTUs) at 97.0% at phylum, class, order, family, genus, and species. The generated OTUs table was then used for downstream analysis. R Studio platform (version 4.0.3) using Phyloseq and Ampvis2 packages together with stamp software were used for visualization of data. Generation of heatmaps and stacked bar plots used highly expressed OTUs at phylum, genus, and species level, using the ggplot2 and Ampvis2 in R version 4.0.3 to visualize the genetic composition of bacterial communities (R Core Team, 2020). Alpha diversity indices variation was determined using ANOVA statistical test to determine their genetic distances and plotted using Phyloseq package. Similarly, comparative metagenomics visuals were plotted and analyzed using Phyloseq and Ampvis2 packages in R.

4.3.7 PICRUSt predicted functional resistance biomarker analysis.

Resistance biomarkers were estimated using a bioinformatics tool PICRUSt v3. The PICRUSt algorithm uses 16S rRNA sequence data and infers to reference databases to assign biomarker gene contents to each OTU. Using the PICRUSt v3 algorithm, COG, and databases, functional resistance biomarkers were identified to Level 2 KEGG using Qiime2. EzBiocloud pipeline (Yoon et al. 2017), was used to predict functional biomarker discovery of the KEGG Orthology functional annotation (KO) genes, using the PICRUSt algorithm and analyzed using LEfSe. Briefly, adapters, noise reads, chimera, cluster, and dereplicate sequences were removed from demultiplexed fast Q files then the resulting OTUs table was normalized with the lowest OTU read and genes predicted using the PICRUSt v3 (Yoon et al. 2017). For visualization of results, STAMP software and R studio were used.

## 4.4 RESULTS

Bacterial DNA was extracted from a total of 28 *R. evertsi evertsi* (n=11), *R. simus* (n=10), and *R. decoloratus* (n=8) collected from the Roodeplaat ARC research farm, RD8 was used as a control sample after its negative bacterial DNA amplification. To determine bacterial communities' genetic composition, 16S rRNA gene amplicon sequencing was performed on a Miseq Illumina. A total of 1326605 reads were generated, after chimera and singletons removal as well as filtering of quality sequences. Sequences were assigned to 33424 operational taxonomic units (OTUs) with each DNA sample having an average of 45745 reads. The saturation of sequences was demonstrated by the rarefaction curve (Figure 4.1), and all samples reached a saturation illustrating that sequenced reads were enough to represent bacterial diversity.

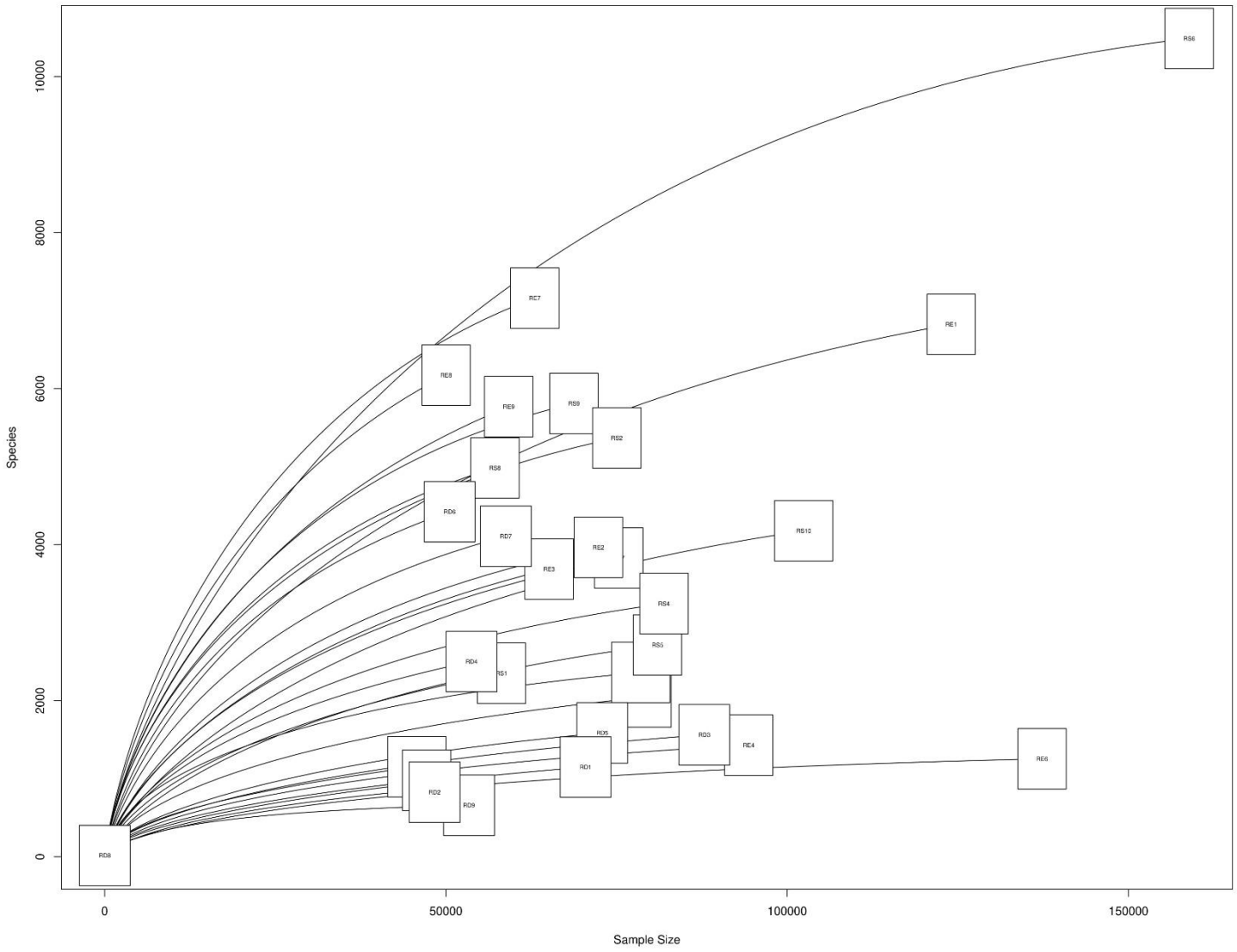



Figure 4.1. Operational taxonomic units (OTUs) per individual sample shown as rarefaction curves from 28 *R. evertsi evertsi, R. simus,* and *R. decoloratus* tick samples. The curves represent OTU reads observed from each tick on the y-axis and species richness on the x-axis.

4.4.1 Diversity and richness indices analysis of bacterial communities.

A comparison of diversity indices from bacterial communities of *R. evertsi evertsi, R. simus,* and *R. decoloratus,* revealed significant differences in diversity measures and richness. *R. evertsi evertsi* bacterial communities were highly diverse in all calculated diversity indices (Observed OTUs, Chao, ACE, Shannon, Simpson, and Fisher) at P>0.005 (Figure 4.2) in comparison with communities of *R. simus* and *R. decoloratus* tick species.

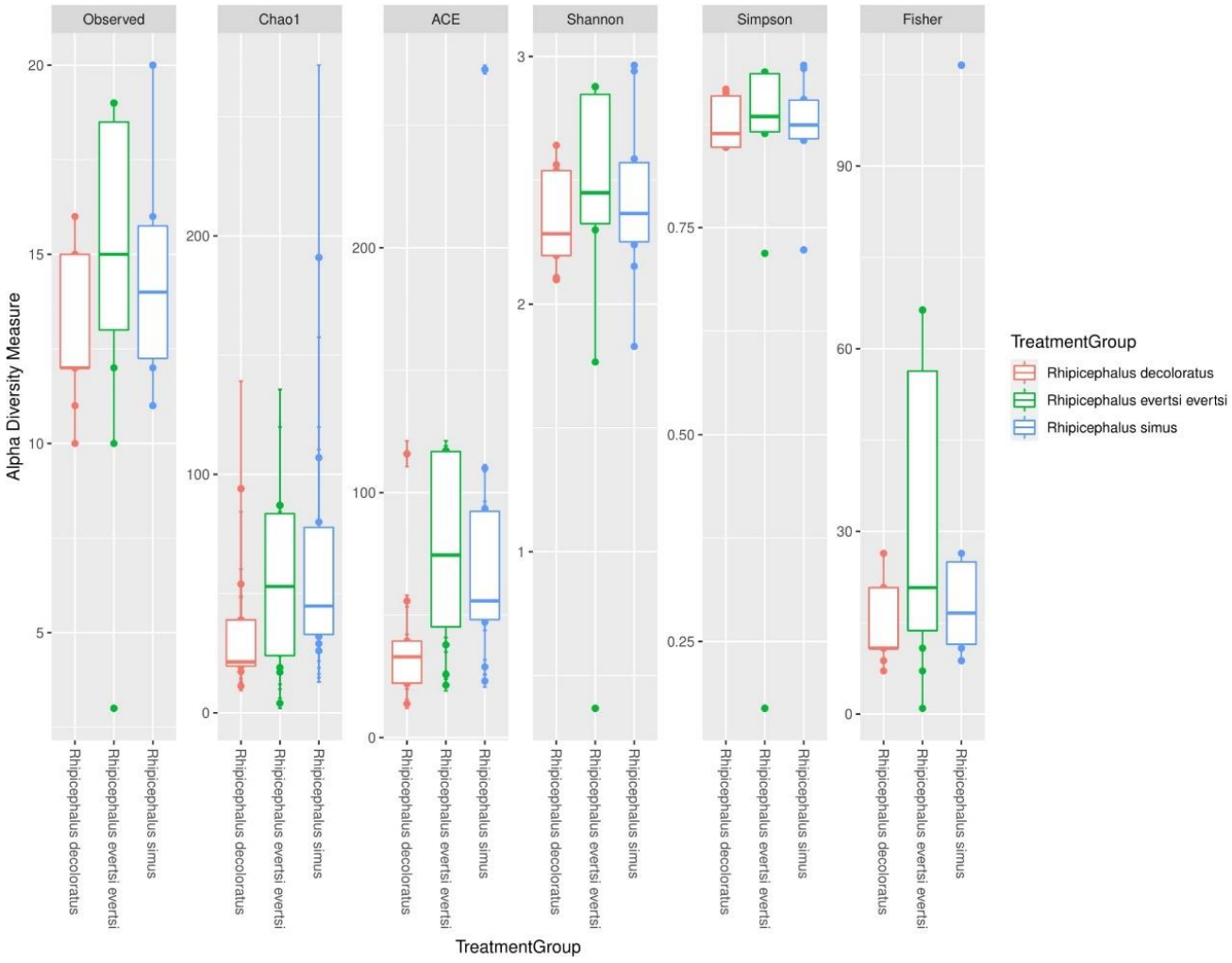



Figure 4.2. Alpha diversity analysis indices calculated to illustrate comparative richness and diversity measures between *R. evertsi evertsi, R. simus,* and *R. decoloratus* tick species, 16S rRNA gene libraries.

4.4.2 Composition of communities of bacteria in *R. evertsi evertsi*, *R. simus*, and *R. decoloratus* tick species

The rarefied data set comprised of 33424 OTUs in total, with 26638 (79.7%) phyla, 26605 (79.6%) classes, 26497 (79.28%) orders, 26224 (78.46%) families, and 25038 (74.91%) genera of bacteria. A stacked bar plot was used to illustrate five of the most abundant phyla from bacterial communities and they included Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, and Proteobacteria (Figure 4.3).

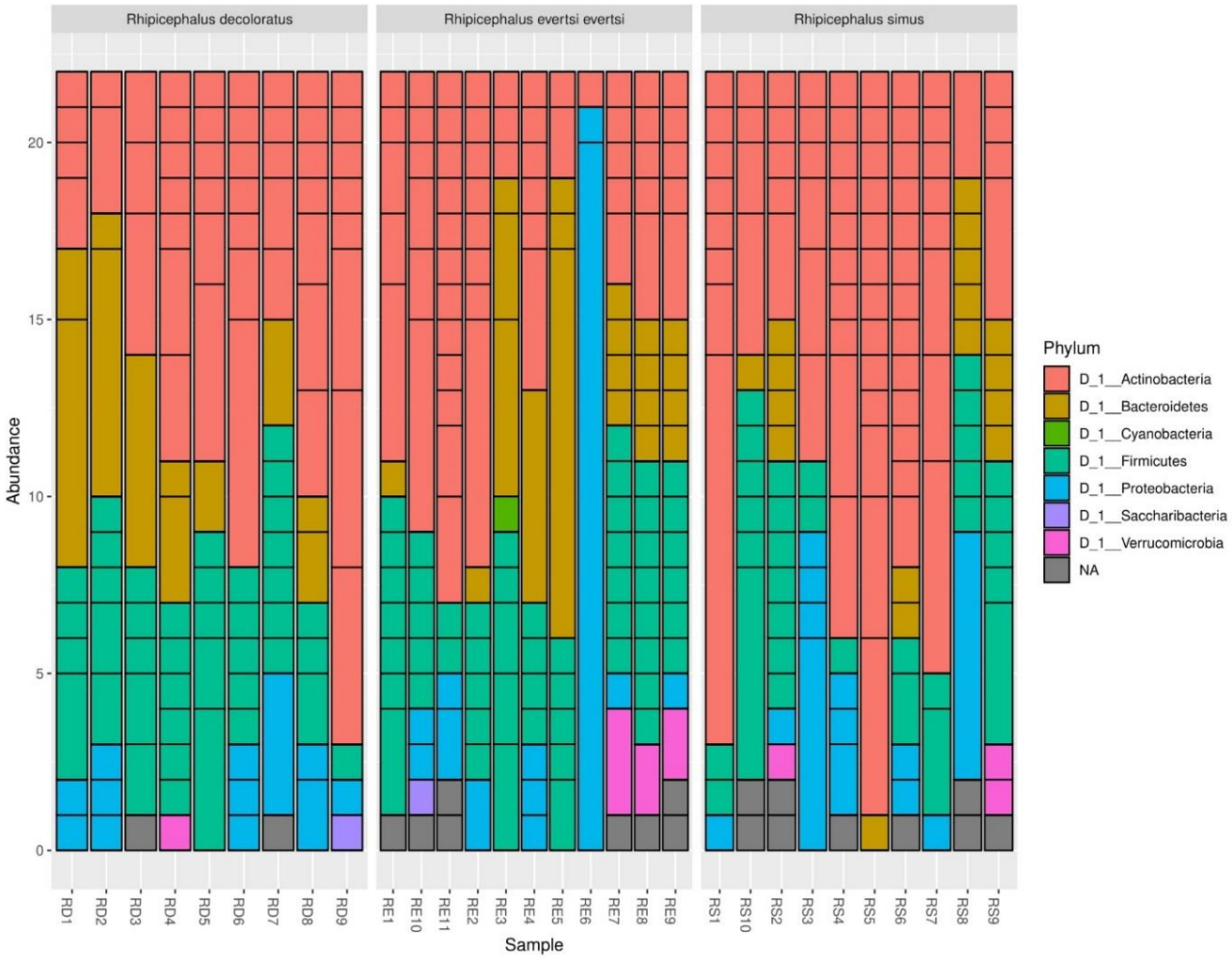



Figure 4.3 A stacked bar plot representing the composition of bacterial communities at phylum level from *R. evertsi evertsi*, *R. simus*, and *R. decoloratus* tick species. Colors represent each phylum composition.

The bar charts showing the percentage abundance of the top 5 bacterial phyla were plotted to illustrate comparative bacterial community composition (Figure 4.4). The Phyla Proteobacteria and Verrucomicrobia were highly dominant in *R. evertsi evertsi* and phyla Actinobacteria and Firmicutes were dominant in *R. simus* while *R. decoloratus* ticks were dominated by Bacteroides (Figure 4.4).

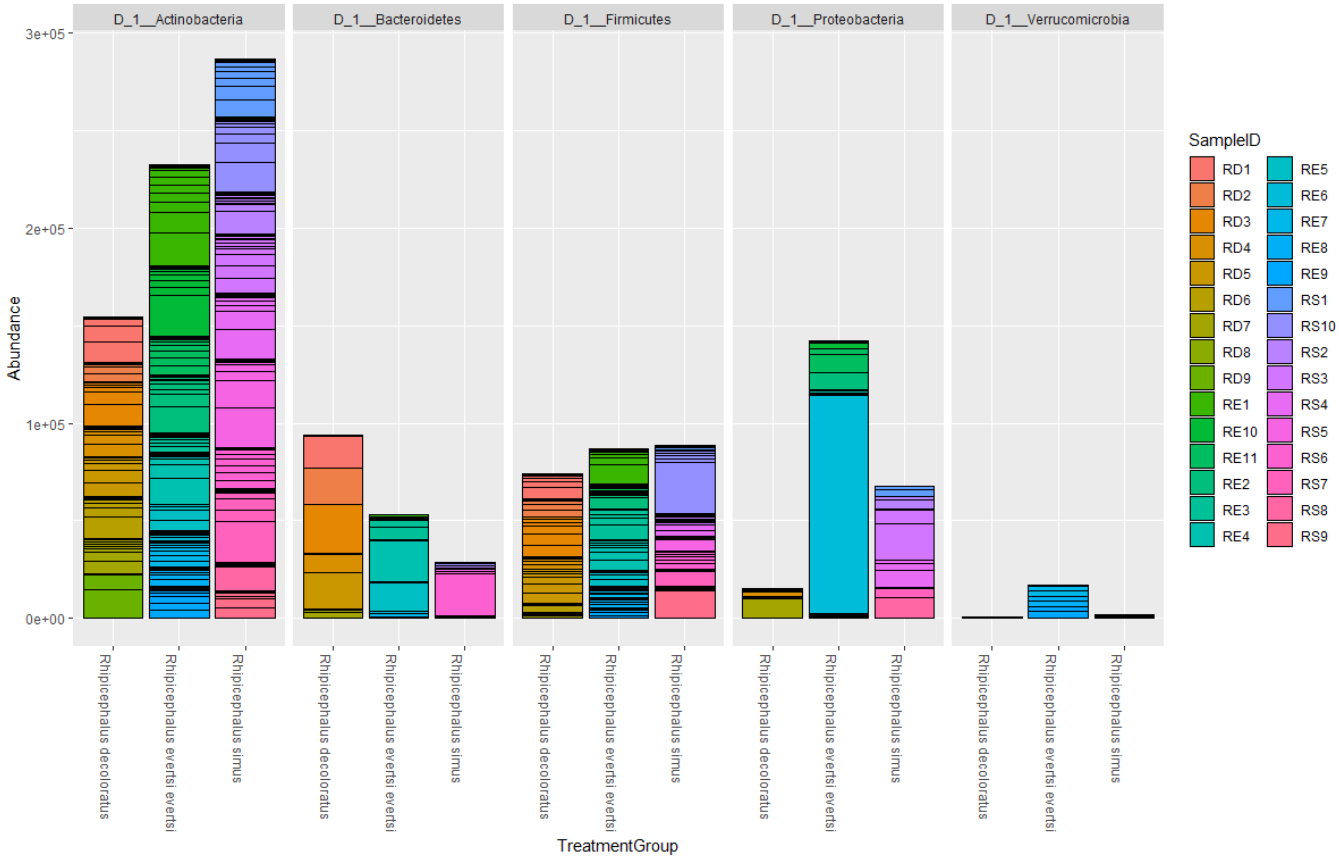



Figure 4.4 Bar plots representing the relative abundance of bacterial composition at phylum level from *R. evertsi evertsi*, *R. simus*, and *R. decoloratus* ticks.

A heatmap was constructed to demonstrate relative percentage abundance per tick of the top 40 bacterial genera from all tick samples (Figure 4.5). At genus level sequences represented 25098 genera OTUs, and the top 10 predominant genera were *Corynebacterium*, *Porphyromonas*, *Anaerococcus*, *Trueperella*, *Enhydrobacteria*, *Rickettsia*, *Helcococcus*, *Coxiella*, *Arthrobacter*, and *Lactobacillus*, varying in percentage distribution as shown in figure 4.5.

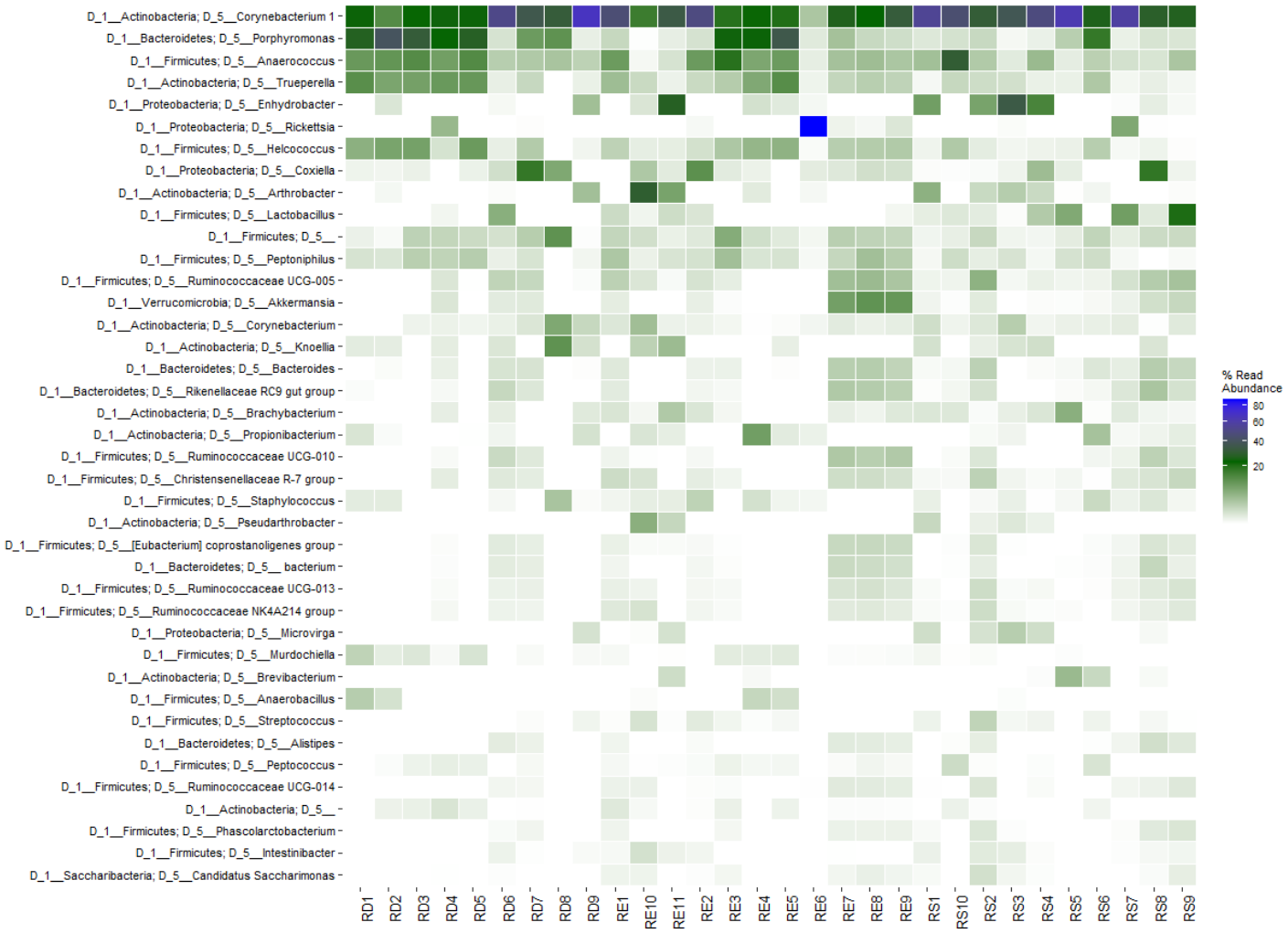



Figure 4.5 Heatmap showing the relative abundance of top 40 genera of bacterial communities associated with individual samples of *R. evertsi evertsi*, *R. simus,* and *R. decoloratus* ticks. Proportions of abundance are based on 16S rRNA gene sequences at the genus level.

