CULTURING, CHARACTERIZATION AND IDENTIFICATION OF CANDIDATE MICROORGANISMS IN CATTLE EAR, RESPONSIBLE FOR PRODUCING VOLATILE CONSTITUENTS ATTRACTIVE TO THE BROWN EAR TICK

Kogo Kipchumba Kenneth (B.Ed. Science)
I56/25569/2011

A Thesis Submitted in Partial Fulfillment of the Requirements for the Award of the Degree of Master of Science (Microbiology) in the School of Pure and Applied Sciences, Kenyatta University

September 2015

Declaration

This thesis is my original work and has not been presented for a degree in any other university or for any other award.

Signature.....Date.....27/10/2015.....

Kogo Kipchumba Kenneth

Department of Microbiology

We confirm that the work reported in this thesis was carried out by the candidate under our supervision.

Signature.....Date.....28/10/2015

Prof. Paul Okemo

Department of Microbiology,
Kenyatta University

Signature.....Date.....28/10/2015

Dr. George Ngondi Michuki

Biotechnology Theme,
International Livestock Research Institute (ILRI),
Nairobi

Signature.....Date.....28/10/2015

Prof. Ahmed Hassanali

Department of Chemistry,
Kenyatta University

Dedication

To my dear mother, Esnas Tirop, for her patience, perseverance and endurance during my education, to all my family members, friends and lastly to Audrey Chepkemoi for the support she gave me all through;

To my supervisors: Your encouragement was outstanding.

Acknowledgement

My sincere and special thanks go to my supervisors Dr George Michuki, Prof. Paul Okemo and Prof. Ahmed Hassanali for their professional guidance offered in supervising the project from inception to completion. Special thanks goes to Dr Rob Skilton of BecA ILRI Hub for his guidance during my placement at the BecA hub, all lab technicians and research fellows of BecA Hub laboratory ILRI for their mentorship and lab guidance.

I further express my gratitude to the African Bioscience Challenge Fund (ABCF) through BecA ILRI Hub for funding this project and Lenana School for providing the research cattle. Special thanks are extended to Mr. Moses Njahira for co-coordinating timely purchase of reagents and other general requirements for use in the laboratory.

Special thanks to the Director BecA ILRI Hub, Dr Djeng Appolonnaire. Thanks too to supervisors and colleagues for constant critic of the manuscript, Stephen Mwaura for supplying the ticks timely.

I am also grateful to members of staff of ILRI for making the working environment conducive. Special thanks go to Prof Steve Kemp for having their time to critic my project.

Special thanks to all my friends, Daniel Ogofu from Ghana, Dr. Wycliffe Wanzala, Judy Njoki, Cecilia Rumberia, Crispus Mbaluto and Sally Wangamati for your patience, encouragement and understanding during the times of my study.

Lastly I sincerely thank all my classmates Towett Gedion, Rono Bernard, Marjorie, Josephine Ngari and Lily Gathu for their personal contributions and encouragement, and above all I thank my Almighty God for the life and grace He gave me throughout the entire course.

Table of contents

List of Figures

List of Tables

List of Plates

List of Appendices

ABBREVIATIONS AND ACRONYMS

AEZ	Agro-Ecological Zone
ANOVA	Analysis of Variance
BecA	Biosciences eastern and Central Africa
CLSI	Clinical and Laboratory Standards Institute
DARLAM	Division of Atmospheric Limited Area Model
DNA	Deoxyribonucleic Acid
DNA	Deoxyribonucleic acid
DRC	Democratic Republic of Congo
ECF	East Coast Fever
EDTA	Ethylenediamine Tetraacetic Acid
ILRAD	International Laboratory for Research on Animal Diseases
ILRI	International Livestock Research Institute
KEPDA	Kenya Economic Pastoralist Development association
MIC	Minimum Inhibitory Concentration
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
RNA	Ribonucleic Acid
USDA	United States Department of Agriculture
DARLAM	Division of Athmospheric Research limited Area Model