A total of 27905 genera were detected in tick-borne bacterial communities, first 40 abundant genera were represented in a heatmap diagram (Figure 4.6). Most of the top genera were generally shared amongst all samples in high proportions and *Corynebacterium* was the most abundant genus with several groups identified.

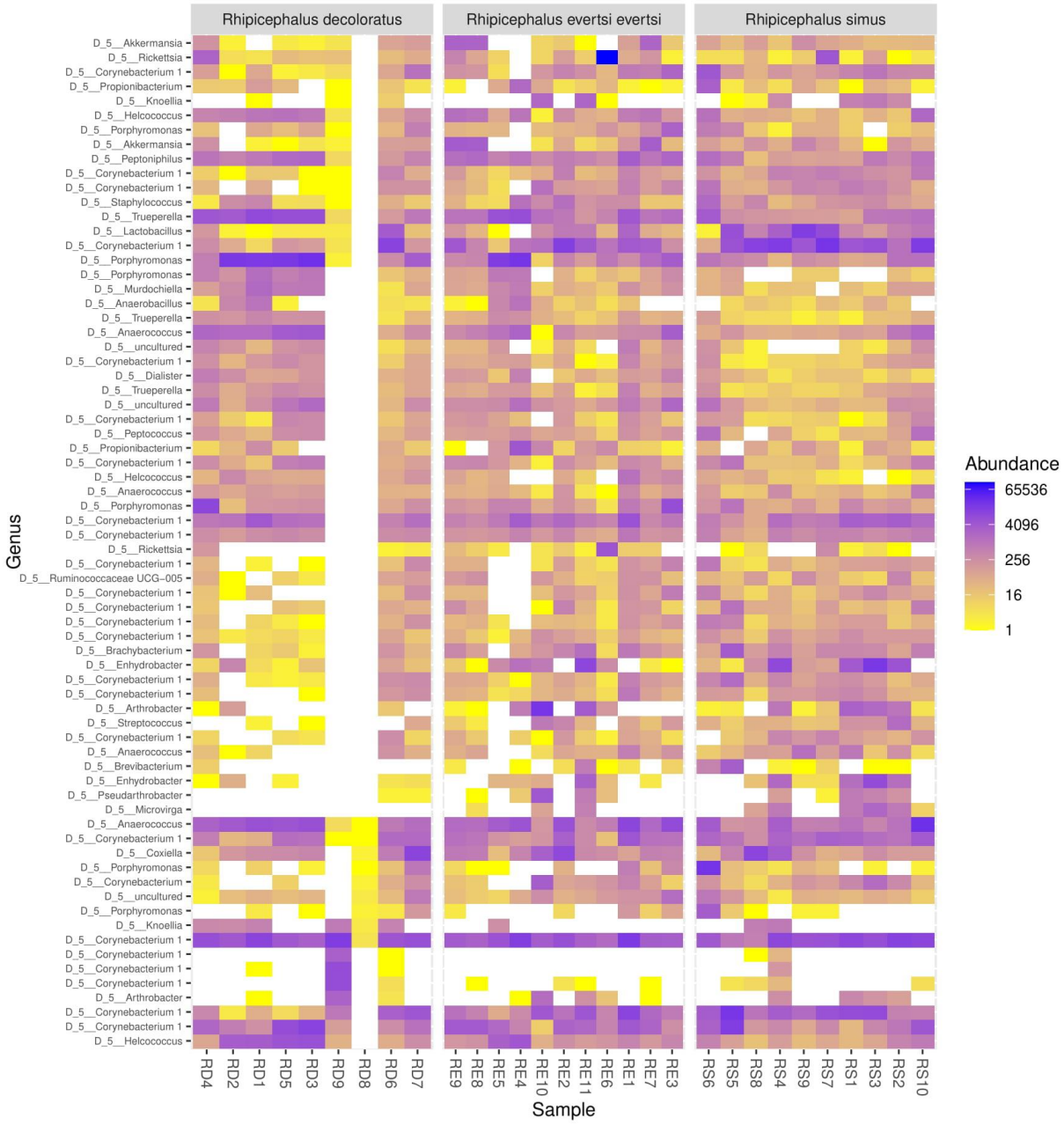



Figure 4.6 Heatmap plot representing grouped individual tick samples bacterial communities at the genus level, from *R. evertsi evertsi, R. simus,* and *R. decoloratus* ticks to show the variable composition of bacteria.

The top ten highly expressed species on an attempt to identify bacterial species in communities were *Corynebacterium diphtheriae group, Porphyromonas levii group, Rickettsia rickettsii group, Corynebacterium auriscanis group, Anaerococcus_uc, Enhydrobacter aerosaccus group, Trueperella pyogenes group, JQ480818 (Coxiella endosymbionts), JN167626,* and *Porphyromonas asaccharolytica groups,* as shown in figure 4.7 and supplementary S4.1.

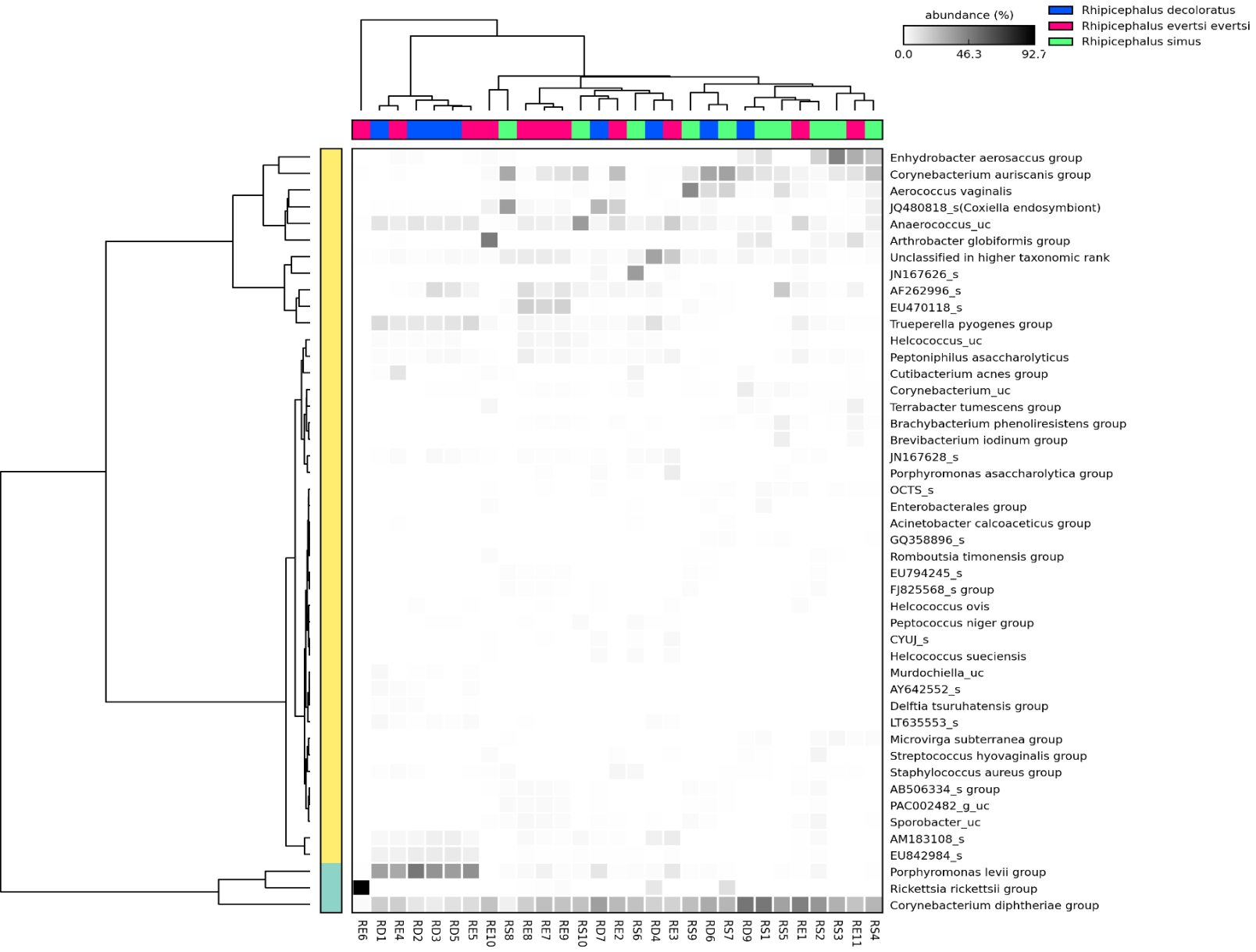



Figure 4.7 Heatmap plot representing individual tick samples bacterial communities at the species level, from *R. evertsi evertsi, R. simus,* and *R. decoloratus* ticks to show the variable composition of top 50 bacterial species.

4.4.3 Comparative metagenomics analysis of communities of bacterial in *R. evertsi evertsi, R. simus,* and *R. decoloratus*

A heatmap was plotted to compare and group the top twenty most expressed genera of bacterial communities from *R. decoloratus, R. evertsi evertsi,* and *R. simus* (Figure 4.8). The most dominantly enrichment seven genera were *Corynebacterium* (33%, 26.9%, and 42,1%), *Porphyromonas* (19,4%, 8.8%, and 2.9%), *Trueperella* (8%, 6.7%, and 5.4%), *Enhydrobacter* (6.1%, 3.7%, and 1.1%), *Rickettsia* (0.7%, 8.2%, and 0.8%), *Helcococcus* (4.5%, 2.8%, and 1%), and *Coxiella* (3.3%, 2.1%, and 2.5%) showing differences in their grouped percentages abundance respectively from each tick.

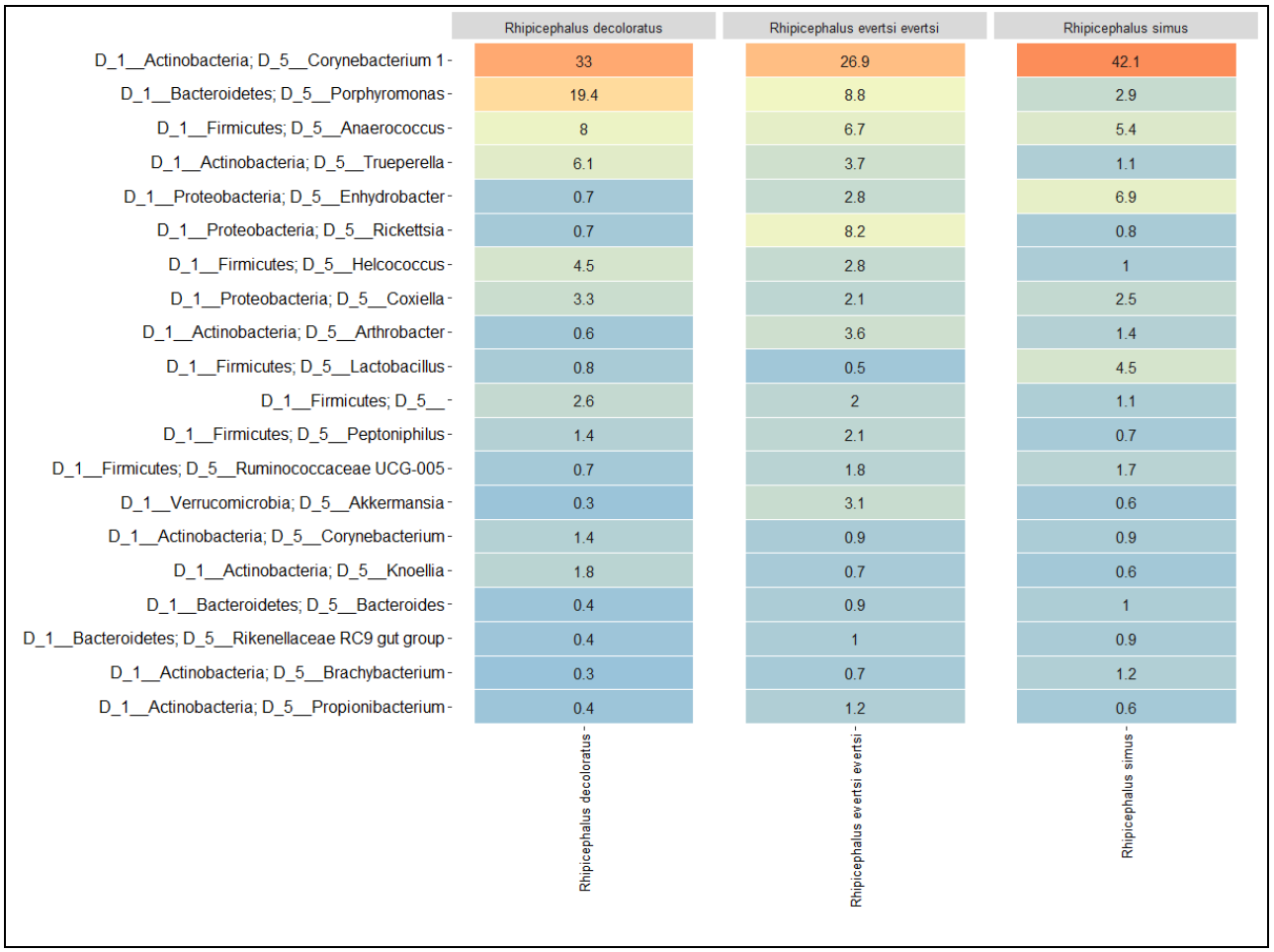



Figure 4.8 Comparative heatmap of the top twenty percentage composition of bacterial genera from communities of bacteria in *R. evertsi evertsi, R. simus,* and *R. decoloratus* ticks combined for comparison.

4.4.4 Pathogenic bacteria detected in bacterial communities.

Known pathogenic tick-borne bacterial genera such as *Corynebacterium*, *Porphyromonas*, *Trueperella*, *Rickettsia*, and *Coxiella* were amongst the most abundant genera illustrated in the stacked bar plot below (Figure 4.9). Among the highly expressed bacteria in ticks were endosymbiotic bacteria in the genera *Rickettsia* and *Coxiella*, with *Rickettsia* highly expressed in *R. evertsi evertsi* while *Coxiella* shared among all tick species.

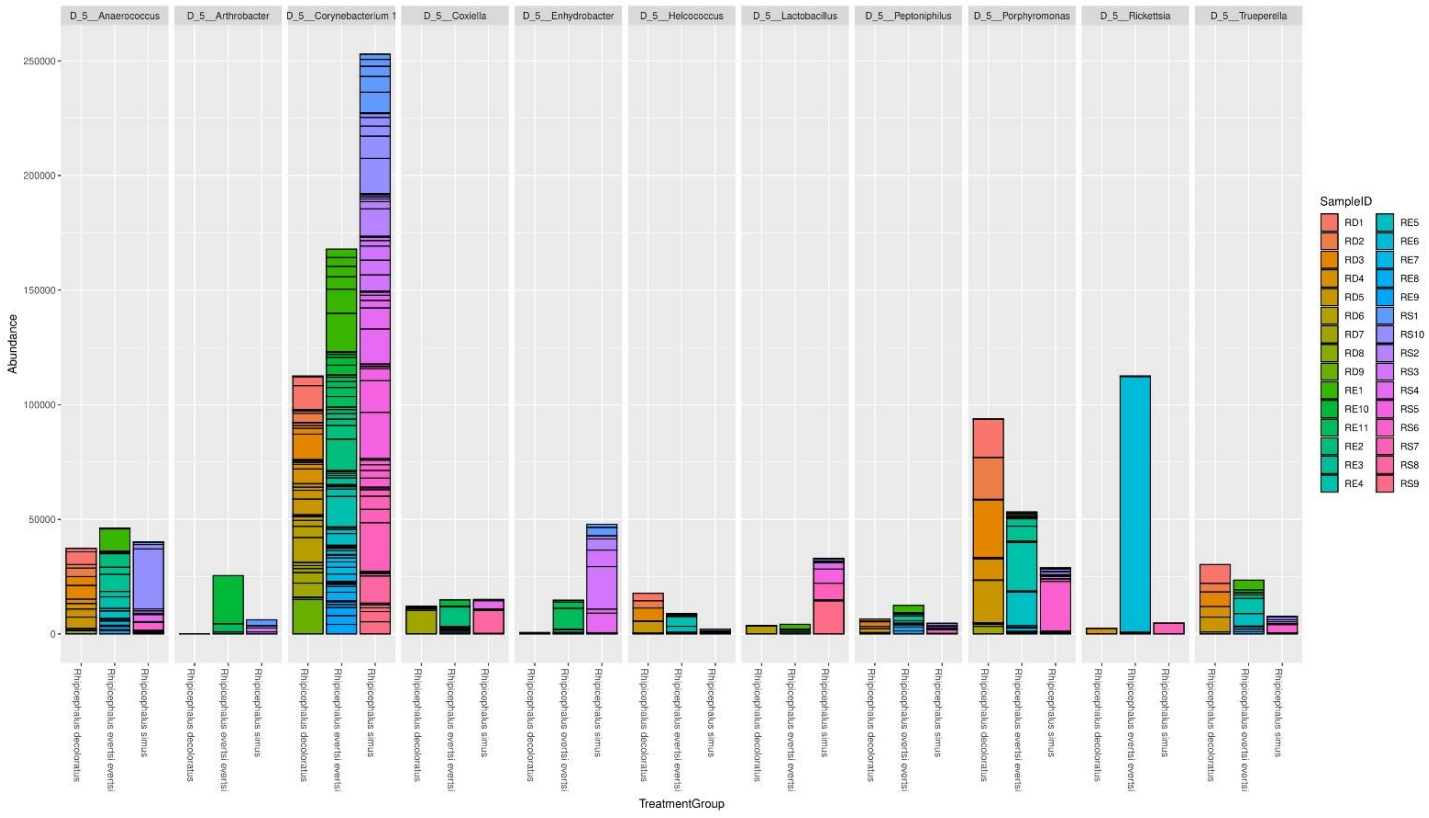



Figure 4.9. Stacked bar plot representing top eleven bacterial genera relative abundance in bacterial communities of *R. evertsi evertsi*, *R. simus*, and *R. decoloratus* ticks. Colors represent tick samples, while heights of bars represent the percentage abundance of OTUs at the genus level.

4.4.5 Shared and core microbial analysis of bacterial communities in ticks

A Venn diagram illustrating unique and shared OTUs was used to assess differences and similarities in bacterial communities identified from tick species (*R. evertsi evertsi, R. simus,* and *R. decoloratus*) (Figure 4.10). A total of 70.8% of core microbial OTUs were shared between all tick species. *R. simus* (4.4%) had the highest number of unique core microbial OTUs followed by *R. decoloratus* (1.2%) and *R. evertsi evertsi* (0.2%) had the least number of unique core microbial OTUs. However, 71.3% of OTUs were shared between; *R. decoloratus* and *R. evertsi evertsi*; while 71.4% were shared between *R. decoloratus* and *R. simus.* 79.3% OTUs were shared between *R. evertsi evertsi* and *R. simus* with 8.5% OTUs shared between *R. evertsi evertsi* and *R. simus* uniquely.

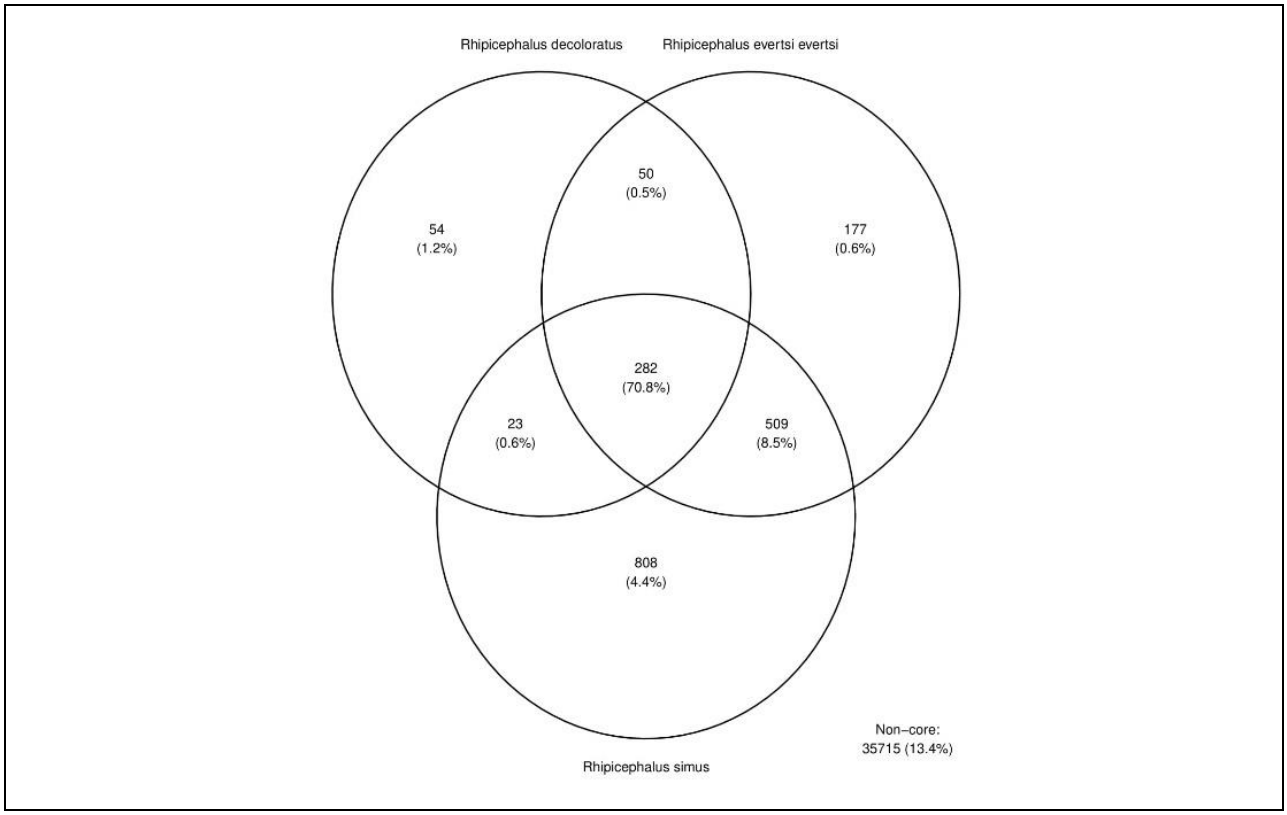



Figure 4.10 Vann Diagram demonstrating shared phylotypes of core microbiome, among *R. evertsi evertsi, R. simus,* and *R. decoloratus* tick species. Percentages represent the shared OTUs between each tick species.

4.4.6 Comparative principal component analysis of bacterial communities at the phylum level

Principal component analysis (PCoA) at (Principal Component 1 = 17% and Principal Component 2 = 10.4%) phylum level, revealed variations in bacterial community composition of *R. evertsi evertsi*, *R. simus*, and *R. decoloratus* individual ticks' samples (Figure 4.11). Close clustering explained the relatedness of individual tick samples at $P < 0.05$.

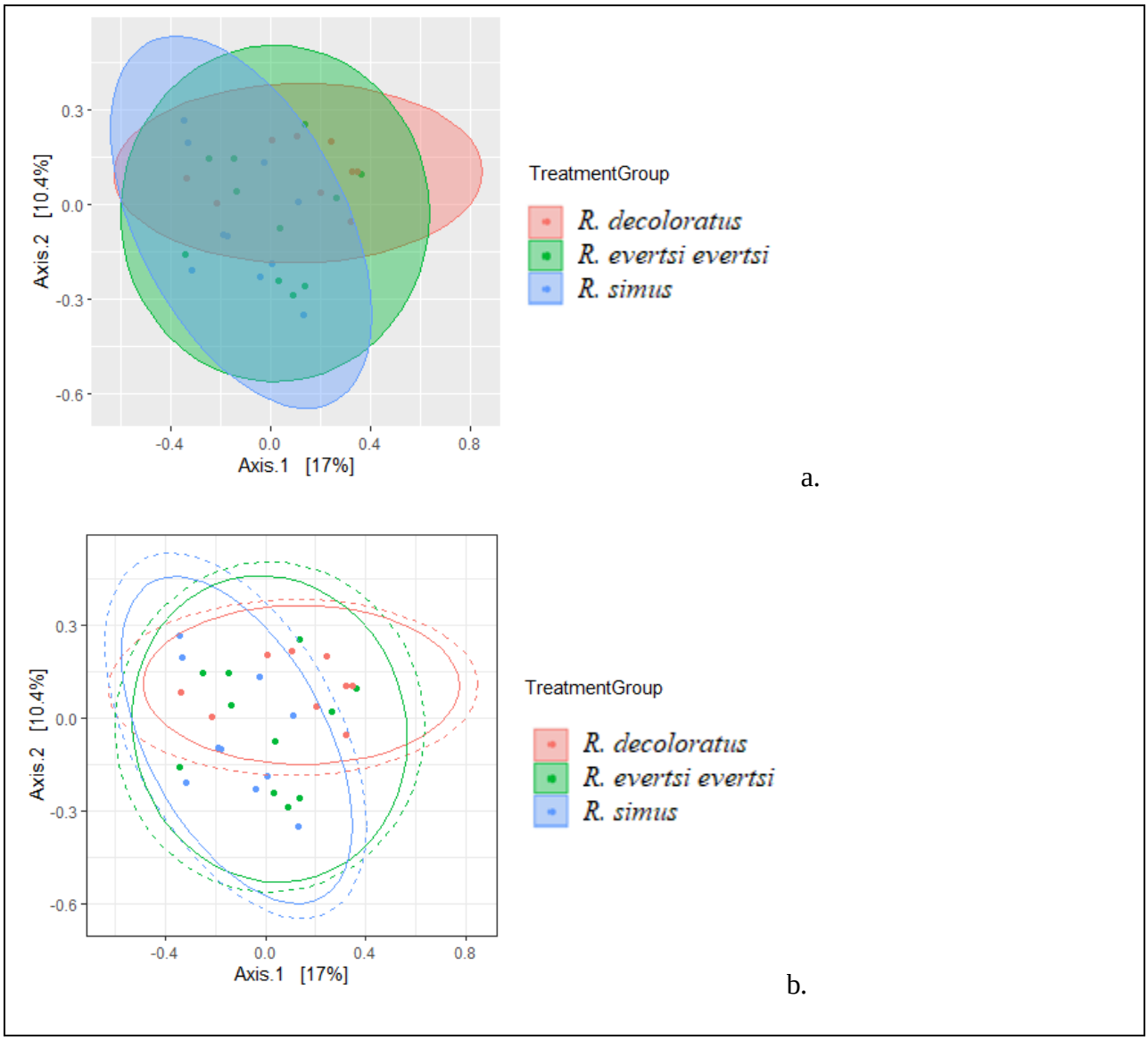



Figure 4.11 A principal component analysis (PCoA) plot showing relatedness of bacterial communities at phylum level from *R. evertsi evertsi*, *R. simus*, and *R. decoloratus* tick species. The total x-axis variances PCoA1 was 17% and Y-axis PCoA2 was 10.4%, with prediction ellipses observed having tick species falling in a different ellipse.

4.4.7 KEGG resistance markers identified in bacterial communities.

The predicted antibiotic resistance biomarkers from *R. evertsi evertsi, R. simus,* and *R. decoloratus* bacterial communities were performed using PICRUSt. A heatmap with hierarchical clustering was constructed based on KEGG level 2 functional biomarker profiles using ANOVA test and showered differences in gene enrichments between bacterial communities identified in individual samples (Figure 4.12). Dendrogram analysis showed how all 27 samples clustered together, with sample (RE6) and (RE10 and RD9) clustering separately and RE6 clustering individually. This illustrates significant differences in expressed KEGG functional genes between bacterial communities in samples. This was further demonstrated by a PCoA analysis of KEGG functional genes, with three samples spreading far wide from a cluster (Figure 4.13).

The most significantly enriched functional pathways in bacterial communities were linked to environmental information processing, genetic information processing, and metabolism. Functional prediction indicated a high abundance in environmental information processing pathways that were involved in the membrane transport systems, secretion, two-component systems, and ABC transporters. Secondly, genetic information processing pathways highly expressed included repair, replication, translation, transcription, and biogenesis genes. Finally, metabolic pathways vastly expressed included families of enzymes, amino acid metabolism, and oxidative metabolism shown in a heatmap (Figure 4.12). These pathways play an important role in antibiotic resistance and are significant resistance.

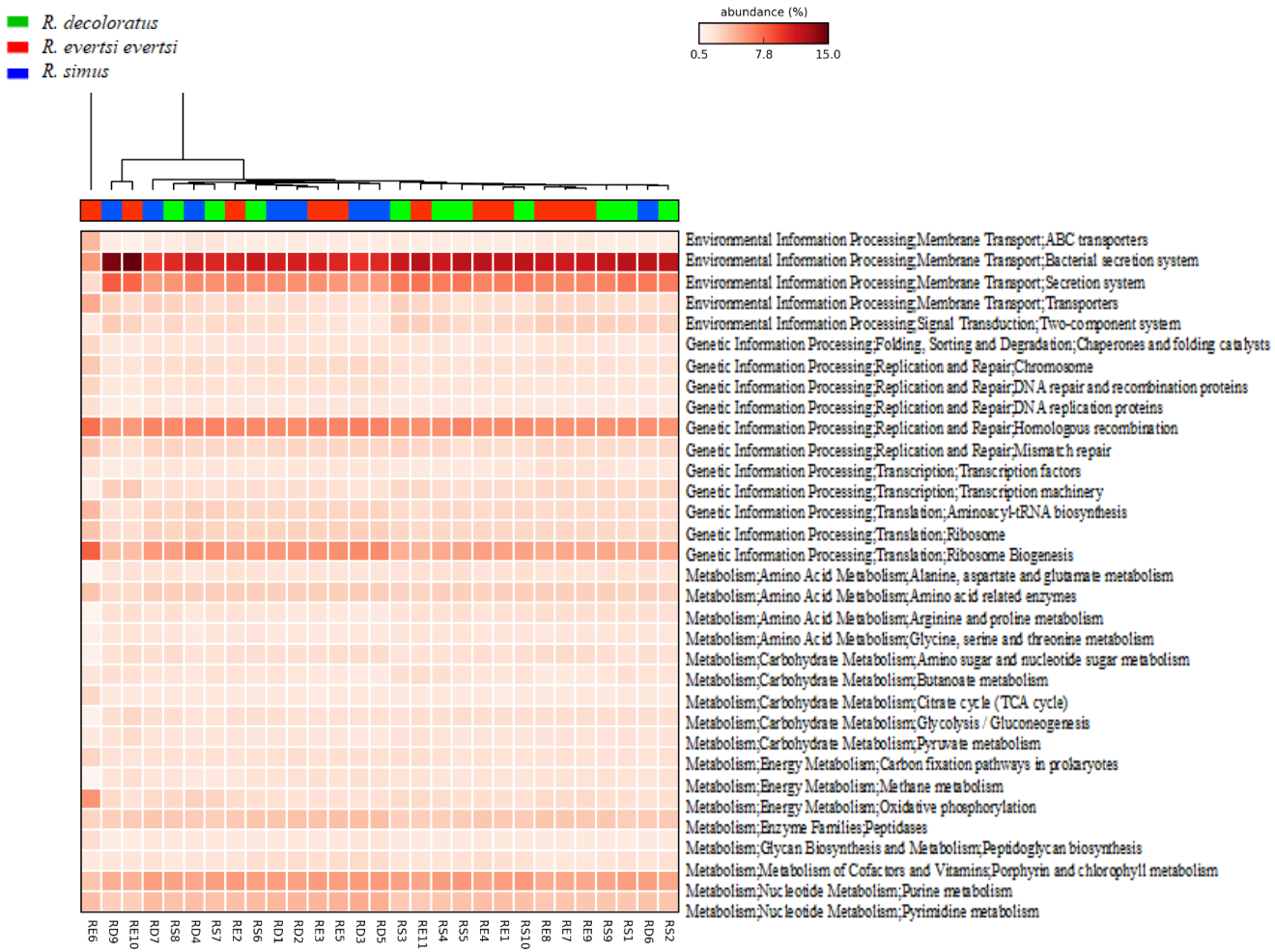



Figure 4.12 Heatmap illustrating PICRUSt predicted KEGG level 2 functional annotations from individual tick bacterial communities.

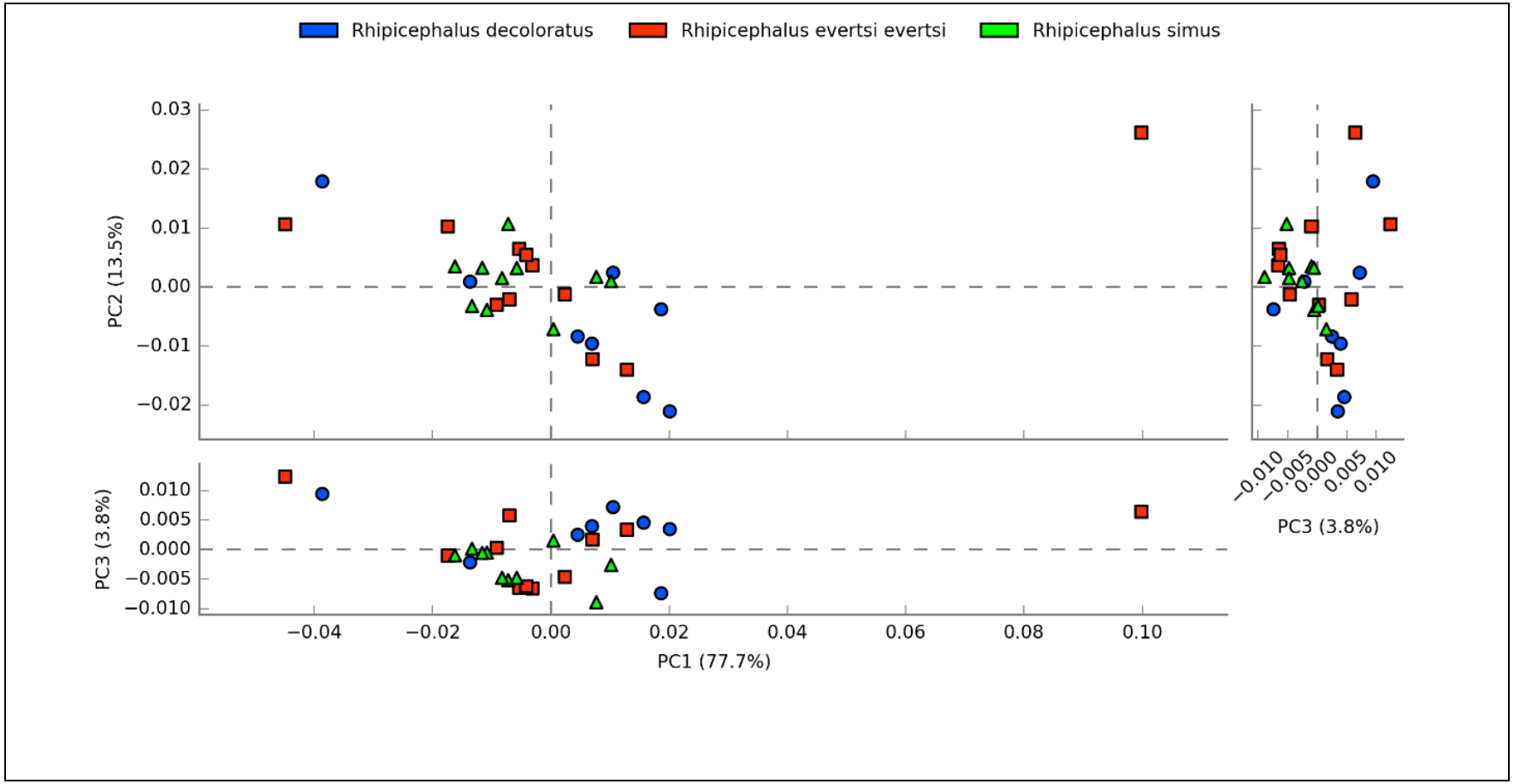



Figure 4.13 A principal component analysis (PCoA) plot based on functional biomarkers in bacterial communities from *Rhipicephalus* tick species. The total x-axis variances PCA1 was 77% and Y-axis PCA2 was 3.8%. There were no significant differences in functional pathways from microbial communities.

4.4.8 KO resistance biomarkers identified from tick-borne bacterial communities.

KEGG Ortholog (KO) count prediction of antimicrobial resistance functional genes from tick-borne microbial communities was performed using a bioinformatics package PICRUSt. The 16S rRNA data Operational taxonomic unit (OTU) were normalized to the minimum OTU count to account for uneven sample total OTUs. Diverse KO antibiotic resistance gene analyzed using LEfSe, in bacterial communities of *R. evertsi evertsi, R. simus,* and *R. decoloratus* tick species were revealed (Figure 4.14) and Appendix 4 (Supplementary 4.2). The mean relative abundance of genes associated with resistance is illustrated by a stacked bar chart in figure 4.14.

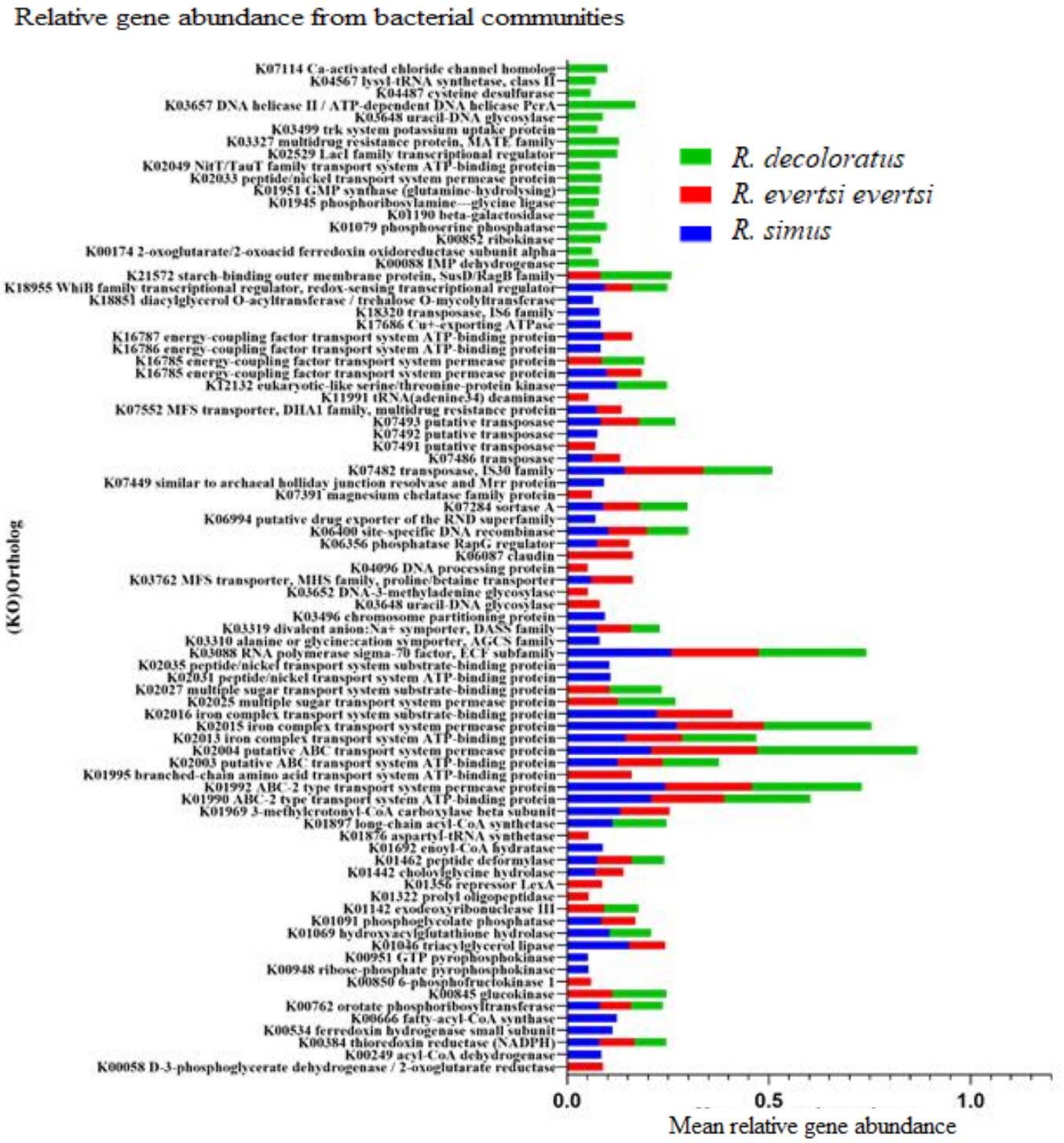



Figure 4.14 A stacked bar plot showing a mean relative gene abundance from bacterial communities of ticks (*R. evertsi evertsi* (red), *R. simus* (blue), and *R. decoloratus* (green)). A comparative analysis of KO Ortholog genes, each color representing communities of Bactria from each tick.

Difficulties in the treatment of tick-borne antibiotics arise from several mechanisms, including multiple-drug efflux pumps that play an important role in the exclusion of antibiotics and toxic substances. Analysis of KO genes for efflux pumps, the ATP binding cassettes (ABC-2 and putative ABC efflux pumps) revealed how they were highly expressed in all bacterial communities of ticks. Major facilitator multiple drug efflux pumps (MFS transport systems) were only highly expressed in communities from *R. evertsi evertsi* and *R. simus* ticks. While multiple drug resistance proteins of the multiple drug and toxic efflux pumps (Mate family) were expressed in bacterial communities of *R. decoloratus*. Also, resistance nodulation (RND putative drug exporters) genes were highly expressed in bacterial communities of *R. simus* tick species.

Transporter systems such as iron complexes, energy coupling, multiple sugars, Cu+- exporters, peptide/nickel, and divalent union/Na+ were highly expressed in all bacterial communities of *Rhipicephalus* tick species. Similarly, organisms can only acquire resistance in the presence of active efflux systems, therefore transporters are significant in antibiotic resistance.

Antibiotic resistance can also be caused by a mechanism involving enzymes that inactivate, degrade, and modify antibiotics. PICRUSt prediction identified enzymes such as hydroxyacylglutathione hydrolase or Metallo-beta-lactamase a beta-lactam enzyme and glucokinase a multiple drug resistance-conferring enzyme in bacterial communities of all *Rhipicephalus* tick species.

Furthermore, autonomous genetic elements such as transposase of several families such as putative and IS30 families were highly expressed in all bacterial communities. Transposases are major antibiotic resistance drivers of spread, as they may carry and laterally transfer resistance genes within bacterial communities.