Abstract

East Coast Fever is a disease of cattle which results in high animal mortality with corresponding economic losses to farmers and to the nation. The disease is caused by a protozoan agent whose vector is *Rhipicephalus appendiculatus* (Brown Ear Tick). The tick feeds dominantly inside the cattle ear. Studies have shown that the adults of *R. appendiculatus* exhibit 'pull-push' effect that guides them to their preferred feeding site. This is linked to the presence of ear volatiles which attracts and anal volatiles which repels the tick. However, there is limited information that describes what produces the volatiles within the cattle ear. This study aims at establishing the role of ear microbes to the brown ear tick behavior while locating its preferred feeding site. In a preliminary observation, release of the ticks on the cattle with one ear sprayed with a broad-spectrum antibiotic showed that most ticks were attracted to the untreated ear, suggesting that the attractant volatiles are derived from specific microbial populations. In the present study where twelve cattle were used, one ear in each cow was treated with vetmycin a broad-spectrum antibiotic while the other ear was left untreated and the orientation behavior of the ticks (*R. appendiculatus)* placed at six different parts of cattle monitored. Most of the ticks moved to the untreated cattle ear ($P<0.001$,t-test). Ear swabs were collected and cultured in a general media to obtain a culture of the microbes for *in-vitro* behavioral tick experiments and genomic studies. In a choice laboratory experiment, ticks were also observed to orient towards cultivable cattle ear microbes relative to a negative control ($P< 0.001$,t-test). General lab media (Nutrient Agar) was then used to culture different micro-organisms. Sensitivity tests carried out to determine the efficacy of the antibiotic spray used on the cattle ears showed that most microbes were sensitive to the antibiotics contained in the spray according to Kirby Buer calibrations. Profiles of aerobically cultivable microbial communities in each of the twelve cows were investigated using pyrosequencing based analysis. Genomic DNA was extracted and amplified using two sets of primers U1F, U1R which targets band lengths of 490bp and 500bp and primers 27F, 1492R which targets bands lengths of 1490bp. Gene sequences of each ear helped to identify the specific genus of each microorganism and profiles of the candidate microorganisms. Pyro-sequencing blast results revealed that there were diverse communities of microbes majority of which were bacteria. The study lays down significant groundwork in identifying the specific ear microbes responsible for the production of the constituents of ear blend that is responsible for attracting the adults of the Brown Ear Tick. This may open up novel ways of manipulating the production of the blend and in controlling the tick.

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

East Coast fever (ECF) is a tick-borne disease of cattle whose etiological agent is a protozoan parasite called *Theileria parva.* The parasite is transmitted cyclo-propagatively and trans-stadially by a three-host tick called *Rhipicephalus appendiculatus,* which drop from infected cattle during the preceding stage of the life cycle (Norval *et al.,* 1992*).* In this transmission pathway, the *T. parva* parasite multiplies and undergoes cyclical changes within two developmental stages (nymphs and adult) of the vector. The epidemiological implication of this kind of transmission is the amplification of the vector's competence in parasite transmission and the ability to infect more than one host during the vector's life cycle (Gachohi *et al.,* 2012). The major factors associated with epidemiology of ECF include agro-ecological zone (AEZ), livestock production system (LPS), animal breed and age.

These factors appear to influence the epidemiology of ECF through structured gradients. These gradients are dynamically shaped by socio-demographic and environmental processes. For a vector-borne disease whose transmission depends on environmental characteristics that influence vector dynamics, a change in the environment implies a change in the epidemiology of the disease (Gachohi *et al.,* 2012).

In 2011, ten countries reported 1,942 outbreaks of bovine theileriosis to the AU-IBAR (African Union-Interafrican Bureau for Animal Resources), with 28,427 cases and 2133 deaths (AU-IBAR, 2011). The number of outbreaks recorded was highest in 2011 compared to previous years. The disease was reported mainly in the eastern part of the continent. Kenya recorded the highest number of outbreaks (1,356), Tanzania, the highest number of cases (14,700) and Zambia, the highest number of deaths (1,444). This was according to report by AU-IBAR, 2011.

Many factors are involved in assessing the economic impact of tick-borne diseases such as the Theilerioses (Mukhebi, 1992). The economic impact of *T. parva* and its control in Eastern and Central Africa are estimated to be US$ 168 million per annum (Mukhebi, 1992). With the increase of cost of production and incidence of the disease, the estimated values have increased too thereby rendering high economic losses incurred in the management of the disease (Mukhebi, 1992). For the case of small holder farmers who have two cows, loss of one cow will be a 50% loss of the herd.