## 4.5 DISCUSSION

Tick infestation on livestock is common worldwide, in South Africa *Rhipicephalus* tick species are the most widespread. In this study *Rhipicephalus* tick species of *R. evertsi evertsi, R. simus,* and *R. decoloratus* were identified parasitizing Nguni cattle from Roodeplaat ARC-Research farm in Gauteng province of South Africa. Similar studies have also confirmed *Rhipicephalus* ticks infestation on Nguni cattle in South Africa (Berggoetz *et al*. 2014; Guo *et al*. 2019; Halajian *et al*. 2016). In the current study, bacterial communities in *Rhipicephalus* ticks were assessed, and the findings revealed a variety of bacteria that are pathogenic, endosymbiotic, and symbiotic bacteria important to tick biology, and those that can be potentially transmitted to livestock. Generally, these results are consistent with previous studies of bacterial communities in ticks (Greay *et al*. 2018; Lim *et al*. 2020; Iweriebor *et al*. 2017), also, bacterial communities' functional makers associated with antibiotic resistance were revealed in this study.

Previous studies that are culture-based and those utilizing molecular techniques have provided limited information on communities of bacteria associated with ticks (Gestin *et al*. 2010; Noh et al. 2017; Latrofa *et al*. 2014). High throughput 16S rRNA metagenomics sequencing has proved to be a very powerful tool in revealing a detailed understanding of the bacterial communities in ticks. To investigate bacterial communities in individual ticks, 16S rRNA next-generation sequencing was performed and a high number of bacterial OTUs that resulted in 26638 phyla and 21038 genera were identified. Numerous pathogenic, endosymbiotic, and symbiotic bacteria were identified, with bacterial community composition differing between tick species and of ticks of the same species, as previously observed in other studies (Weinert *et al*., 2009; Machado-Ferreira *et al.*, 2016; Chicana *et al.*, 2019). Next-generation sequencing revealed tick-borne pathogen's

coinfections within individual ticks, seen also in previous findings by other researchers (Zaura *et al*. 2009; Guo et al. 2019; J. Yang *et al*. 2015).

The predominant phyla identified in *R. evertsi evertsi, R. decoloratus,* and *R. simus* tick species bacterial communities belonged to Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, and Proteobacteria. This is in agreement with previous studies of tick-borne bacterial communities (Berggoetz et al. 2014; Z. Chen et al. 2014; Portillo et al. 2019; Papa et al. 2020) that reported five phyla as the most dominant bacterial phyla in *Rhipicephalus* ticks.

The top ten most dominant genera identified in tick species were *Corynebacterium, Porphyromonas, Anaerococcus, Trueperella, Enhydrobacteria, Rickettsia, Helcoccoccus, Coxiella, Arthrobacter,* and *lactobacillus* (Figure 4.4). Many studies have also reported these bacteria genera associated with ticks (Portillo *et al*. 2019; Noh *et al*. 2017; Papa *et al*. 2020; Andreotti *et al*. 2011), suggesting that these genera may have an important role in tick biology and transmission of pathogenic bacteria. Bacterial communities identified in *R. evertsi evertsi, R. decoloratus,* and *R. simus* were not significantly distinct in comparison, indicated by the diversity indices analysis (Figure 4.2), and further, illustrated by their close relatedness at phylum level from a PCA analysis (Figure 4.11). the 16S rRNA has a conserved nature that gives it a low resolution to classify bacteria to species level (Papa *et al*. 2020), in this study, the most dominant species groups were *Corynebacterium diphtheriae, Corynebacterium auriscanis, Porphyromonas levii, Porphyromonas asaccharolytica, Rickettsia rickettsii, Anaerococcus_uc, Enhydrobacter aerosaccus, Trueperella pyogenes, AF262996_s, Arthrobacter globiformis,* and *JQ480818_s* (*Coxiella* endosymbiont).

The most common genera were *Corynebacterium* in all individual tick samples, this confirmed the possibility that ticks are a vector of these emerging pathogens, as described by Lim et al (2018).

Even though most species of *Corynebacterium* are innocuous, some species such as *C. lactis* and *C. pseudotuberculosis* have been confirmed as opportunistic pathogens of veterinary importance causing abscesses (Lim *et al*. 2018), secondary meningitis as well as Otitis media-interna (Leask, Blignaut, and Grobler 2013) respectively. Most importantly some pathogenic groups such as *C. diphtheriae* a primary cause of diphtheria (Bernard 2012). However, the most dominant *Corynebacterium* genera remain reported as uncultured, more studies on isolation and whole-genome sequencing are required. *Corynebacterium* genus was the most frequently detected genus in all tick samples, their high proportion in bacterial communities suggests that they are stronger competitors to other bacteria and could be well adapted to the environment ticks provide.

A consortium of pathogenic bacteria have been reported with portions of their life cycles in ticks, these include *Anaplasma, Borrelia, Coxiella, Ehrlichia, Francisella,* and *Rickettsia* (Guo et al. 2019; Halajian *et al*. 2016; Iweriebor *et al.* 2017; Mtshali *et al*. 2017; Ringo *et al*. 2018a). Equally, Gamma-proteobacteria genera *Coxiella* and Alpha-proteobacteria genera *Rickettsia* have been reported containing endosymbiotic bacteria closely related to pathogenic bacteria (Brinkmann *et al.*, 2019; *Seo et al.*, 2020). Tick-borne bacterial pathogens in the genera *Rickettsia, Coxiella, Trueperella,* and *Porphyromonas* were identified in this study. These pathogens were previously reported from cattle in different South African provinces (Halajian *et al*., 2018; Mtshali *et al*., 2017; Guo *et al.*, 2019). The *Coxiella* genus was identified from all ticks' bacterial communities, *Coxiella burnetii* has been implicated as a Q-fever agent, suggesting the need for surveys in cattle to bring clarity on the risk levels. Similarly, all samples contained *Rickettsia* genus, implicated as spotted and rocky mountain fever agents, and identification of these organisms in *R. evertsi evertsi, R. decoloratus,* and *R. simus* ticks corroborate previous findings (Guo *et al.*, 2019; Halajian *et al*., 2016).

Ticks bacterial endosymbionts have not been studied much extensively in comparison with other arthropods such as insects. Endosymbionts have been proven to be conferring fitness advantage to ticks against hosts, providing essential nutrients not found in blood meals, and are important in reproduction as well as during developmental stages of ticks (Rounds *et al*., 2012), providing a mutualistic, parasitic and commensal relationship with ticks (Andreotti *et al*., 2011). In this study, high OTUs of known endosymbionts in the *Rickettsia* and *Coxiella* genera were recorded. However, *Francisella*-like and *Candidatus* symbiont endosymbionts were not detected in *Rhipicephalus* ticks species as in line with previous reports (Brinkmann *et al.*, 2019; Vila *et al*., 2019; Seo *et al*., 2020). The results nonetheless suggested both endosymbionts and pathogenic *Rickettsia, Coxiella* groups were detected in this study. The findings of this nature allow us to channel our efforts on future investigations towards *Rhipicephalus* ticks for vector competence, epidemiological associations, and zoonotic potential associated with bacterial communities identified.

A bioinformatics tool (PICRUSt) was employed to predict functional resistance biomarkers from normalized metagenomics data. It has been reported that PICRUSt functional predictions in microorganism accuracy can range between 85% to 90% (Park *et al*., 2020). Functional resistance markers are genes involved in resistance mechanisms, that involve the ability to produce enzymes that deactivate antibiotics, mutations of the antibiotic target sites, reducing antibiotic permeability, and acquiring very active efflux pumps (Biswas *et al*.,2008; Aldred *et al*., 2014). These genes can also be shared amongst bacterial communities through horizontal gene transfer using mobile chromosomal element phages, plasmids, and transposons (Lerminiaux and Cameron 2019). The key findings in the present study was a very high relative abundance of functional biomarkers associated with antibiotic resistance (Figure 4.14).

Multiple drug efflux pumps are significant determinants of antibiotics resistance, provided for by acquired or phenotypic genes specific to drug-resistance and mutational hyperexpression of efflux pump genes (Blanco *et al.*, 2016). Efflux pumps extrude antibiotics, heavy metals, organic pollutants, and a wide range of toxins. In our study, ABC, ABC-2, MATE, RND, and MFS efflux transport systems were highly expressed in *R. decoloratus*, *R. evertsi evertsi*, and *R. simus* ticks bacterial communities (Figure 4.14). Several reports have documented these efflux systems and their role in antimicrobial resistance (J. M. Rolain 2005; Webber and Piddock 2003; Venter *et al*., 2015; Zárate *et al*., 2019).

Rolain (2005) reported how ABC efflux pumps conferred resistance to antibiotics such as beta-lactams and macrolides to tick-borne *Rickettsia* species groups, while Kuley (2017) reported macrolide-specific carrier ABC efflux pumps in *Coxiella* species (J. M. Rolain 2005; Kuley 2017). Multiple drug-resistant bacterial strains have also been reviewed with overexpression of ABC efflux pumps (Poole 2005). MFS efflux pumps have been reported in conferring tetracycline resistance in *Coxiella burnetii* (Vranakis *et al*., 2012), and quinolone resistance (Vranakis *et al*., 2010). Moreover, *Rickettsia* has been characterized to possess multiple drug efflux pumps of the MFS superfamily (Renesto *et al*., 2005). Hyper-expression of MFS efflux pumps in our findings confirm this and raises concerns as farmers use tetracycline as their main antibiotic of choice.

Putative drug exporters of the RND superfamily genes were highly expressed in communities of bacteria from *R. simus*, these have been documented playing an important role in raising antibiotics MIC in Gram-negative bacteria (Nikaido and Pagès, 2012; Li *et al.*, 2015; Venter *et al.*, 2015). Furthermore, the RND efflux pumps have been implicated in fluoroquinolone-resistance in *Coxiella burnetii* (Vranakis *et al*., 2010) and *Rickettsia* (Renesto *et al*., 2005). Communities of bacteria identified in *R. decoloratus* highly expressed genes of MATE superfamily, a multiple

drugs and toxic exclusion transport system that has been implicated in fluoroquinolone-resistance in *Coxiella burnetii*.

In addition to efflux pumps, transport systems such as iron complexes, energy coupling, multiple sugars, Cu+/- exporters, peptide/nickel, and divalent union/Na+ were highly expressed in all bacterial communities. Thus, presenting evidence of metal and antibiotic co-resistance in tick-borne bacterial communities. When screening and, improving current drugs or developing novel antimicrobial agents, it is imperative to investigate efflux pumps, utilizing efflux pump inhibitors such as paroxetine, phenylpiperidine SSRIs, and many others.

Genes of enzymes that degrade or modify beta-lactam hydroxyacylglutathione hydrolase or Metallo-beta-lactamase and glucokinase a multiple drug resistance-conferring enzyme were highly expressed in communities of bacteria identified in the current study. This is in line with previous findings looking at tick-borne bacteria such as *Ehrlichia* (P Brouqui and Raoult 1990; Philippe Brouqui and Raoult 1992), *Rickettsia* (J. M. Rolain *et al*., 1998), and *Corynebacterium* (Lim *et al*., 2018) that showed resistance to beta-lactams.

This information extends novel insights and understanding of antimicrobial biomarkers associated with tick-borne bacterial communities. The findings from the current study are important to stimulate future bioinformatics studies focusing on antibiotic resistance biomarkers aimed at the improvement of therapeutic drugs used in veterinary and medical sectors.

## 4.6 CONCLUSION

In conclusion, the results of this study provide an exploratory look into bacterial communities associated with *R. decoloratus, R. evertsi evertsi,* and *R. simus* ticks to better understand pathogens, endosymbiotic, and symbionts. The predominant genera identified included *Corynebacterium, Porphyromonas, Anaerococcus, Trueperella, Enhydrobacteria, Rickettsia, Helcoccoccus, Coxiella, Arthrobacter,* and *Lactobacillus*. This insight allows additional molecular studies to further understand their role in tick biology. Moreover, this study demonstrates that using 16S rRNA gene amplicon sequencing and PICRUSt functional annotation are efficient enough to detect resistance biomarkers associated with antimicrobial resistance. Studies in resistance biomarkers allow the improvement of current agents to achieve a maximum therapeutic effect in the treatment and discovery of new drugs. In the current study, functional antibiotic-resistant biomarkers, composed of efflux pumps (ABC, MFS, and MDR), drug degrading and modifying enzymes, ribosomal protection proteins pathways as well as secretion systems were conclusively identified. These functional resistance markers confer resistance to antibiotics such as fluoroquinolones, beta-lactam, tetracyclines, and macrolides. This contributes novel insights into complex intercellular microbial communities as well as providing their analytical and visualization of antimicrobial resistance biomarkers.

REFERENCES


Al-Adhami, Batol, W Brad Scandrett, Vladislav A Lobanov, and Alvin A Gajadhar. 2011. “Serological Cross-Reactivity between \textit{{Anaplasma} Marginale} and an \textit{{Ehrlichia}} Species in Naturally and Experimentally Infected Cattle.” *Journal of Veterinary Diagnostic Investigation* 23 (6): 1181–88. https://doi.org/10.1177/1040638711425593.

Aldred, Katie J, Robert J Kerns, and Neil Osheroff. 2014. “Mechanism of {Quinolone} {Action} and {Resistance}.” *Biochemistry* 53 (10): 1565–74. https://doi.org/10.1021/bi5000564.

Allsopp, B A. 2009. “Trends in the Control of Heartwater,” August, 8.

Aminov, R I, J C Chee-Sanford, N Garrigues, B Teferedegne, I J Krapac, B A White, and R I Mackie. 2002. “Development, {Validation}, and {Application} of {PCR} {Primers} for {Detection} of {Tetracycline} {Efflux} {Genes} of {Gram}-{Negative} {Bacteria}.” *Applied and Environmental Microbiology* 68 (4): 1786–93. https://doi.org/10.1128/AEM.68.4.1786-1793.2002.

Andreotti, Renato, Adalberto A de León, Scot E Dowd, Felix D Guerrero, Kylie G Bendele, and Glen A Scoles. 2011. “Assessment of Bacterial Diversity in the Cattle Tick {Rhipicephalus} ({Boophilus}) Microplus through Tag-Encoded Pyrosequencing.” *BMC Microbiology* 11 (1): 6. https://doi.org/10.1186/1471-2180-11-6.

Askoura, Momen, Walid Mottawea, Turki Abujamel, and Ibrahim Taher. 2011. “Efflux Pump Inhibitors (EPIs) as New Antimicrobial Agents against Pseudomonas Aeruginosa.” *Libyan Journal of Medicine* 6 (1): 1–8. https://doi.org/10.3402/ljm.v6i0.5870.

Aslam, Saadia, Muhammad Shahzad Latif, Muhammad Daud, Zia Ur Rahman, Bushra Tabassum, Muhammad Sohail Riaz, Anwar Khan, Muhammad Tariq, and Tayyab Husnain. 2016. “Crimean-{Congo} Hemorrhagic Fever: {Risk} Factors and Control Measures for the Infection Abatement.” *Biomedical Reports* 4 (1): 15–20. https://doi.org/10.3892/br.2015.545.

Aubry, Pascale, and Dorothy W Geale. 2011. “A Review of Bovine Anaplasmosis.” *Transboundary and Emerging Diseases* 58 (1): 1–30. https://doi.org/10.1111/j.1865-1682.2010.01173.x.

Baauw, Anna H, Heloise Heyne, Kathryn S Williams, Russell A Hill, Ignas M A Heitkönig, and Samual T Williams. 2019. “First Records of {Hyalomma} Rufipes and {Ixodes} Neitzi ({Acari}: {Ixodidae}) Found on Large Carnivores in {South} {Africa}.” *Ticks and Tick-Borne Diseases* 10 (1): 128–31. https://doi.org/10.1016/j.ttbdis.2018.08.011.

Baldridge, Gerald D., Nicole Y. Burkhardt, Roderick F. Felsheim, Timothy J. Kurtti, and Ulrike G. Munderloh. 2008. “Plasmids of the PRM/PRF Family Occur in Diverse Rickettsia Species.” *Applied and Environmental Microbiology* 74 (3): 645–52. https://doi.org/10.1128/AEM.02262-07.

Barker, Stephen C, and Alan R Walker. 2014. “Ticks of {Australia}. {The} Species That Infest Domestic Animals and Humans.” *Zootaxa* 3816 (1): 1. https://doi.org/10.11646/zootaxa.3816.1.1.

Bell-Sakyi, Lesley, Erich Zweygarth, Edmour F Blouin, Ernest A Gould, and Frans Jongejan. 2007. “Tick Cell Lines: Tools for Tick and Tick-Borne Disease Research.” *Trends in Parasitology* 23 (9): 450–57. https://doi.org/10.1016/j.pt.2007.07.009.

Bente, Dennis A, Naomi L Forrester, Douglas M Watts, Alexander J McAuley, Chris A Whitehouse, and Mike Bray. 2013. “Crimean-{Congo} Hemorrhagic Fever: {History}, Epidemiology, Pathogenesis, Clinical Syndrome and Genetic Diversity.” *Antiviral Research* 100 (1): 159–89. https://doi.org/10.1016/j.antiviral.2013.07.006.

Berggoetz, M., M. Schmid, D. Ston, V. Wyss, C. Chevillon, A. M. Pretorius, and L. Gern. 2014. “Protozoan and Bacterial Pathogens in Tick Salivary Glands in Wild and Domestic Animal Environments in South Africa.” *Ticks and Tick-Borne Diseases* 5 (2): 176–85. https://doi.org/10.1016/j.ttbdis.2013.10.003.

Bernard, Kathryn. 2012. “The Genus Corynebacterium and Other Medically Relevant” 50 (10): 3152–58. https://doi.org/10.1128/JCM.00796-12.

Bielawska-Drózd, Agata, Piotr Cieślik, Tomasz Mirski, Michał Bartoszcze, Józef Piotr Knap, Jerzy Gaweł, and Dorota Zakowska. 2013. “Q Fever - Selected Issues.” *Annals of Agricultural and Environmental Medicine* 20 (2): 222–32.

Biswas, Silpak, Didier Raoult, and Jean-Marc Rolain. 2008. “A Bioinformatic Approach to Understanding Antibiotic Resistance in Intracellular Bacteria through Whole Genome Analysis.” *International Journal of Antimicrobial Agents* 32 (3): 207–20. https://doi.org/10.1016/j.ijantimicag.2008.03.017.

Blanco, Paula, Sara Hernando-Amado, Jose Reales-Calderon, Fernando Corona, Felipe Lira, Manuel Alcalde-Rico, Alejandra Bernardini, Maria Sanchez, and Jose Martinez. 2016. “Bacterial Multidrug Efflux Pumps: Much More Than Antibiotic Resistance Determinants.” *Microorganisms* 4 (1): 14. https://doi.org/10.3390/microorganisms4010014.

Boulanger, N, P Boyer, E Talagrand-Reboul, and Y Hansmann. 2019. “Ticks and Tick-Borne Diseases.” *Médecine et Maladies Infectieuses* 49 (2): 87–97. https://doi.org/10.1016/j.medmal.2019.01.007.

Branger, S, J M Rolain, and D Raoult. 2004. “Evaluation of {Antibiotic} {Susceptibilities} of {Ehrlichia} Canis, {Ehrlichia} Chaffeensis, and {Anaplasma} Phagocytophilum by {Real}-{Time} {PCR}.” *Antimicrobial Agents and Chemotherapy* 48 (12): 4822–28. https://doi.org/10.1128/AAC.48.12.4822-4828.2004.

Brennan, Robert E., and James E. Samuel. 2003. “Evaluation of Coxiella Burnetii Antibiotic Susceptibilities by Real-Time PCR Assay.” *Journal of Clinical Microbiology* 41 (5): 1869–74. https://doi.org/10.1128/JCM.41.5.1869-1874.2003.

Brinkmann, Annika, Olcay Hekimoğlu, Ender Dinçer, Peter Hagedorn, Andreas Nitsche, and Koray Ergünay. 2019. “A Cross-Sectional Screening by next-Generation Sequencing Reveals {Rickettsia}, {Coxiella}, {Francisella}, {Borrelia}, {Babesia}, {Theileria} and {Hemolivia} Species in Ticks from {Anatolia}.” *Parasites & Vectors* 12 (1). https://doi.org/10.1186/s13071-018-3277-7.

Brolund, Alma, Martin Sundqvist, Gunnar Kahlmeter, and Malin Grape. 2010. “Molecular {Characterisation} of {Trimethoprim} {Resistance} in {Escherichia} Coli and {Klebsiella} Pneumoniae during a {Two} {Year} {Intervention} on {Trimethoprim} {Use}.” Edited by Eleftherios Mylonakis. *PLoS ONE* 5 (2): e9233. https://doi.org/10.1371/journal.pone.0009233.

Brouqui, P, and D Raoult. 1990. “In Vitro Susceptibility of {Ehrlichia} Sennetsu to Antibiotics.” *Antimicrobial Agents and Chemotherapy* 34 (8): 1593–96. https://doi.org/10.1128/AAC.34.8.1593.

Brouqui, Philippe, and Didier Raoult. 1992. “In {Vitro} {Antibiotic} {Susceptibility} of the {Newly} {Recognized} {Agent} of {Ehrlichiosis} in {Humans}, {Ehrlichia} Chaffeensis” 36: 5.

Camp, Pieter Jan Van, David B. Haslam, and Aleksey Porollo. 2020. “Bioinformatics Approaches to the Understanding of Molecular Mechanisms in Antimicrobial Resistance.” *International Journal of Molecular Sciences* 21 (4). https://doi.org/10.3390/ijms21041363.

Castro, Julio J de. 1997. “Sustainable Tick and Tickborne Disease Control in Livestock Improvement in Developing Countries.” *Veterinary Parasitology* 71 (2–3): 77–97. https://doi.org/10.1016/S0304-4017(97)00033-2.

Chen, Zhao Jin, Yang Shao, Ying Jun Li, Li An Lin, Yan Chen, Wei Tian, Bai Lian Li, and Yu Ying Li. 2020. “Rhizosphere Bacterial Community Structure and Predicted Functional Analysis in the Water-Level Fluctuation Zone of the Danjiangkou Reservoir in China during the Dry Period.” *International Journal of Environmental Research and Public Health* 17 (4). https://doi.org/10.3390/ijerph17041266.

Chen, Zhuo, Qin Liu, Ji-Qi Liu, Bian-Li Xu, Shan Lv, Shang Xia, and Xiao-Nong Zhou. 2014. “Tick-Borne Pathogens and Associated Co-Infections in Ticks Collected from Domestic Animals in Central {China}.” *Parasites & Vectors* 7 (1): 237. https://doi.org/10.1186/1756-3305-7-237.