In Kenya, ECF control has previously relied predominantly on tick control using acaricides and chemotherapy while ECF immunization is steadily being disseminated. Gachohi *et al.* (2012) highlighted the contribution of ECF epidemiology and economics in the design of production system or geographical area-specific integrated control strategies based on both the dynamic epidemiological risk of the disease and economic impacts of control strategies. In all production systems (except marginal areas),

economic analyses suggest that integrated control that target both the vector as well as the parasite can play an important role in the overall control of the disease.

The control of ticks by accaricides, chemotherapy and immunization involves high cost implication which most small scale farmers cannot afford. Use of chemicals also increases toxicity levels on the environment and on the animal hence leading to high levels of environmental pollution. This therefore calls for a need to change the ways of controlling on the cattle.

Studies on host behaviour of adults of the brown ear tick *R. appendiculatus* (Neumann, 1901) and the red-legged tick *R. evertsi* (Neumann, 1897), show that both ticks prefer to feed mainly inside the ears and the anal regions of bovids, respectively. This odour-based 'push-pull' pair of stimuli may largely account for efficient orientation behavior of the two tick species to their respective feeding sites. Such concurrent deployment of repulsive and attractive cues may be quite widespread among arthropods and related organisms that specialize on specific hosts or microenvironments in the performance of their biological functions (Wanzala *et al.,* 2002). This shows that the two species have evolved to exist together in the same host and reduce competition in time and space. The operation of avoidance (closer to the feeding site of the other) and attraction (closer to its own feeding site) responses of the ticks were due to chemical blends, and laboratory odor trapped from cattle ears attracted *R. appendiculatus* but repelled *R. evertsi*, whereas that from the anal region had an opposite effect on the two tick species.

1.2 Problem Statement and justification

ECF is the cause of great economic loss due to its high mortality in livestock especially cattle, and the cost of disease surveillance, control and treatment. According to United States Department of Agriculture report (Fuglie *et al.,* 2013) developing regions like Sub-Saharan Africa, livestock producers are often faced with the challenge of managing diseases that infect animals, making it difficult to sustain herds and farm productivity. In Kenya the highest number of outbreaks (1,356), was experienced with the number increasing each year (AU-IBAR, 2011).

Due to the rapid development of tick resistance to synthetic chemical acaricides and the potential risk posed by these chemicals to non-target species, efforts are intensifying towards the development of alternative tick control strategy. The main objective of this study was to elucidate the role of microbes in production of volatile blends attractive to *R. appendiculatus* by screening for the microorganisms found in the cattle ear, which could open up a novel approach to manipulate the behaviour of the vector.

1.3 Hypotheses

i. Microbes in the cattle ear play a critical role in production of volatiles that attract brown ear tick to cattle ears.
ii. Certain populations of microbes which reside in cattle ear are cultivable.
iii. Some cultivable microbes in the cattle ear are resistant or resistant to antibiotics.
iv. Genomic profiling of microbes in the cattle ear could help identify particular genera of microbes that are responsible for production of volatiles.

1.4 Objectives of the study

1.4.1 General objective

To determine the role of microbes in the production of volatiles attractive to *R. appendiculatus* to cattle ear, their sensitivity to different antibiotics and to genetically profile cultivable aerobic microbes for identification.

1.4.2 Specific objectives

i. To compare the behaviour of brown ear ticks released at different locations on cattle with ears treated or untreated with a broad-spectrum antibiotic.
ii. To culture microbes from the ear swabs to generate a community of microbes.
iii. To determine antibiotic sensitivity of aerobic microbes cultured from swabs obtained from different cattle ears to commonly used antibiotics.
iv. To genetically characterize the profiles of the culturable microbes.

1.5 Significance of the study

This study was aimed at identifying and profiling of candidate microbes that may be responsible for the production of volatiles attracting *R. appendiculatus* to cattle ears. In addition, the cultivated microbes were genetically profiled. The study was designed to lay down groundwork in identifying and controlling the microbes responsible for the production of the blend attractive to the brown ear tick. The attractive blend could be used as bait in the grazing field to reduce the number of the ticks to a manageable level that can be controlled easily, more over this can also be used to design a push pull mechanism within the cow and it will help repel the ticks away from the ear. This will thereby play a role in reduction and avoidance of the ticks in the cattle ears hence reducing the menace of east Coast fever. This will therefore profit the farmer by producing healthy cattle free from disease due to reduced cost of production in terms of disease control and loss of cattle.

CHAPTER TWO

LITERATURE REVIEW

2.1 Distribution of Tick species and the diseases transmitted

The tick species *R. appendiculatus* is found to be well distributed in areas where the disease (ECF) is prevalent across the eastern, central, and southern parts of Africa (Figure 1) (De Deken *et al.,* 2007). East Coast Fever was also reported in Comoros between 2003 and 2004 for the first time. This incident was as a result of importation of immunized cattle from Tanzania, which were fed upon by naive ticks that subsequently transmitted the infection to a susceptible local cattle population.

The climatological anomalies for the 2020s vs. the 1990s as predicted by DARLAM shows that January minimum and maximum temperatures are simulated to increase over certain regions of the subcontinent (Olwoch *et al.,* 2007). Much of the eastern regions are expected to become drier with an associated pattern of higher sea-level pressure, whilst the western subcontinent is expected to become wetter. An interesting feature of the July anomaly fields is that parts of the central subcontinent are simulated to become cooler and wetter.

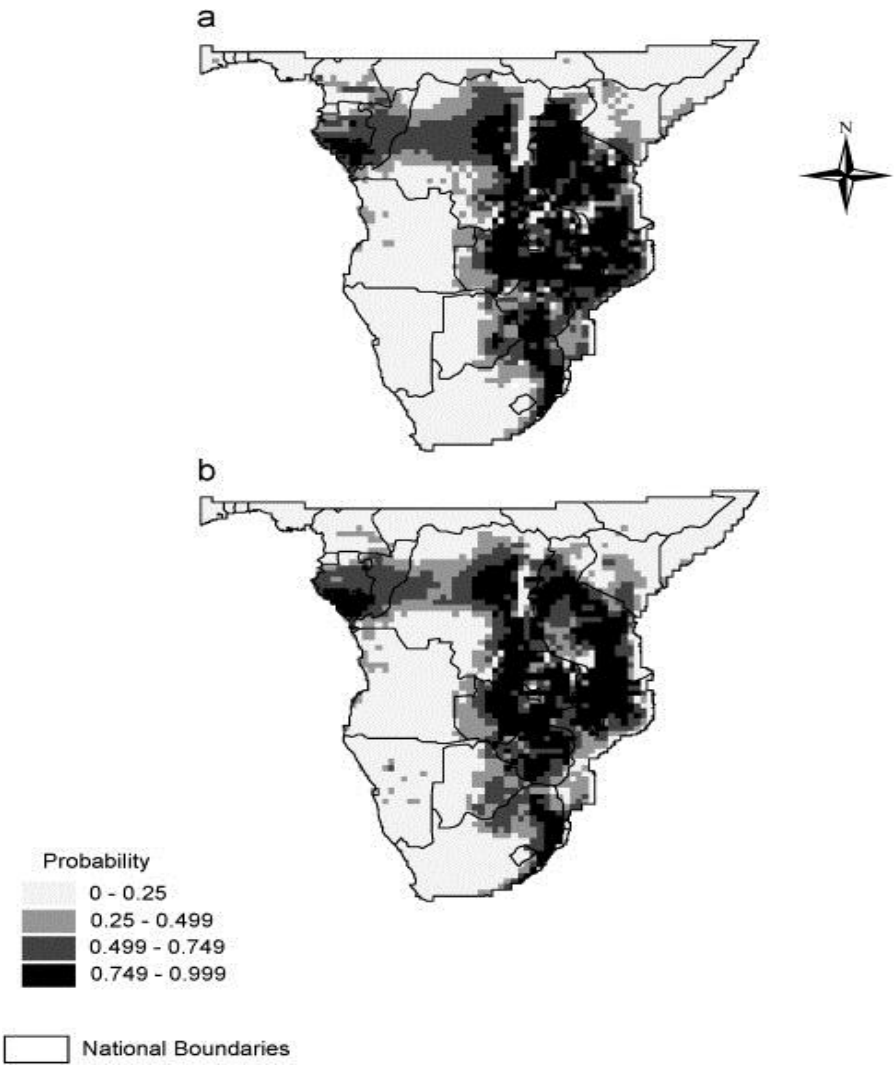


Figure 2.1: The map shows (a) the current probability of *R. appendiculatus* occurrence; (b) the latter (year 2030) based on predictive species model and DARLAM climate surfaces. Dark regions show high probability of *R. appendiculatus* occurrence.

Figure courtesy of (Erasmus *et al.,* 2002).