Chicana, Betsabel, Lisa I. Couper, Jessica Y. Kwan, Enxhi Tahiraj, and Andrea Swei. 2019. “Comparative Microbiome Profiles of Sympatric Tick Species from the Far-Western United States.” *Insects* 10 (10): 1–12. https://doi.org/10.3390/insects10100353.

Chitanga, Simbarashe, Holly Gaff, and Samson Mukaratirwa. 2014. “Tick-Borne Pathogens of Potential Zoonotic Importance in the Southern {African} {Region}.” *Journal of the South African Veterinary Association* 85 (1): 3 pages. https://doi.org/10.4102/jsava.v85i1.1084.

Choi, Umji, and Chang Ro Lee. 2019. “Distinct Roles of Outer Membrane Porins in Antibiotic Resistance and Membrane Integrity in Escherichia Coli.” *Frontiers in Microbiology* 10 (APR): 1–9. https://doi.org/10.3389/fmicb.2019.00953.

Choopa, Chimvwele Namantala. 2015. “{IDENTIFICATION} {OF} {THEILERIA} {PARVA} {CARRIERS},” 170.

Crowder, Chris D, Megan A Rounds, Curtis A Phillipson, John M Picuri, Justina Halverson, Steven E Schutzer, David J Ecker, and Mark W Eshoo. 2011. “Extraction of {Total} {Nucleic} {Acids} {From} {Ticks} for the {Detection} of {Bacterial} and {Viral} {Pathogens},” 10.

Dantas-Torres, Filipe. 2010. “Biology and Ecology of the Brown Dog Tick, {Rhipicephalus} Sanguineus.” *Parasites & Vectors* 3 (1): 26. https://doi.org/10.1186/1756-3305-3-26.

Davidson, M M, R Evans, C L Ling, A D Wiseman, A W L Joss, and D O Ho-Yen. 1999. “Isolation of {Borrelia} Burgdorferi from Ticks in the {Highlands} of {Scotland}.” *Journal of Medical Microbiology* 48 (1): 59–65. https://doi.org/10.1099/00222615-48-1-59.

Dube, Joel P., Angel Valverde, Joachim M. Steyn, Don A. Cowan, and Jacqueline E. van der Waals. 2019. “Differences in Bacterial Diversity, Composition and Function Due to Long-Term Agriculture in Soils in the Eastern Free State of South Africa.” *Diversity* 11 (4). https://doi.org/10.3390/d11040061.

Elad, Daniel, Orly Friedgut, Nir Alpert, Yehuda Stram, Dan Lahav, Doron Tiomkin, Miriam Avramson, Kalia Grinberg, and Michael Bernstein. 2004. “Bovine Necrotic Vulvovaginitis Associated with Porphyromonas Levii.” *Emerging Infectious Diseases* 10 (3): 505–7. https://doi.org/10.3201/eid1003.020592.

Eldin, Carole, Cléa Mélenotte, Oleg Mediannikov, Eric Ghigo, Matthieu Million, Sophie Edouard, Jean Louis Mege, Max Maurin, and Didier Raoult. 2017. “From Q Fever to Coxiella Burnetii Infection: A Paradigm Change.” *Clinical Microbiology Reviews* 30 (1): 115–90. https://doi.org/10.1128/CMR.00045-16.

Espinaze, Marcela P A, Eléonore Hellard, Ivan G Horak, and Graeme S Cumming. 2018. “Domestic Mammals Facilitate Tick-Borne Pathogen Transmission Networks in {South} {African} Wildlife.” *Biological Conservation* 221 (May): 228–36. https://doi.org/10.1016/j.biocon.2018.03.017.

Estrada-peña, A., A.R Walker, A Bouattour, J.L Camicas, I.G Horak, A.a Latif, R.G Pegram, and P.M Preston. 2003. *Ticks of Domestic Animals in Africa: A Guide to Identification of Species*. *The*

*University of Edinburgh*. http://www.researchgate.net/publication/259641898_Ticks_of_domestic_animals_in_Africa_a_guide_to_identification_of_species/file/5046352d0429878d7f.pdf.

Eyer, Ludek, Hirofumi Kondo, Darina Zouharova, Minato Hirano, James J Valdés, Memi Muto, Tomas Kastl, et al. 2017. "Escape of {Tick}-{Borne} {Flavivirus} from 2′- \textit{{C}} -{Methylated} {Nucleoside} {Antivirals} {Is} {Mediated} by a {Single} {Conservative} {Mutation} in {NS}5 {That} {Has} a {Dramatic} {Effect} on {Viral} {Fitness}." Edited by Terence S Dermody. *Journal of Virology* 91 (21). https://doi.org/10.1128/JVI.01028-17.

Falvey, Lindsay. 2015. "Food Security: The Contribution of Livestock." *Chiang Mai University Journal of Natural Sciences* 14 (1): 103–18. https://doi.org/10.12982/cmujns.2015.0074.

Fedorina, Ekaterina A, Anna L Arkhipova, Gleb Y Kosovskiy, and Svetlana N Kovalchuk. 2019. "Molecular Survey and Genetic Characterization of {Anaplasma} Marginale Isolates in Cattle from Two Regions of {Russia}." *Ticks and Tick-Borne Diseases* 10 (2): 251–57. https://doi.org/10.1016/j.ttbdis.2018.10.011.

Fyfe, Corey, Trudy H Grossman, Kathy Kerstein, and Joyce Sutcliffe. 2016. "Resistance to Macrolide Antibiotics in Public Health Pathogens," 1–37.

Fyumagwa, Robert D, Pascale Simmler, Marina L Meli, Richard Hoare, Regina Hofmann-Lehmann, and Hans Lutz. 2011. "Molecular {Detection} of \textit{{Anaplasma}, {Babesia}} and \textit{{Theileria}} {Species} in a {Diversity} of {Tick} {Species} from {Ngorongoro} {Crater}, {Tanzania}." *South African Journal of Wildlife Research* 41 (1): 79–86. https://doi.org/10.3957/056.041.0109.

Galay, Remil L, Anna Angelica L Manalo, Sidney Lyndon D Dolores, Irene Pearl M Aguilar, Kristina Andrea C Sandalo, Kathlyn B Cruz, Billy P Divina, Masako Andoh, Tatsunori Masatani, and Tetsuya Tanaka. 2018. "Molecular Detection of Tick-Borne Pathogens in Canine Population and {Rhipicephalus} Sanguineus (Sensu Lato) Ticks from Southern {Metro} {Manila} and {Laguna}, {Philippines}." *Parasites & Vectors* 11 (1). https://doi.org/10.1186/s13071-018-3192-y.

Gavin M. Douglas1, Vincent J. Maffei2, Jesse Zaneveld3, Svetlana N. Yurgel4, James R. Brown5, Christopher M. Taylor2, Curtis Huttenhower6, Morgan G. I. Langille1, 7. 1991. "PICRUSt2: An Improved and Extensible Approach for Metagenome Inference Gavin." *Journal of Home Economics of Japan* 42 (2): 189–91. https://doi.org/10.11428/jhej1987.42.189.

Gestin, Brieuc, Eric Valade, François Thibault, Dominique Schneider, and Max Maurin. 2010. "Phenotypic and Genetic Characterization of Macrolide Resistance in {Francisella} Tularensis Subsp. Holarctica Biovar {I}." *Journal of Antimicrobial Chemotherapy* 65 (11): 2359–67. https://doi.org/10.1093/jac/dkq315.

Ghoneim, Nahed H, Khaled A Abdel-Moein, and Hala M Zaher. 2017. "Molecular {Detection} of \textit{{Francisella}} Spp. {Among} {Ticks} {Attached} to {Camels} in {Egypt}." *Vector-Borne and Zoonotic Diseases* 17 (6): 384–87. https://doi.org/10.1089/vbz.2016.2100.

Greay, Telleasha L, Alexander W Gofton, Andrea Paparini, Una M Ryan, Charlotte L Oskam, and Peter J Irwin. 2018. "Recent Insights into the Tick Microbiome Gained through Next-Generation Sequencing." *Parasites & Vectors* 11 (1). https://doi.org/10.1186/s13071-017-2550-5.

Guo, Huanping, Paul Franck Adjou Moumouni, Oriel Thekisoe, Yang Gao, Mingming Liu, Jixu Li, Eloiza May Galon, et al. 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): 875–82. https://doi.org/10.1016/j.ttbdis.2019.04.008.

Halajian, Ali, Ana M Palomar, Aránzazu Portillo, Heloise Heyne, Wilmien J Luus-Powell, and José A Oteo. 2016. “Investigation of {Rickettsia}, {Coxiella} Burnetii and {Bartonella} in Ticks from Animals in {South} {Africa}.” *Ticks and Tick-Borne Diseases* 7 (2): 361–66. https://doi.org/10.1016/j.ttbdis.2015.12.008.

Halajian, Ali, Ana M Palomar, Aránzazu Portillo, Heloise Heyne, Lourdes Romero, and José A Oteo. 2018. “Detection of Zoonotic Agents and a New {Rickettsia} Strain in Ticks from Donkeys from {South} {Africa}: {Implications} for Travel Medicine.” *Travel Medicine and Infectious Disease* 26 (November): 43–50. https://doi.org/10.1016/j.tmaid.2018.10.007.

Hedayatianfard, Keshvad, Mostafa Akhlaghi, and Hassan Sharifiyazdi. 2014. “Detection of Tetracycline Resistance Genes in Bacteria Isolated from Fish Farms Using Polymerase Chain Reaction,” October, 7.

Hwang, Wontae, and Sang Sun Yoon. 2019. “Virulence Characteristics and an Action Mode of Antibiotic Resistance in Multidrug-Resistant Pseudomonas Aeruginosa.” *Scientific Reports* 9 (1): 1–15. https://doi.org/10.1038/s41598-018-37422-9.

Illumina, Inc., San Diego, CA, USA. 2013. “16S Metagenomic Sequencing Library.” *Illumina.Com*, no. B: 1–28. http://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf.

Iweriebor, Benson C, Elia J Mmbaga, Abiodun Adegborioye, Aboi Igwaran, Larry C Obi, and Anthony I Okoh. 2017. “Genetic Profiling for {Anaplasma} and {Ehrlichia} Species in Ticks Collected in the {Eastern} {Cape} {Province} of {South} {Africa}.” *BMC Microbiology* 17 (1). https://doi.org/10.1186/s12866-017-0955-0.

Johnson, Jethro. 2017. “Full-Length 16S RRNA Gene Sequencing.” https://doi.org/10.1371/journal.pone.0039315.g001.

Junquera, P. 2018. “Biology and Life Cycle of {Rhipicephalus} Ticks.”

Jurado-Rabadán, Sonia, Ricardo de la Fuente, José A. Ruiz-Santa-Quiteria, José A. Orden, Lisbeth E. de Vries, and Yvonne Agersø. 2014. “Detection and Linkage to Mobile Genetic Elements of Tetracycline Resistance Gene Tet(M) in Escherichia Coli Isolates from Pigs.” *BMC Veterinary Research* 10 (M): 1–7. https://doi.org/10.1186/1746-6148-10-155.

Karkman, Antti, Timothy A Johnson, Christina Lyra, Robert D Stedtfeld, Manu Tamminen, James M Tiedje, and Marko Virta. 2016. “High-Throughput Quantification of Antibiotic Resistance Genes from an Urban Wastewater Treatment Plant.” Edited by Edward Topp. *FEMS Microbiology Ecology* 92 (3): fiw014. https://doi.org/10.1093/femsec/fiw014.

Kersh. 2015. “Antimicrobial Therapies for Q Fever.” *Expert Rev Anti Infect Ther.* 8 (5): 1207–14. https://doi.org/10.1586/14787210.2013.840534.

Keskin, Adem, Ahmet Bursali, David E. Snow, Scot E. Dowd, and Saban Tekin. 2017. “Assessment of Bacterial Diversity in Hyalomma Aegyptium, H. Marginatum and H. Excavatum Ticks through Tag-Encoded Pyrosequencing.” *Experimental and Applied Acarology* 73 (3–4): 461–75. https://doi.org/10.1007/s10493-017-0186-y.

Khumalo, Zamantungwa Thobeka Happiness. 2018. “Anaplasma Centrale in {South} {Africa}: Occurrence, Phylogeny and Genetic Diversity.” university of pretoria.

Kim, Hong, Sun-Hyun Kim, Yuan-Hai Ying, Hyun-Ju Kim, Young-Hwan Koh, Chang-Jin Kim, Sueng-Hyun Lee, Chang-Yong Cha, Yoon-Hoh Kook, and Bum-Joon Kim. 2005. “Mechanism of Natural

Rifampin Resistance of {Streptomyces} Spp." *Systematic and Applied Microbiology* 28 (5): 398–404. https://doi.org/10.1016/j.syapm.2005.02.009.

Kimberlin, David W, and Richard J Whitley. 1996. "Antiviral Resistance: Mechanisms, Clinical Significance, and Future Implications." *Journal of Antimicrobial Chemotherapy* 37 (3): 403–21. https://doi.org/10.1093/jac/37.3.403.

Kirkan, Sükrü, Göksel Erbas, and Ugur Parin. 2017. "Bacterial {Tick}-{Borne} {Diseases} of {Livestock} {Animals}." In *Livestock {Science}*, edited by Selim Sekkin. InTech. https://doi.org/10.5772/66122.

Klindworth, Anna, Elmar Pruesse, Timmy Schweer, Jörg Peplies, Christian Quast, Matthias Horn, and Frank Oliver Glöckner. 2013. "Evaluation of General 16S Ribosomal {RNA} Gene {PCR} Primers for Classical and Next-Generation Sequencing-Based Diversity Studies." *Nucleic Acids Research* 41 (1): e1--e1. https://doi.org/10.1093/nar/gks808.

Klitgaard, Kirstine, Jesper Højgaard, Anastasia Isbrand, Jesper J Madsen, Kasper Thorup, and Rene Bødker. 2019. "Screening for Multiple Tick-Borne Pathogens in {Ixodes} Ricinus Ticks from Birds in {Denmark} during Spring and Autumn Migration Seasons." *Ticks and Tick-Borne Diseases* 10 (3): 546–52. https://doi.org/10.1016/j.ttbdis.2019.01.007.

Kocan, K M, J de la Fuente, A A Guglielmone, and R D Melendez. 2003. "Antigens and {Alternatives} for {Control} of {Anaplasma} Marginale {Infection} in {Cattle}." *Clinical Microbiology Reviews* 16 (4): 698–712. https://doi.org/10.1128/CMR.16.4.698-712.2003.

Kozich, James J., Sarah L. Westcott, Nielson T. Baxter, Sarah K. Highlander, and Patrick D. Schloss. 2013. "Development of a Dual-Index Sequencing Strategy and Curation Pipeline for Analyzing Amplicon Sequence Data on the Miseq Illumina Sequencing Platform." *Applied and Environmental Microbiology* 79 (17): 5112–20. https://doi.org/10.1128/AEM.01043-13.

Kuley, Runa. 2017. *Characterization of Coxiella Burnetii Outbreak Strains. Characterization of Coxiella Burnetii Outbreak Strains*. http://edepot.wur.nl/410829.

Kumsa, Bersissa, Cristina Socolovschi, Didier Raoult, and Philippe Parola. 2015. "New {Borrelia} Species Detected in Ixodid Ticks in {Oromia}, {Ethiopia}." *Ticks and Tick-Borne Diseases* 6 (3): 401–7. https://doi.org/10.1016/j.ttbdis.2015.03.009.

Langille, Morgan G.I., Jesse Zaneveld, J. Gregory Caporaso, Daniel McDonald, Dan Knights, Joshua A. Reyes, Jose C. Clemente, et al. 2013a. "Predictive Functional Profiling of Microbial Communities Using 16S RRNA Marker Gene Sequences." *Nature Biotechnology* 31 (9): 814–21. https://doi.org/10.1038/nbt.2676.

———. 2013b. "Predictive Functional Profiling of Microbial Communities Using 16S RRNA Marker Gene Sequences." *Nature Biotechnology* 31 (9): 814–21. https://doi.org/10.1038/nbt.2676.

Latif, Abdalla A., John F. Putterill, Daniel G. de Klerk, Ronel Pienaar, and Ben J. Mans. 2012. "Nuttalliella Namaqua (Ixodoidea: Nuttalliellidae): First Description of the Male, Immature Stages and Re-Description of the Female." *PLoS ONE* 7 (7). https://doi.org/10.1371/journal.pone.0041651.

Latrofa, Maria Stefania, Filipe Dantas-Torres, Alessio Giannelli, and Domenico Otranto. 2014. "Molecular Detection of Tick-Borne Pathogens in {Rhipicephalus} Sanguineus Group Ticks." *Ticks and Tick-Borne Diseases* 5 (6): 943–46. https://doi.org/10.1016/j.ttbdis.2014.07.014.

Laws, Mark, Ali Shaaban, and Khondaker Miraz Rahman. 2019. "Antibiotic Resistance Breakers: Current Approaches and Future Directions." *FEMS Microbiology Reviews* 43 (5): 490–516. https://doi.org/10.1093/femsre/fuz014.

Leask, Rhoda, David J.C. Blignaut, and Maria J. Grobler. 2013. “Corynebacterium Pseudotuberculosis Associated with Otitis Media-Interna in Goats.” *Journal of the South African Veterinary Association* 84 (1): 1–3. https://doi.org/10.4102/jsava.v84i1.969.

Lejal, Emilie, Sara Moutailler, Ladislav Šimo, Muriel Vayssier-Taussat, and Thomas Pollet. 2019. “Tick-Borne Pathogen Detection in Midgut and Salivary Glands of Adult Ixodes Ricinus.” *Parasites and Vectors* 12 (1): 1–8. https://doi.org/10.1186/s13071-019-3418-7.

Lerminiaux, Nicole A, and Andrew D S Cameron. 2019. “Horizontal Transfer of Antibiotic Resistance Genes in Clinical Environments.” *Canadian Journal of Microbiology* 65 (1): 34–44. https://doi.org/10.1139/cjm-2018-0275.

Lew-Tabor, A. E., and M. Rodriguez Valle. 2016. “A Review of Reverse Vaccinology Approaches for the Development of Vaccines against Ticks and Tick Borne Diseases.” *Ticks and Tick-Borne Diseases* 7 (4): 573–85. https://doi.org/10.1016/j.ttbdis.2015.12.012.

Li, Xian Zhi, Patrick Plésiat, and Hiroshi Nikaido. 2015. “The Challenge of Efflux-Mediated Antibiotic Resistance in Gram-Negative Bacteria.” *Clinical Microbiology Reviews* 28 (2): 337–418. https://doi.org/10.1128/CMR.00117-14.

Lim, Fang Shiang, Jing Jing Khoo, Kim Kee Tan, Nurhafiza Zainal, Shih Keng Loong, Chee Sieng Khor, and Sazaly AbuBakar. 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). https://doi.org/10.1016/j.ttbdis.2019.101352.

Lim, Fang Shiang, Shih Keng Loong, Jing Jing Khoo, Kim Kee Tan, Nurhafiza Zainal, Muhammad Firdaus Abdullah, Chee Sieng Khor, and Sazaly AbuBakar. 2018. “Identification and Characterization of Corynebacterium Lactis Isolated from Amblyomma Testudinarium of Sus Scrofa in Malaysia.” *Systematic and Applied Acarology* 23 (9): 1838–44. https://doi.org/10.11158/saa.23.9.10.

Lingzhi, Li, Ge Haojie, Gu Dan, Meng Hongmei, Li Yang, Jia Mengdie, Zheng Chengkun, and Zhou Xiaohui. 2018. “The Role of Two-Component Regulatory System in β-Lactam Antibiotics Resistance.” *Microbiological Research* 215 (October): 126–29. https://doi.org/10.1016/j.micres.2018.07.005.

Livanova, Natalia N, Natalia V Fomenko, Ivan A Akimov, Mikhail J Ivanov, Nina V Tikunova, Rob Armstrong, and Sergey V Konyaev. 2018. “Dog Survey in {Russian} Veterinary Hospitals: Tick Identification and Molecular Detection of Tick-Borne Pathogens.” *Parasites & Vectors* 11 (1). https://doi.org/10.1186/s13071-018-3161-5.

Machado-Ferreira, Erik, Vinicius F. Vizzoni, Emilia Balsemão-Pires, Leonardo Moerbeck, Gilberto S. Gazeta, Joseph Piesman, Carolina M. Voloch, and Carlos A.G. Soares. 2016. “Coxiella Symbionts Are Widespread into Hard Ticks.” *Parasitology Research* 115 (12): 4691–99. https://doi.org/10.1007/s00436-016-5230-z.

Makarov, G I, and T M Makarova. 2018. “A Noncanonical Binding Site of Chloramphenicol Revealed via Molecular Dynamics Simulations.” *Biochimica et Biophysica Acta (BBA) - General Subjects* 1862 (12): 2940–47. https://doi.org/10.1016/j.bbagen.2018.09.012.

Mansfield, Karen L, Charlotte Cook, Richard J Ellis, Lesley Bell-Sakyi, Nicholas Johnson, Pilar Alberdi, José de la Fuente, and Anthony R Fooks. 2017. “Tick-Borne Pathogens Induce Differential Expression of Genes Promoting Cell Survival and Host Resistance in {Ixodes} Ricinus Cells.” *Parasites & Vectors* 10 (1). https://doi.org/10.1186/s13071-017-2011-1.

Mansueto, Pasquale, Giustina Vitale, Antonio Cascio, Aurelio Seidita, Ilenia Pepe, Antonio Carroccio,

Salvatore Di Rosa, Giovam Battista Rini, Enrico Cillari, and David H. Walker. 2012. "New Insight into Immunity and Immunopathology of Rickettsial Diseases." *Clinical and Developmental Immunology* 2012. https://doi.org/10.1155/2012/967852.

Mapholi, Ntanganedzeni O., Munyaradzi C. Marufu, Azwihangwisi Maiwashe, Cuthbert B. Banga, Voster Muchenje, Michael D. MacNeil, Michael Chimonyo, and Kennedy Dzama. 2014. "Towards a Genomics Approach to Tick (Acari: Ixodidae) Control in Cattle: A Review." *Ticks and Tick-Borne Diseases* 5 (5): 475–83. https://doi.org/10.1016/j.ttbdis.2014.04.006.

Mapiye, C., M. Chimonyo, K. Dzama, J. G. Raats, and M. Mapekula. 2009. "Opportunities for Improving Nguni Cattle Production in the Smallholder Farming Systems of South Africa." *Livestock Science* 124 (1–3): 196–204. https://doi.org/10.1016/j.livsci.2009.01.013.

Martinez, Eric, Franck Cantet, Laura Fava, Isobel Norville, and Matteo Bonazzi. 2014. "Identification of OmpA, a Coxiella Burnetii Protein Involved in Host Cell Invasion, by Multi-Phenotypic High-Content Screening." *PLoS Pathogens* 10 (3). https://doi.org/10.1371/journal.ppat.1004013.

Marufu, Munyaradzi Christopher. 2008. "Prevalence of {Ticks} and {Tick}-Borne {Diseases} in {Cattle} on {Communal} {Rangelands} in the {Highland} {Areas} of the {Eastern} {Cape} {Province}, {South} {Africa}," 104.

Mastropaolo, Mariano, L Fabián Beltrán-Saavedra, and Alberto A Guglielmone. 2014. "The Ticks ({Acari}: {Ixodida}: {Argasidae}, {Ixodidae}) of {Bolivia}." *Ticks and Tick-Borne Diseases* 5 (2): 186–94. https://doi.org/10.1016/j.ttbdis.2013.10.005.

Maurin, M, C Abergel, and D Raoult. 2001. "{DNA} {Gyrase}-{Mediated} {Natural} {Resistance} to {Fluoroquinolones} in {Ehrlichia} Spp." *Antimicrobial Agents and Chemotherapy* 45 (7): 2098–2105. https://doi.org/10.1128/AAC.45.7.2098-2105.2001.

Mohd Shukri, Munirah, Kai Ling Kho, Masoumeh Ghane Kisomi, Rafidah Lani, Suria Marlina, Siti Fatimah Muhd Radzi, Sun Tee Tay, et al. 2015. "Seroprevalence Report on Tick-Borne Encephalitis Virus and {Crimean}-{Congo} Hemorrhagic Fever Virus among {Malaysian}'s Farm Workers." *BMC Public Health* 15 (1). https://doi.org/10.1186/s12889-015-1901-4.

Moses, Abraham S., Jess A. Millar, Matteo Bonazzi, Paul A. Beare, and Rahul Raghavan. 2017. "Horizontally Acquired Biosynthesis Genes Boost Coxiella Burnetii's Physiology." *Frontiers in Cellular and Infection Microbiology* 7 (MAY): 1–9. https://doi.org/10.3389/fcimb.2017.00174.

Mtshali, Khethiwe, Ryo Nakao, Chihiro Sugimoto, and Oriel Thekisoe. 2017. "Occurrence of Coxiella Burnetii, Ehrlichia Canis, Rickettsia Species and Anaplasma Phagocytophilum-like Bacterium in Ticks Collected from Dogs and Cats in South Africa." *Journal of the South African Veterinary Association* 88 (May). https://doi.org/10.4102/jsava.v88i0.1390.

Mukherjee, Arghya, Bobby Chettri, James S. Langpoklakpam, Pijush Basak, Aravind Prasad, Ashis K. Mukherjee, Maitree Bhattacharyya, Arvind K. Singh, and Dhrubajyoti Chattopadhyay. 2017. "Bioinformatic Approaches Including Predictive Metagenomic Profiling Reveal Characteristics of Bacterial Response to Petroleum Hydrocarbon Contamination in Diverse Environments." *Scientific Reports* 7 (1): 1–22. https://doi.org/10.1038/s41598-017-01126-3.

Narasimhan, Sukanya, and Erol Fikrig. 2015. "Tick Microbiome: The Force Within." *Trends in Parasitology* 31 (7): 315–23. https://doi.org/10.1016/j.pt.2015.03.010.

Nguyen, Christine C., Cody N. Hugie, Molly L. Kile, and Tala Navab-Daneshmand. 2019. "Association between Heavy Metals and Antibiotic-Resistant Human Pathogens in Environmental Reservoirs: A Review." *Frontiers of Environmental Science and Engineering* 13 (3).

https://doi.org/10.1007/s11783-019-1129-0.

Nikaido, Hiroshi, and Jean Marie Pagès. 2012. “Broad-Specificity Efflux Pumps and Their Role in Multidrug Resistance of Gram-Negative Bacteria.” *FEMS Microbiology Reviews* 36 (2): 340–63. https://doi.org/10.1111/j.1574-6976.2011.00290.x.

Noh, Yoontae, Yeong Seon Lee, Heung-Chul Kim, Sung-Tae Chong, Terry A Klein, Ju Jiang, Allen L Richards, Hae Kyeong Lee, and Su Yeon Kim. 2017. “Molecular Detection of {Rickettsia} Species in Ticks Collected from the Southwestern Provinces of the {Republic} of {Korea}.” *Parasites & Vectors* 10 (1). https://doi.org/10.1186/s13071-016-1955-x.

Nyangiwe, N, M Yawa, and V Muchenje. 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): 829. https://doi.org/10.4314/sajas.v48i5.4.

Onyiche, ThankGod E, Keisuke Suganuma, Ikuo Igarashi, Naoaki Yokoyama, Xuenan Xuan, and Oriel Thekisoe. 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): 1736. https://doi.org/10.3390/ijerph16101736.

Pachori, Preeti, Ragini Gothalwal, and Puneet Gandhi. 2019. “Emergence of Antibiotic Resistance Pseudomonas Aeruginosa in Intensive Care Unit; a Critical Review.” *Genes and Diseases* 6 (2): 109–19. https://doi.org/10.1016/j.gendis.2019.04.001.

Palanisamy, Navaneethan, Dario Akaberi, Johan Lennerstrand, and Åke Lundkvist. 2018. “Comparative Genome Analysis of {Alkhumra} Hemorrhagic Fever Virus with {Kyasanur} Forest Disease and Tick-Borne Encephalitis Viruses by the \textit{in Silico} Approach.” *Pathogens and Global Health* 112 (4): 210–26. https://doi.org/10.1080/20477724.2018.1471187.

Pan, Yu, Jiaxiong Zeng, Liguan Li, Jintao Yang, Ziyun Tang, Wenguang Xiong, and Yafei Li. 2020. “Crossm Coexistence of Antibiotic Resistance Genes and Virulence Factors Deciphered by Large-Scale Complete Genome Analysis” 5 (3): 1–15.

Papa, Anna, Katerina Tsioka, Maria Antonia Daskou, Fani Minti, Elpida Papadopoulou, Ageliki Melidou, and Nektarios Giadinis. 2020. “Application of 16S RRNA next Generation Sequencing in Ticks in Greece.” *Heliyon* 6 (7): e04542. https://doi.org/10.1016/j.heliyon.2020.e04542.

Park, Jong-hun, Jeong-hoon Park, Sang-hoon Lee, Jeong-jun Yoon, and Sang-hyoun Kim. 2020. “Bioresource Technology Metabolic Fl Ux and Functional Potential of Microbial Community in an Acidogenic Dynamic Membrane Bioreactor.” *Bioresource Technology* 305 (December 2019): 123060. https://doi.org/10.1016/j.biortech.2020.123060.

Perry, Julie A, and Gerard D Wright. 2014. “Forces Shaping the Antibiotic Resistome: {Prospects} & {Overviews}.” *BioEssays* 36 (12): 1179–84. https://doi.org/10.1002/bies.201400128.

Poole, Keith. 2005. *Efflux-Mediated Antimicrobial Resistance*. *Journal of Antimicrobial Chemotherapy*. Vol. 56. https://doi.org/10.1093/jac/dki171.

Portillo, Aránzazu, Ana M Palomar, María de Toro, Sonia Santibáñez, Paula Santibáñez, and José A Oteo. 2019. “Exploring the Bacteriome in Anthropophilic Ticks: {To} Investigate the Vectors for Diagnosis.” Edited by Ben J Mans. *PLOS ONE* 14 (3): e0213384. https://doi.org/10.1371/journal.pone.0213384.

Quiroz-Castañeda, Rosa Estela, Itzel Amaro-Estrada, and Sergio Darío Rodríguez-Camarillo. 2016. “Anaplasma Marginale: Diversity, Virulence, and Vaccine Landscape through a Genomics Approach.” *BioMed Research International* 2016: 9032085. https://doi.org/10.1155/2016/9032085.

Ramaiah, Arunachalam, and Gregory A. Dasch. 2018. “Genome Sequence of Coxiella-like Endosymbiont Strain CLERmD, a Bacterial Agent in the Cattle Tick (Rhipicephalus Microplus) Deutsch Strain.” *Genome Announcements* 6 (13): 18–19. https://doi.org/10.1128/genomeA.00003-18.

Ramanathan, Babu, Hassan Mahmood Jindal, Cheng Foh Le, Ranganath Gudimella, Arif Anwar, Rozaimi Razali, Johan Poole-Johnson, Rishya Manikam, and Shamala Devi Sekaran. 2017. “Next Generation Sequencing Reveals the Antibiotic Resistant Variants in the Genome of {Pseudomonas} Aeruginosa.” Edited by Chiyu Zhang. *PLOS ONE* 12 (8): e0182524. https://doi.org/10.1371/journal.pone.0182524.

Renesto, Patricia, Hiroyuki Ogata, Stéphane Audic, Jean-Michel Claverie, and Didier Raoult. 2005. “Some Lessons from Rickettsia Genomics.” *FEMS Microbiology Reviews* 29 (1): 99–117. https://doi.org/10.1016/j.femsre.2004.09.002.

Rezanejad, Mobin, Sepideh Karimi, and Hassan Momtaz. 2019. “Phenotypic and Molecular Characterization of Antimicrobial Resistance in Trueperella Pyogenes Strains Isolated from Bovine Mastitis and Metritis.” *BMC Microbiology* 19 (1): 1–9. https://doi.org/10.1186/s12866-019-1630-4.

Rikihisa, Yasuko. 2011. “Mechanisms of Obligatory Intracellular Infection with Anaplasma Phagocytophilum.” *Clinical Microbiology Reviews* 24 (3): 469–89. https://doi.org/10.1128/CMR.00064-10.

Ringo, Aaron Edmond, Paul Franck Adjou Moumouni, Moeti Taioe, Charoonluk Jirapattharasate, Mingming Liu, Guanbo Wang, Yang Gao, et al. 2018a. “Molecular Analysis of Tick-Borne Protozoan and Rickettsial Pathogens in Small Ruminants from Two {South} {African} Provinces.” *Parasitology International* 67 (2): 144–49. https://doi.org/10.1016/j.parint.2017.11.002.

———. 2018b. “Molecular Analysis of Tick-Borne Protozoan and Rickettsial Pathogens in Small Ruminants from Two {South} {African} Provinces.” *Parasitology International* 67 (2): 144–49. https://doi.org/10.1016/j.parint.2017.11.002.

Roberts, Marilyn C. 2005. “Update on Acquired Tetracycline Resistance Genes.” *FEMS Microbiology Letters* 245 (2): 195–203. https://doi.org/10.1016/j.femsle.2005.02.034.

Rolain, J.-M., L Stuhl, M Maurin, and D Raoult. 2002. “Evaluation of {Antibiotic} {Susceptibilities} of {Three} {Rickettsial} {Species} {Including} {Rickettsia} Felis by a {Quantitative} {PCR} {DNA} {Assay}.” *Antimicrobial Agents and Chemotherapy* 46 (9): 2747–51. https://doi.org/10.1128/AAC.46.9.2747-2751.2002.

Rolain, J. M., and Didier Raoult. 2005. “Prediction of Resistance to Erythromycin in the Genus Rickettsia by Mutations in L22 Ribosomal Protein.” *Journal of Antimicrobial Chemotherapy* 56 (2): 396–98. https://doi.org/10.1093/jac/dki242.

Rolain, J M. 2005. “Genome {Comparison} {Analysis} of {Molecular} {Mechanisms} of {Resistance} to {Antibiotics} in the {Rickettsia} {Genus}.” *Annals of the New York Academy of Sciences* 1063 (1): 222–30. https://doi.org/10.1196/annals.1355.035.

Rolain, J M, M Maurin, G Vestris, and D Raoult. 1998. “In {Vitro} {Susceptibilities} of 27 {Rickettsiae} to 13 {Antimicrobials}.” *Antimicrobial Agents and Chemotherapy* 42 (7): 1537–41. https://doi.org/10.1128/AAC.42.7.1537.

Rounds, Megan A, Christopher D Crowder, Heather E Matthews, Curtis A Philipson, Glen A Scoles, David J Ecker, Steven E Schutzer, and Mark W Eshoo. 2012. “Identification of Endosymbionts in Ticks by Broad-Range Polymerase Chain Reaction and Electrospray Ionization Mass Spectrometry.” *Journal of Medical Entomology* 49 (4): 843–50. https://doi.org/10.1603/me12038.

Rzewuska, Magdalena, Ewelina Kwiecień, Dorota Chrobak-Chmiel, Magdalena Kizerwetter-Świda, Ilona

Stefańska, and Małgorzata Gieryńska. 2019. “Pathogenicity and Virulence of Trueperella Pyogenes: A Review.” *International Journal of Molecular Sciences* 20 (11). https://doi.org/10.3390/ijms20112737.

Sánchez-Montes, Sokani, Ana Belem Isaak-Delgado, Carmen Guzmán-Cornejo, Emilio Rendón-Franco, Claudia I. Muñoz-García, Sergio Bermúdez, Jorge Morales-Diaz, et al. 2019. “Rickettsia Species in Ticks That Parasitize Amphibians and Reptiles: Novel Report from Mexico and Review of the Worldwide Record.” *Ticks and Tick-Borne Diseases*. https://doi.org/10.1016/j.ttbdis.2019.04.013.

Sanjuán, Rafael, and Pilar Domingo-Calap. 2016. “Mechanisms of Viral Mutation.” *Cellular and Molecular Life Sciences* 73 (23): 4433–48. https://doi.org/10.1007/s00018-016-2299-6.

Santos, E. V., G. Silva, G. P. Cardozo, T. A. Bitencourt, S. C. França, A. L. Fachin, and M. Marins. 2012. “In Silico Characterization of Three Two-Component Systems of Ehrlichia Canis and Evaluation of a Natural Plant-Derived Inhibitor.” *Genetics and Molecular Research* 11 (4): 3576–84. https://doi.org/10.4238/2012.October.4.5.

Santos, Paula S., Angela A.S. Sena, Rafael Nascimento, Thaise G. Araújo, Mirian M. Mendes, João R.S. Martins, Tiago W.P. Mineo, José R. Mineo, and Luiz R. Goulart. 2013. “Epitope-Based Vaccines with the Anaplasma Marginale MSP1a Functional Motif Induce a Balanced Humoral and Cellular Immune Response in Mice.” *PLoS ONE* 8 (4): 1–9. https://doi.org/10.1371/journal.pone.0060311.

Scally, S. Z. de, T. P. Makhalanyane, A. Frossard, I. D. Hogg, and D. A. Cowan. 2016. “Antarctic Microbial Communities Are Functionally Redundant, Adapted and Resistant to Short Term Temperature Perturbations.” *Soil Biology and Biochemistry* 103: 160–70. https://doi.org/10.1016/j.soilbio.2016.08.013.

Scherler, Aurélie, Nicolas Jacquier, and Gilbert Greub. 2018. “Chlamydiales, {Anaplasma} and {Bartonella}: Persistence and Immune Escape of Intracellular Bacteria.” *Microbes and Infection* 20 (7–8): 416–23. https://doi.org/10.1016/j.micinf.2017.11.002.

Schroeder, Meredith, Benjamin D Brooks, and Amanda E Brooks. 2017. “Antibiotic Resistance.” https://doi.org/10.3390/genes8010039.

Segata, Nicola, Jacques Izard, Levi Waldron, Dirk Gevers, Larisa Miropolsky, Wendy S. Garrett, and Curtis Huttenhower. 2011. “Metagenomic Biomarker Discovery and Explanation.” *Genome Biology* 12 (6): R60. https://doi.org/10.1186/gb-2011-12-6-r60.

Selvarajan, Ramganesh, Timothy Sibanda, Siddarthan Venkatachalam, Henry J.O. Ogola, Chinedu Christopher Obieze, and Titus A. Msagati. 2019. “Distribution, Interaction and Functional Profiles of Epiphytic Bacterial Communities from the Rocky Intertidal Seaweeds, South Africa.” *Scientific Reports* 9 (1): 1–13. https://doi.org/10.1038/s41598-019-56269-2.

Seo, Min Goo, Oh Deog Kwon, and Dongmi Kwak. 2020. “Molecular and Phylogenetic Analysis of Tick-Borne Pathogens in Ticks Parasitizing Native Korean Goats (Capra Hircus Coreanae) in South Korea.” *Pathogens* 9 (2). https://doi.org/10.3390/pathogens9020071.

Seshadri, Rekha, Ian T. Paulsen, Jonathan A. Eisen, Timothy D. Read, Karen E. Nelson, William C. Nelson, Naomi L. Ward, et al. 2003. “Complete Genome Sequence of the Q-Fever Pathogen Coxiella Burnetii.” *Proceedings of the National Academy of Sciences of the United States of America* 100 (9): 5455–60. https://doi.org/10.1073/pnas.0931379100.

Siebert, Claire, Helena Lindgren, Sabrina Ferré, Corinne Villers, Sandrine Boisset, Julien Perard, Anders Sjöstedt, et al. 2019. “*Francisella Tularensis*: FupA Mutation Contributes to Fluoroquinolone Resistance by Increasing Vesicle Secretion and Biofilm Formation.” *Emerging Microbes & Infections*

8 (1): 808–22. https://doi.org/10.1080/22221751.2019.1615848.

Speer, B S, N B Shoemaker, and A A Salyers. 1992. “Bacterial Resistance to Tetracycline: Mechanisms, Transfer, and Clinical Significance.” *Clinical Microbiology Reviews* 5 (4): 387–99. https://doi.org/10.1128/CMR.5.4.387.

Spengler, Jessica R, and Agustin Estrada-Peña. 2018. “Host Preferences Support the Prominent Role of {Hyalomma} Ticks in the Ecology of {Crimean}-{Congo} Hemorrhagic Fever.” Edited by Hans-Peter Fuehrer. *PLOS Neglected Tropical Diseases* 12 (2): e0006248. https://doi.org/10.1371/journal.pntd.0006248.

Spickett, Arthur M., I. Heloise Heyne, and Roy Williams. 2011. “Survey of the Livestock Ticks of the North West Province, South Africa.” *The Onderstepoort Journal of Veterinary Research* 78 (1): 305. https://doi.org/10.4102/ojvr.v78i1.305.

Spyridaki, I. 2002. “Mechanisms of Resistance to Fluoroquinolones in Coxiella Burnetii.” *Journal of Antimicrobial Chemotherapy* 49 (2): 379–82. https://doi.org/10.1093/jac/49.2.379.

Sreekumar, C., R. Anandan, S. Balasundaram, and G. Rajavelu. 1996. “Morphology and Staining Characteristics of Ehrlichia Bovis.” *Comparative Immunology, Microbiology and Infectious Diseases*. https://doi.org/10.1016/0147-9571(95)00011-9.

Strasfeld, Lynne, and Sunwen Chou. 2010. “Antiviral {Drug} {Resistance}: {Mechanisms} and {Clinical} {Implications}.” *Infectious Disease Clinics of North America* 24 (2): 413–37. https://doi.org/10.1016/j.idc.2010.01.001.

Szigeti, Alexandra, Zsuzsa Kreizinger, Sándor Hornok, Getachew Abichu, and Miklós Gyuranecz. 2014. “Detection of {Francisella}-like Endosymbiont in {Hyalomma} Rufipes from {Ethiopia}.” *Ticks and Tick-Borne Diseases* 5 (6): 818–20. https://doi.org/10.1016/j.ttbdis.2014.06.002.

Te, Helen S, Glenn Randall, and Donald M Jensen. 2007. “Mechanism of {Action} of {Ribavirin} in the {Treatment} of {Chronic} {Hepatitis} {C}.” *Gastroenterology & Hepatology* 3: 8.

Theiler, A. 1909. “Anaplasma Marginale (Gen. Spec. Nov.). {The} Marginal Points in the Blood of Cattle Suffering from a Specific Disease.” *Report of the Government Veterinary Bacteriologist,*.

———. 1910. “Further Investigations into Anaplasmosis of {South} {African} Cattle.” *First Report of the Director of Veterinary Research*.

———. 1912. “Gall Sickness of Imported Cattle and the Protective Inoculation against This Disease.” *Agricultural Journal of the Union of South Africa* 3:7-46.

Troyer, Jill Michelle, Suzana Radulovic, Siv G E Andersson, and Abdu F Azad. 1998. “Detection of Point Mutations in RpoB Gene of Rifampin-Resistant Rickettsia Typhi.” *Antimicrobial Agents and Chemotherapy* 42 (7): 1845–46. https://doi.org/10.1128/AAC.42.7.1845.

Tyson, Gregory H, Patrick F McDermott, Cong Li, Yuansha Chen, Daniel A Tadesse, Sampa Mukherjee, Sonya Bodeis-Jones, et al. 2015. “{WGS} Accurately Predicts Antimicrobial Resistance in \textit{{Escherichia} Coli}.” *Journal of Antimicrobial Chemotherapy* 70 (10): 2763–69. https://doi.org/10.1093/jac/dkv186.

Venter, Henrietta, Rumana Mowla, Thelma Ohene-Agyei, and Shutao Ma. 2015. “RND-Type Drug Efflux Pumps from Gram-Negative Bacteria: Molecular Mechanism and Inhibition.” *Frontiers in Microbiology* 6 (APR): 1–11. https://doi.org/10.3389/fmicb.2015.00377.

Vila, A., A. Estrada-Peña, L. Altet, A. Cusco, S. Dandreano, O. Francino, L. Halos, and X. Roura. 2019.

"Endosymbionts Carried by Ticks Feeding on Dogs in Spain." *Ticks and Tick-Borne Diseases* 10 (4): 848–52. https://doi.org/10.1016/j.ttbdis.2019.04.003.

Vranakis, Iosif, Pieter Jan de Bock, Anastasia Papadioti, Yannis Tselentis, Kris Gevaert, Georgios Tsiotis, and Anna Psaroulaki. 2012. "Quantitative Proteome Profiling of C. Burnetii under Tetracycline Stress Conditions." *PLoS ONE* 7 (3). https://doi.org/10.1371/journal.pone.0033599.

Vranakis, Iosif, Vassilios Sandalakis, Dimosthenis Chochlakis, Yannis Tselentis, and Anna Psaroulaki. 2010. "DNA Gyrase and Topoisomerase IV Mutations in an in Vitro Fluoroquinolone-Resistant Coxiella Burnetii Strain." *Microbial Drug Resistance* 16 (2): 111–17. https://doi.org/10.1089/mdr.2010.0015.

Wang, Wei, Muhammad Imran Arshad, Mohsin Khurshid, Muhammad Hidayat Rasool, Muhammad Atif Nisar, Muhammad Aamir Aslam, and Muhammad Usman Qamar. 2018. "Antibiotic Resistance : A Rundown of a Global Crisis." *Infection and Drug Resistance*, 1645–58.

Watts, J L, D E Lowery, J F Teel, and S Rossbach. 2000. "Identification of Corynebacterium Bovis and Other Coryneforms Isolated from Bovine Mammary Glands." *Journal of Dairy Science* 83 (10): 2373–79. https://doi.org/10.3168/jds.S0022-0302(00)75126-5.

Webber, M. A., and L. J V Piddock. 2003. "The Importance of Efflux Pumps in Bacterial Antibiotic Resistance." *Journal of Antimicrobial Chemotherapy* 51 (1): 9–11. https://doi.org/10.1093/jac/dkg050.

Weber, Nick, David Liou, Jennifer Dommer, Philip Macmenamin, Mariam Quiñones, Ian Misner, Andrew J. Oler, et al. 2018. "Nephele: A Cloud Platform for Simplified, Standardized and Reproducible Microbiome Data Analysis." *Bioinformatics* 34 (8): 1411–13. https://doi.org/10.1093/bioinformatics/btx617.

Weinert, Lucy A., John J. Welch, and Francis M. Jiggins. 2009. "Conjugation Genes Are Common throughout the Genus Rickettsia and Are Transmitted Horizontally." *Proceedings of the Royal Society B: Biological Sciences* 276 (1673): 3619–27. https://doi.org/10.1098/rspb.2009.0875.

Wright, Edward, and Engin H. Serpersu. 2005. "Enzyme-Substrate Interactions with an Antibiotic Resistance Enzyme: Aminoglycoside Nucleotidyltransferase(2″)-Ia Characterized by Kinetic and Thermodynamic Methods." *Biochemistry* 44 (34): 11581–91. https://doi.org/10.1021/bi050797c.

Xu, W, L Beati, and D Raoult. 1997. "Characterization of and Application of Monoclonal Antibodies against {Rickettsia} Africae, a Newly Recognized Species of Spotted Fever Group Rickettsia." 35: 8.

Yabsley, Michael J, Nola J Parsons, Elizabeth C Horne, Barbara C Shock, and Michaelle Purdee. 2012. "Novel Relapsing Fever {Borrelia} Detected in {African} Penguins ({Spheniscus} Demersus) Admitted to Two Rehabilitation Centers in {South} {Africa}." *Parasitology Research* 110 (3): 1125–30. https://doi.org/10.1007/s00436-011-2602-2.

Yang, Jifei, Youquan Li, Zhijie Liu, Junlong Liu, Qingli Niu, Qiaoyun Ren, Ze Chen, Guiquan Guan, Jianxun Luo, and Hong Yin. 2015. "Molecular Detection and Characterization of {Anaplasma} Spp. in Sheep and Cattle from {Xinjiang}, Northwest {China}." *Parasites & Vectors* 8 (1): 108. https://doi.org/10.1186/s13071-015-0727-3.

Yang, Xiao Jun, Yan Bo Wang, Zhi Wei Zhou, Guo Wei Wang, Xiao Hong Wang, Qing Fu Liu, Shu Feng Zhou, and Zhen Hai Wang. 2015. "High-Throughput Sequencing of 16S RDNA Amplicons Characterizes Bacterial Composition in Bronchoalveolar Lavage Fluid in Patients with Ventilator-Associated Pneumonia." *Drug Design, Development and Therapy* 9: 4883–96. https://doi.org/10.2147/DDDT.S87634.

Yoon, Seok Hwan, Sung Min Ha, Soonjae Kwon, Jeongmin Lim, Yeseul Kim, Hyungseok Seo, and Jongsik Chun. 2017. "Introducing EzBioCloud: A Taxonomically United Database of 16S RRNA Gene Sequences and Whole-Genome Assemblies." *International Journal of Systematic and Evolutionary Microbiology* 67 (5): 1613–17. https://doi.org/10.1099/ijsem.0.001755.

Zárate, Sandra G., Paula Morales, Katarzyna Swiderek, Victor M. Bolanos-Garcia, and Agatha Bastida. 2019. "A Molecular Modeling Approach to Identify Novel Inhibitors of the Major Facilitator Superfamily of Efflux Pump Transporters." *Antibiotics* 8 (1). https://doi.org/10.3390/antibiotics8010025.

Zaura, Egija, Bart Jf Keijser, Susan M. Huse, and Wim Crielaard. 2009. "Defining the Healthy 'Core Microbiome' of Oral Microbial Communities." *BMC Microbiology* 9: 1–12. https://doi.org/10.1186/1471-2180-9-259.

Zuñiga-Navarrete, Fernando, Gabriela Flores-Ramirez, Maksym Danchenko, Oldrich Benada, Anton Skriba, and Ludovit Skultety. 2019. "Proteomic Analysis Revealed the Survival Strategy of Coxiella Burnetii to Doxycycline Exposure." *Journal of Proteomics* 208 (July): 103479. https://doi.org/10.1016/j.jprot.2019.103479.

# CHAPTER 5 FINAL CONCLUSIONS AND RECOMMENDATIONS

The general conclusions from this study are as follows:

Tick-borne microbial communities are of economic importance presenting a major economic challenge to cattle in South Africa. Farmers rely mainly on acaricides for tick control; however, the infestation is still evident with increasing tick resistance to acaricides.

- In the current study, species of *Hyalomma* (8%), *Amblyomma* (30%), and *Rhipicephalus* (62%) ticks were identified. Highly expressed tick-borne pathogens associated with *Hyalomma, Amblyomma,* and *Rhipicephalus* ticks included species from the genera of *Rickettsia, Coxiella, Porphyromonas, Trueperella*, *Corynebacterium,* and *Ehrlichia*. Variability in abundance and diversity of bacterial profiles reflected differences in bacterial communities harbored by tick species.
- Next-generation sequencing provided a clear understanding of bacterial communities associated with ticks. Ticks are not only vectors but also act as reservoirs of pathogens to reinfect herds of cattle. In the current study, an overabundance of tick-associated bacteria including bacteriome and endosymbionts were identified. More studies to characterize the identified bacteria, and other genera such as *Porphyromonas, Helcoccoccus, Arthrobacter, Anaerococcus, Aerococcus, Helcococcus, Acinetobacter*, *Peptoniphilus,* and *Trueperella* are necessary.
- A consortium of antibiotic resistance genes amongst tick-borne bacterial communities, coding for drug efflux pumps; drug degrading and modifying enzymes; secretion systems proteins; and ribosomal protection proteins was revealed. These resistance genes are known to confer resistance to several antibiotics. Future studies looking at the pharmacological aspects of these genes are important in improving the treatment of tick-borne infections.

This was a preliminary study to elucidate bacterial communities associated with local ticks, and ticks used for the study were collected from cattle only and no ticks were collected from the surrounding environment which is a limitation we identified in the current study. As a result, the overall number of ticks was low and given that, it may be difficult to make comparisons and

conclusions based on the microbial populations present in each tick species. Only a variable region V3 and V4 of 16S rRNA were amplified, this limited the number of species groups associated with these variable regions identified in the current study. NGS is a very powerful tool that identifies the whole community of bacteria including non-pathogenic species that were not of interest in the current study, however, 16S rRNA NGS provided insights on the interaction between endosymbionts, pathogens, and ticks. Furthermore, predicted antimicrobial resistance pathways require further phenotypic studies for conclusiveness of the results.

The results from the current investigation support the hypothesis that tick-borne bacterial communities are highly drug-resistant, with a high abundance of resistance biomarkers. Follow-up molecular studies are necessary to understand the distribution and composition of microbial communities in ticks, and the full spectrum of antimicrobial-resistant genes they continue to acquire as they evolve.

APPENDICES

APPENDIX 1 UNIVERSITY OF SOUTH AFRICA ETHICS RESEARCH COMMITTEE CLEARANCE (REFERENCE NUMBER: 2019/CAES_AREC/152)

UNISA university of south africa

**UNISA-CAES ANIMAL RESEARCH ETHICS COMMITTEE**

Date: 09/09/2019

Dear Mr Chigwada

NHREC Registration # : N/A
REC Reference # : 2019/CAES_AREC/152
Name : Mr AD Chigwada
Student #: 61366943

**Decision: Ethics Approval from 06/09/2019 to completion**

**Researcher(s):** Mr AD Chigwada
aubreychigwada@yahoo.com

**Supervisor (s):** Dr TM Masebe
masebtm@unisa.ac.za; 011-471-2268

**Working title of research:**

Investigation of tick-borne pathogens resistance markers using next generation sequencing

**Qualification:** MSc Life Science

Thank you for the application for research ethics clearance by the UNISA-CAES Animal Research Ethics Committee for the above mentioned research. Ethics approval is granted until the completion of the project, **subject to submission of yearly progress reports. Failure to submit the progress report will lead to withdrawal of the ethics clearance until the report has been submitted.**

**Due date for progress report: 31 August 2020**

*The **low risk application** was **reviewed** by the UNISA-CAES Animal Research Ethics Committee on 06 September 2019 in compliance with the Unisa Policy on Research Ethics and the Standard Operating Procedure on Research Ethics Risk Assessment.*

The proposed research may now commence with the provisions that:

1. The researcher(s) will ensure that the research project adheres to the values and principles expressed in the UNISA Policy on Research Ethics.

University of South Africa
Preller Street, Muckleneuk Ridge, City of Tshwane
PO Box 392 UNISA 0003 South Africa
Telephone: +27 12 429 3111 Facsimile: +27 12 429 4150
www.unisa.ac.za

2. Any adverse circumstance arising in the undertaking of the research project that is relevant to the ethicality of the study should be communicated in writing to the Committee.
3. The researcher(s) will conduct the study according to the methods and procedures set out in the approved application.
4. Any changes that can affect the study-related risks for the research participants, particularly in terms of assurances made with regards to the protection of participants' privacy and the confidentiality of the data, should be reported to the Committee in writing, accompanied by a progress report.
5. The researcher will ensure that the research project adheres to any applicable national legislation, professional codes of conduct, institutional guidelines and scientific standards relevant to the specific field of study. Adherence to the following South African legislation is important, if applicable: Protection of Personal Information Act, no 4 of 2013; Children's act no 38 of 2005 and the National Health Act, no 61 of 2003.
6. Only de-identified research data may be used for secondary research purposes in future on condition that the research objectives are similar to those of the original research. Secondary use of identifiable human research data require additional ethics clearance.
7. No field work activities may continue after the expiry date. Submission of a completed research ethics progress report will constitute an application for renewal of Ethics Research Committee approval.

*Note:*
*The reference number **2019/CAES_AREC/152** should be clearly indicated on all forms of communication with the intended research participants, as well as with the Committee.*

Yours sincerely,

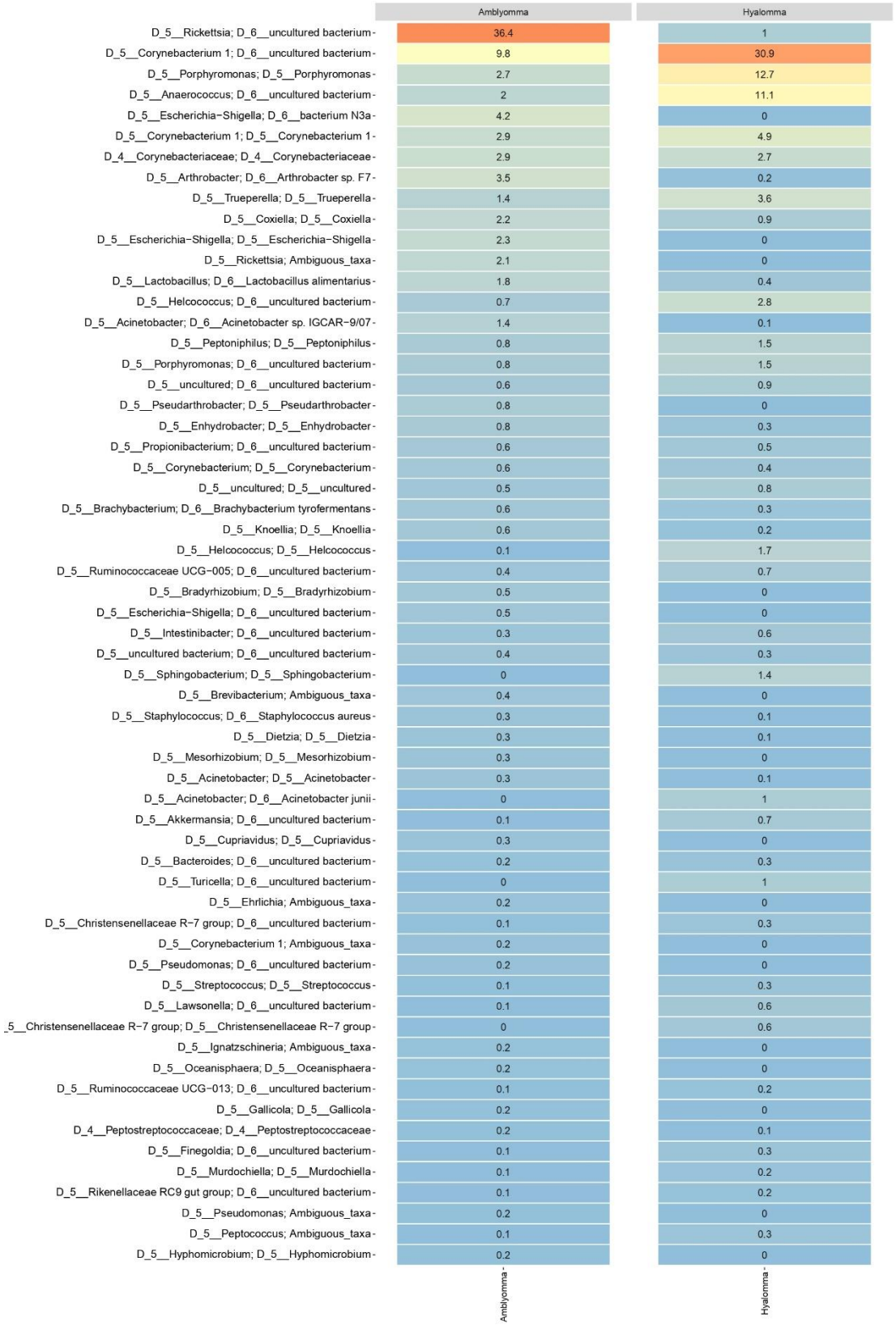
**Prof EL Kempen**
**Chair of UNISA-CAES Animal REC**
E-mail: kempeel@unisa.ac.za
Tel: (011) 471-2241

**Prof MJ Linington**
**Executive Dean : CAES**
E-mail: lininmj@unisa.ac.za
Tel: (011) 471-3806



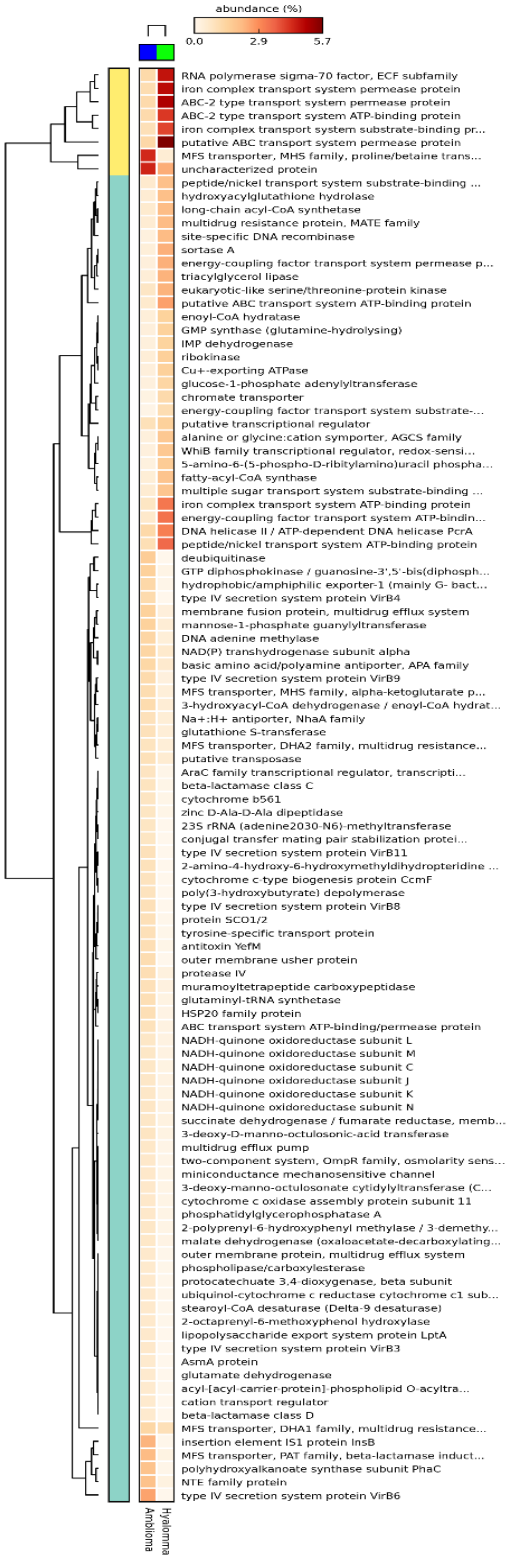
University of South Africa
Preller Street, Muckleneuk Ridge, City of Tshwane
PO Box 392 UNISA 0003 South Africa
Telephone: +27 12 429 3111 Facsimile: +27 12 429 4150
www.unisa.ac.za

# APPENDIX 2 SUPPLEMENTARY 3.1 DATA TOP 60 BACTERIAL SPECIES IDENTIFIED



| | Amblyomma | Hyalomma |
|---|---|---|
| D_5__Rickettsia; D_6__uncultured bacterium | 36.4 | 1 |
| D_5__Corynebacterium 1; D_6__uncultured bacterium | 9.8 | 30.9 |
| D_5__Porphyromonas; D_5__Porphyromonas | 2.7 | 12.7 |
| D_5__Anaerococcus; D_6__uncultured bacterium | 2 | 11.1 |
| D_5__Escherichia−Shigella; D_6__bacterium N3a | 4.2 | 0 |
| D_5__Corynebacterium 1; D_5__Corynebacterium 1 | 2.9 | 4.9 |
| D_4__Corynebacteriaceae; D_4__Corynebacteriaceae | 2.9 | 2.7 |
| D_5__Arthrobacter; D_6__Arthrobacter sp. F7 | 3.5 | 0.2 |
| D_5__Trueperella; D_5__Trueperella | 1.4 | 3.6 |
| D_5__Coxiella; D_5__Coxiella | 2.2 | 0.9 |
| D_5__Escherichia−Shigella; D_5__Escherichia−Shigella | 2.3 | 0 |
| D_5__Rickettsia; Ambiguous_taxa | 2.1 | 0 |
| D_5__Lactobacillus; D_6__Lactobacillus alimentarius | 1.8 | 0.4 |
| D_5__Helcococcus; D_6__uncultured bacterium | 0.7 | 2.8 |
| D_5__Acinetobacter; D_6__Acinetobacter sp. IGCAR−9/07 | 1.4 | 0.1 |
| D_5__Peptoniphilus; D_5__Peptoniphilus | 0.8 | 1.5 |
| D_5__Porphyromonas; D_6__uncultured bacterium | 0.8 | 1.5 |
| D_5__uncultured; D_6__uncultured bacterium | 0.6 | 0.9 |
| D_5__Pseudarthrobacter; D_5__Pseudarthrobacter | 0.8 | 0 |
| D_5__Enhydrobacter; D_5__Enhydrobacter | 0.8 | 0.3 |
| D_5__Propionibacterium; D_6__uncultured bacterium | 0.6 | 0.5 |
| D_5__Corynebacterium; D_5__Corynebacterium | 0.6 | 0.4 |
| D_5__uncultured; D_5__uncultured | 0.5 | 0.8 |
| D_5__Brachybacterium; D_6__Brachybacterium tyrofermentans | 0.6 | 0.3 |
| D_5__Knoellia; D_5__Knoellia | 0.6 | 0.2 |
| D_5__Helcococcus; D_5__Helcococcus | 0.1 | 1.7 |
| D_5__Ruminococcaceae UCG−005; D_6__uncultured bacterium | 0.4 | 0.7 |
| D_5__Bradyrhizobium; D_5__Bradyrhizobium | 0.5 | 0 |
| D_5__Escherichia−Shigella; D_6__uncultured bacterium | 0.5 | 0 |
| D_5__Intestinibacter; D_6__uncultured bacterium | 0.3 | 0.6 |
| D_5__uncultured bacterium; D_6__uncultured bacterium | 0.4 | 0.3 |
| D_5__Sphingobacterium; D_5__Sphingobacterium | 0 | 1.4 |
| D_5__Brevibacterium; Ambiguous_taxa | 0.4 | 0 |
| D_5__Staphylococcus; D_6__Staphylococcus aureus | 0.3 | 0.1 |
| D_5__Dietzia; D_5__Dietzia | 0.3 | 0.1 |
| D_5__Mesorhizobium; D_5__Mesorhizobium | 0.3 | 0 |
| D_5__Acinetobacter; D_5__Acinetobacter | 0.3 | 0.1 |
| D_5__Acinetobacter; D_6__Acinetobacter junii | 0 | 1 |
| D_5__Akkermansia; D_6__uncultured bacterium | 0.1 | 0.7 |
| D_5__Cupriavidus; D_5__Cupriavidus | 0.3 | 0 |
| D_5__Bacteroides; D_6__uncultured bacterium | 0.2 | 0.3 |
| D_5__Turicella; D_6__uncultured bacterium | 0 | 1 |
| D_5__Ehrlichia; Ambiguous_taxa | 0.2 | 0 |
| D_5__Christensenellaceae R−7 group; D_6__uncultured bacterium | 0.1 | 0.3 |
| D_5__Corynebacterium 1; Ambiguous_taxa | 0.2 | 0 |
| D_5__Pseudomonas; D_6__uncultured bacterium | 0.2 | 0 |
| D_5__Streptococcus; D_5__Streptococcus | 0.1 | 0.3 |
| D_5__Lawsonella; D_6__uncultured bacterium | 0.1 | 0.6 |
| _5__Christensenellaceae R−7 group; D_5__Christensenellaceae R−7 group | 0 | 0.6 |
| D_5__Ignatzschineria; Ambiguous_taxa | 0.2 | 0 |
| D_5__Oceanisphaera; D_5__Oceanisphaera | 0.2 | 0 |
| D_5__Ruminococcaceae UCG−013; D_6__uncultured bacterium | 0.1 | 0.2 |
| D_5__Gallicola; D_5__Gallicola | 0.2 | 0 |
| D_4__Peptostreptococcaceae; D_4__Peptostreptococcaceae | 0.2 | 0.1 |
| D_5__Finegoldia; D_6__uncultured bacterium | 0.1 | 0.3 |
| D_5__Murdochiella; D_5__Murdochiella | 0.1 | 0.2 |
| D_5__Rikenellaceae RC9 gut group; D_6__uncultured bacterium | 0.1 | 0.2 |
| D_5__Pseudomonas; Ambiguous_taxa | 0.2 | 0 |
| D_5__Peptococcus; Ambiguous_taxa | 0.1 | 0.3 |
| D_5__Hyphomicrobium; D_5__Hyphomicrobium | 0.2 | 0 |
| | Amblyomma | Hyalomma |

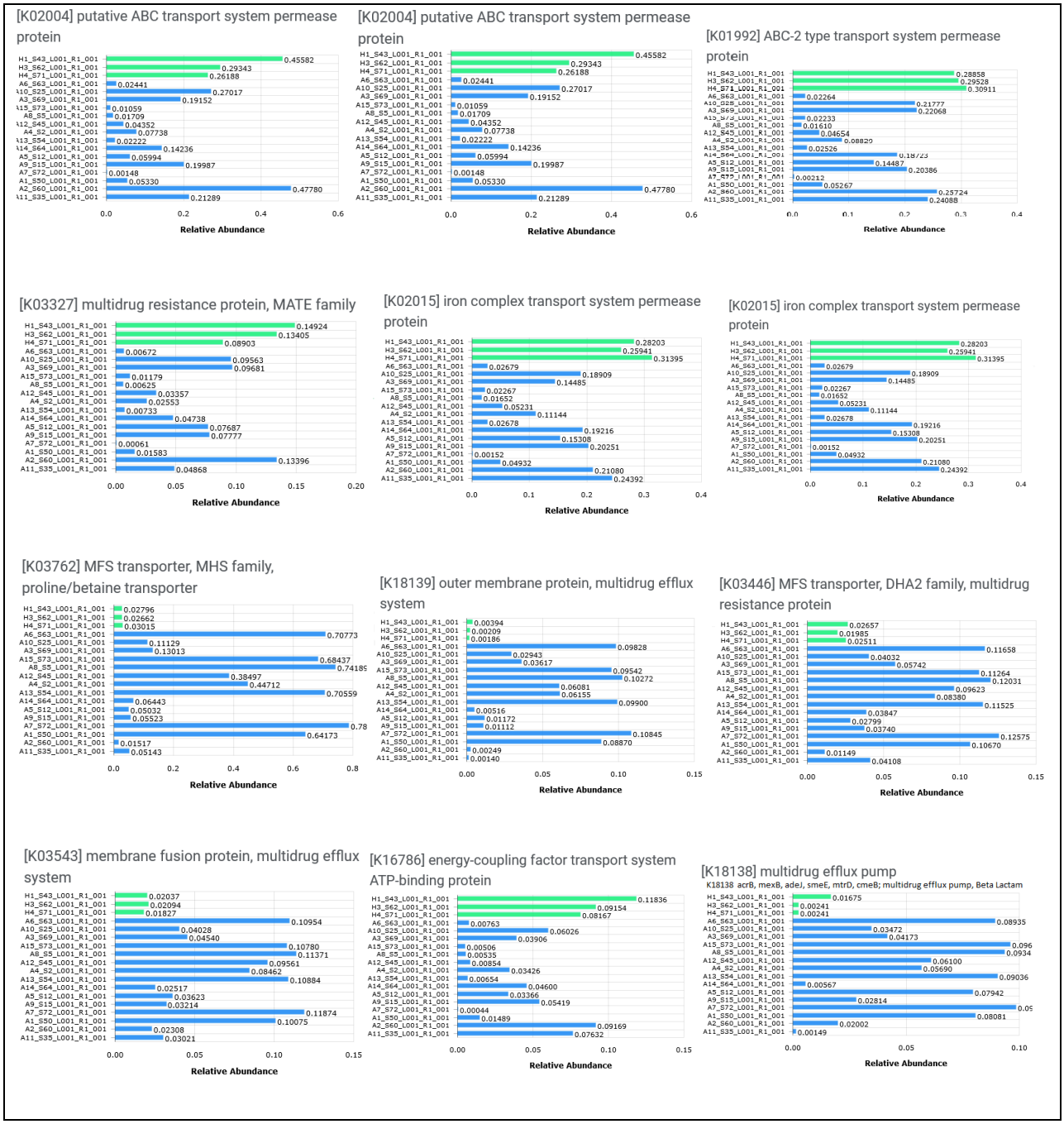
APPENDIX 3 CHAPTER 3 SUPPLEMENTARY 3.2 RESISTANCE BIOMARKERS




Supplementary 3.2. Top 100 functional biomarkers associated with bacterial communities of Hyalomma and Amblyomma ticks, resistance to antibiotics, identified using PICRUSt algorithm analyzed using LEfSe analysis.

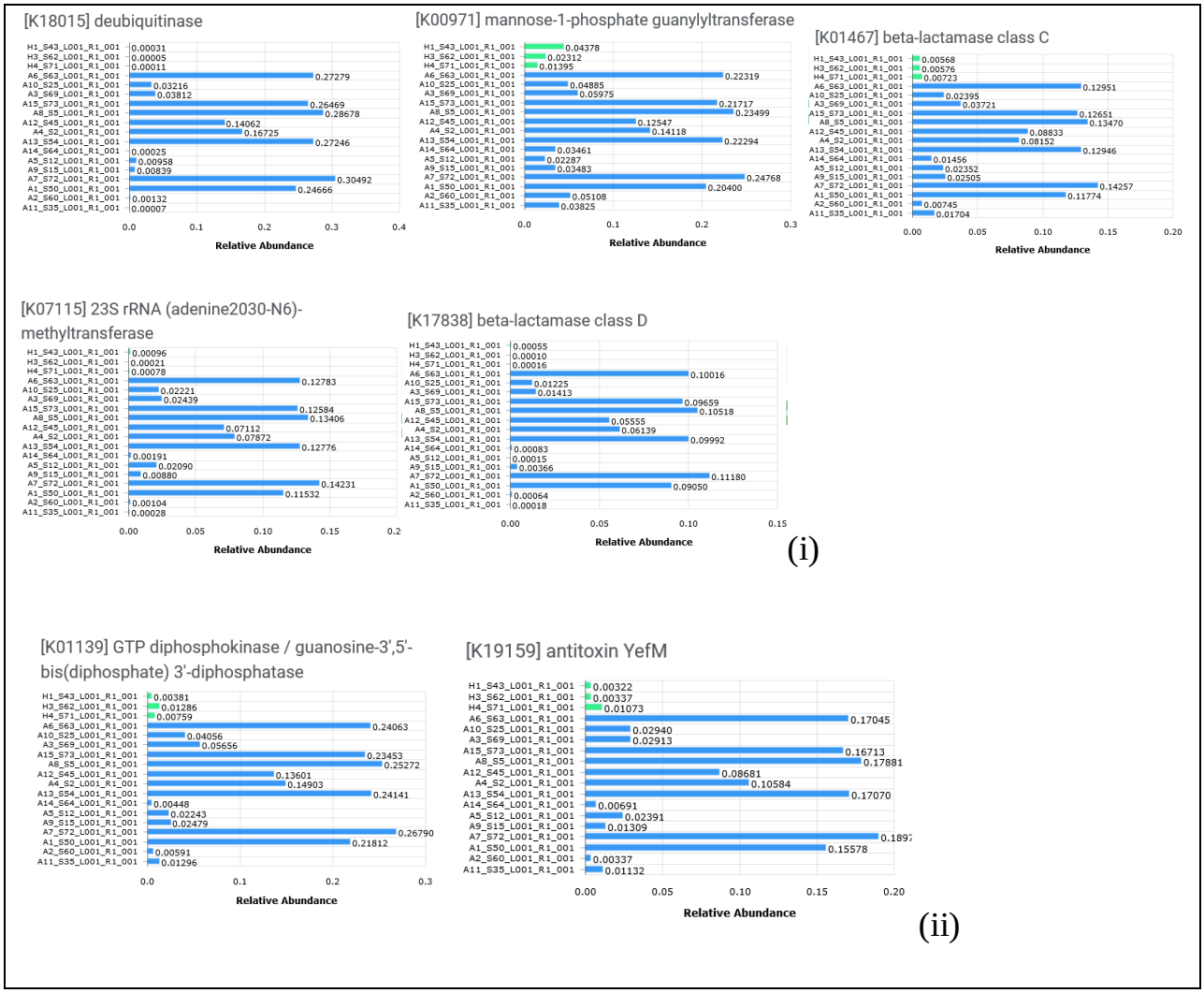
Supplementary data 3.3 illustrating individual resistance genes relative abundance.

Efflux pumps and transport proteins

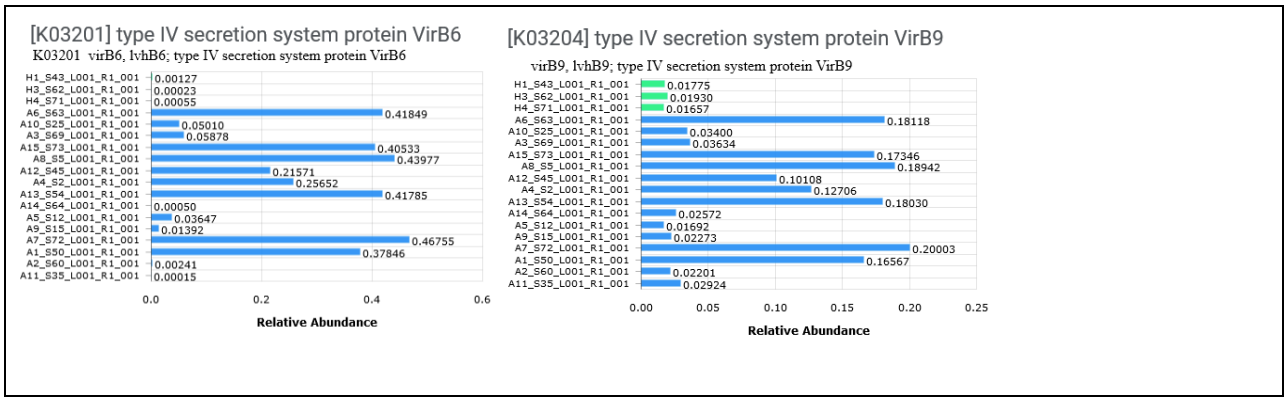



Supplementary 3.3.2. Resistance genes detected associated with drug efflux pumps

Drug degrading enzymes and ribosomal protection proteins

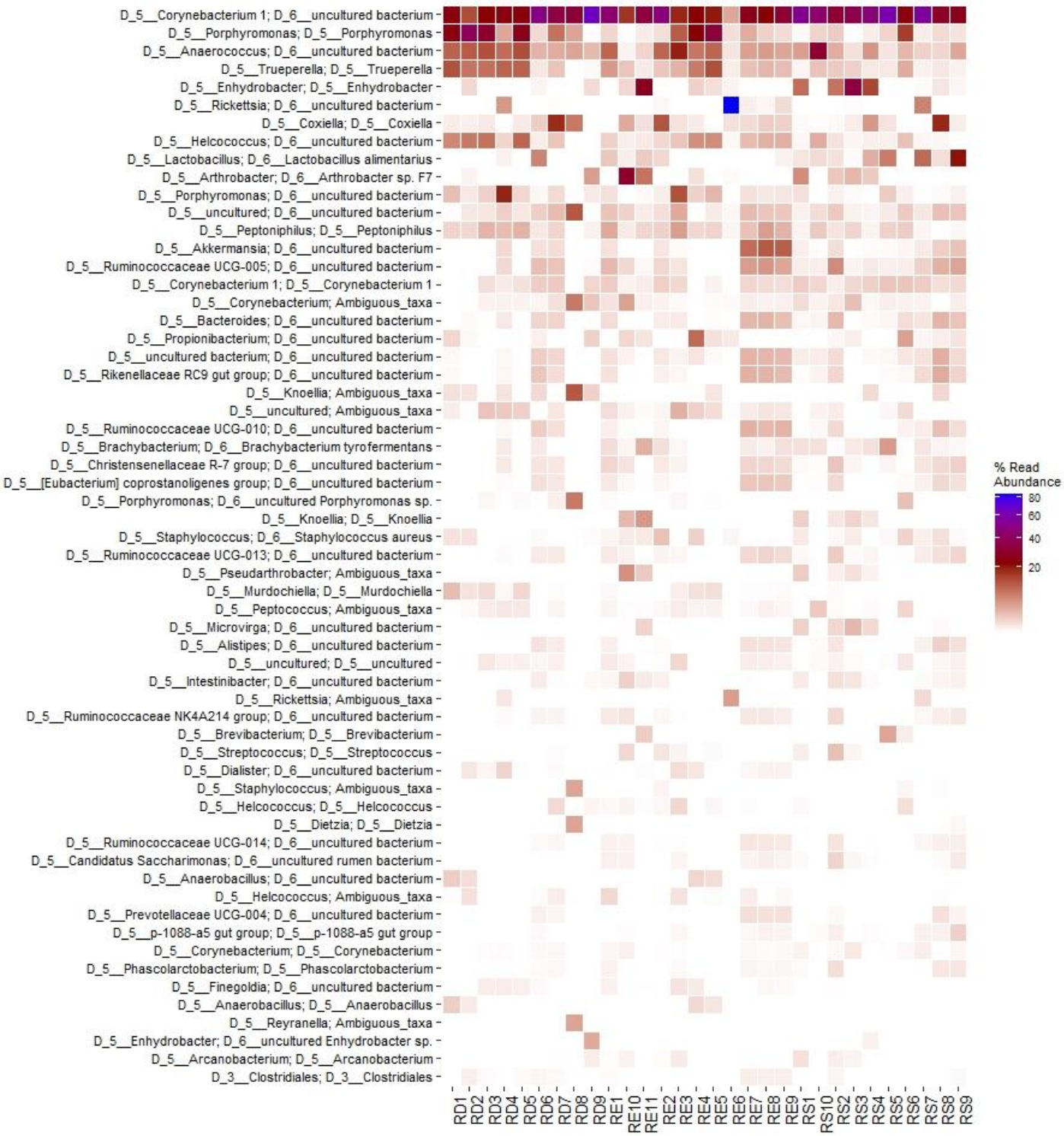



Supplementary 3.3.3. Drug degrading enzymes (i) and ribosomal protection proteins (ii)



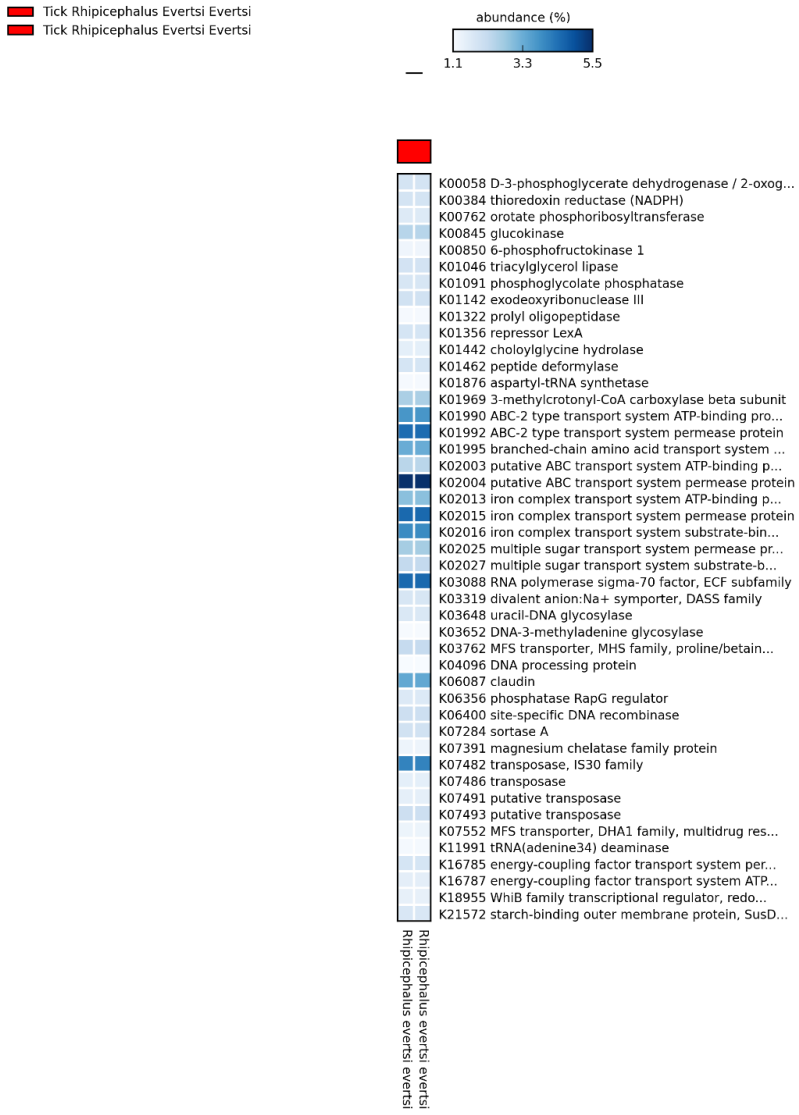

Supplementary 3.3.4 secretion proteins

APPENDIX 4. CHAPTER 4 SUPPLEMENTARY 4.1 SPECIES SUPPLEMENTARY DATA



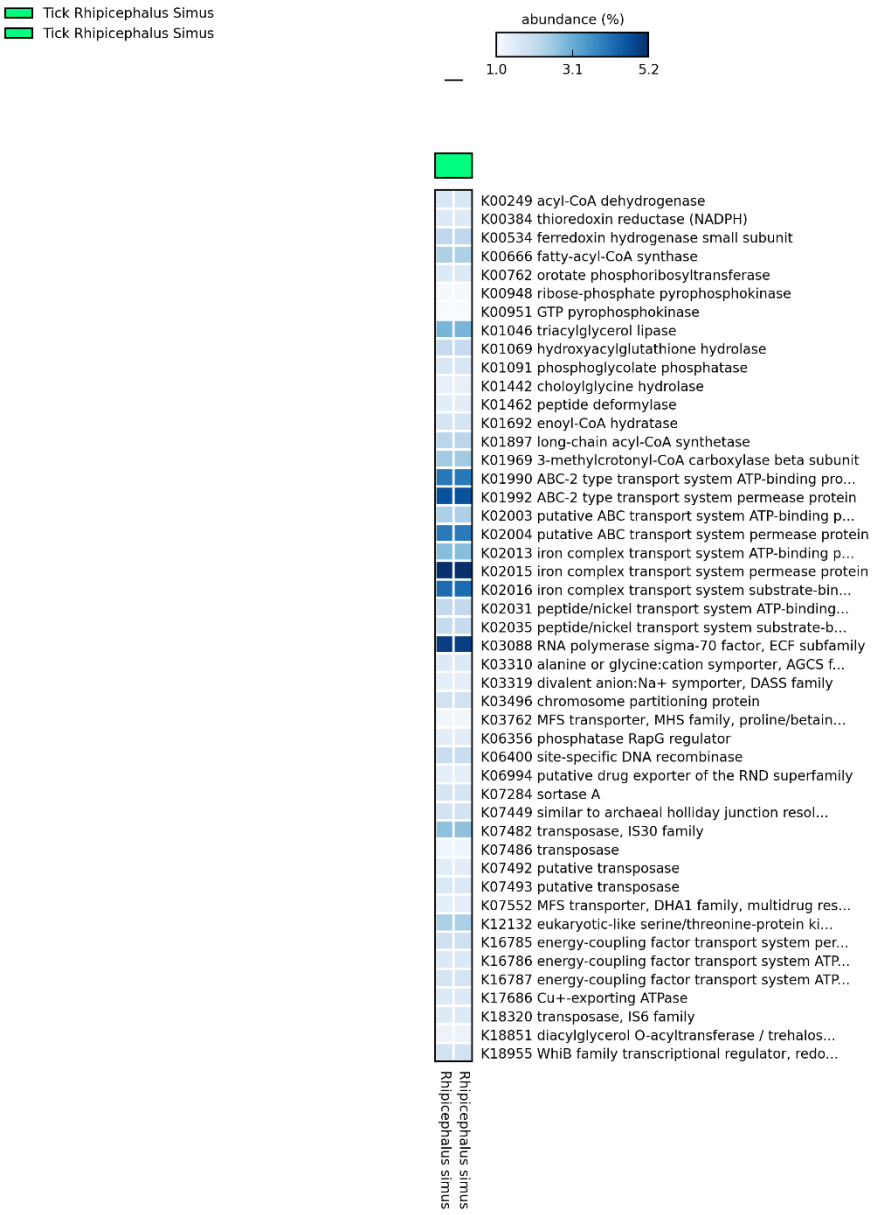

Supplementary 4.1. heatmap representing individual tick samples bacterial communities at the species level, from *R. evertsi evertsi*, *R. simus*, and *R. decoloratus* ticks.

Supplementary 4.2 Antibiotic resistance biomarkers




Supplementary 4.2.1. Heatmap of top 40 PICRUSt predicted KEGG KO functional resistance biomarkers identified from communities of bacteria from *Rhipicephalus evertsi evertsi* tick species.


Supplementary 4.2.2 Heatmap of top 40 PICRUSt predicted KEGG KO functional resistance biomarkers identified from communities of bacteria from *Rhipicephalus decoloratus* tick species.




Supplementary 4.2.3 Heatmap of top 40 PICRUSt predicted KEGG KO functional resistance biomarkers identified from communities of bacteria from *Rhipicephalus simus* tick species.