These range alterations are in response to the predicted general change in mean minimum, maximum temperatures and rainfall in the months of January and July. The ECF sub-Saharan risk map provided is a useful tool to complement existing traditional control methods (Eramus *et al.,* 2002).

These results, based on the predicted distribution of the main tick vector species (*R. appendiculatus*) and its host, cattle shows increased infection of ECF in areas of favorable warm and moist climatic conditions. Understanding and mapping changes in space and time of this disease are a prerequisite to sustainable disease reduction since it is then possible for current and future disease control programs to be timely and directed at specific areas based on risk maps (Eramus *et al.*, 2002).

In Kenya, *T. parva* infection poses a significant threat to the livestock sector in two ways: through the economic impact of the disease from cattle morbidity and mortality and production losses in all production systems, as well as from the costs of the measures taken to control ticks and the disease. The costs of acaricide application, which is the primary means of tick control, was estimated to range between US$6 and US$36 per adult animal in Kenya, Tanzania and Uganda (Minjauw *et al.,* 2003). There are other indirect losses which can be attributed to ECF. One being depletion of scarce foreign exchange arising from expenditure for importing livestock products in short supply. In addition, a loss in beef milk and hides due to disease which reduces the supply of these products as raw materials and thereby retards the development of the livestock product processing industry (Yacob *et al.,* 2008).

In the epidemiological studies of ECF in Tanzania it showed that the application of the capture–recapture method, where the estimated number of clinical cases and deaths was 625 ($CI_{95\%}$ 617–633) and 401 ($CI_{95\%}$ 384–418), respectively. The respective prevalence and case fatality rates were 45% ($CI_{95\%}$ 41–48%) and 64% ($CI_{95\%}$ 60–68%). The estimates obtained using the capture–recapture method are

higher than those identified by traditional cross-sectional studies conducted in the same study area, and probably provide a more accurate epidemiological picture of ECF in this region of Tanzania (Kivaria *et al.*, 2010).

Table1. 1: East coast fever prevalence, incidence and case-fatality rates from studies conducted in intensive/semi-intensive smallholder dairy systems in Kenya.

Region	District /area	Prevalence (cattle ages sampled)	Annual incidence rates	Case-fatality rates	Epidemiological factors
Central highlands	Kiambu	41%-55%			Age
	Murang'a	18%[a], 72%[b] (6–18 months)	54%[c] 74%[d] 86%[e], 110%[f]	6%[c], 5%[d] 9%[e], 16%[f]	AEZ suitability for tick vector, age, breed, grazing system
Coastal lowlands	Kaloleni/ Kilifi	57%[g], 79%[h] (adult)			Age, AEZ, grazing system
	Kaloleni/ Kilifi	18%[g] 48%[h] (<18 months)	6.0%[g] - 50.4%[g], 10.8%[h] - 87.6%[h]	13%[g], 31%[h]	Age, AEZ, grazing system
	Kwale		23%*	11%*	Age, grazing system
Central Rift Valley	Nakuru		22%[j], 33%[k]		Grazing system

a: higher elevation AEZ; b: lower elevation AEZ; c: zero grazing/higher AEZ elevation stratum; d: zero grazing stratum/lower AEZ elevation stratum; e: free grazing/higher AEZ elevation stratum; f: free grazing/ lower AEZ elevation stratum; g: zero grazing; h: free grazing; j: semi-zero grazing; k: free grazing; *parasitological data.

Data obtained from (Gachohi *et al*., 2012).

Theileria parasites are of considerable biological interest, as the only eukaryotic pathogens known to transform lymphocytes. Parasite sporozoites invade lymphocytes, escape from the invasion vacuole, interact with the host cell cytoskeleton, and alter cellular signaling pathways through mechanisms that are incompletely understood (Shaw, 2003). Further insight into their fascinating biology is described in the complete genome sequences for *T. parva* (Gardner *et al.*, 2005; Pain *et al.*, 2005; Hayashida *et al.*, 2013).

2.2 Genus Rhipicephalus

Ticks in the genus *Rhipicephalu*s include many important vectors of animal and human pathogens, but many species are notoriously difficult to identify, particularly at immature stage. Identification keys for adult ticks from the Afro-tropical regions and elsewhere is therefore important. For the nymphs and larvae, unique plates have been compiled in which line drawings of the capitula of similar species are grouped together to facilitate identification (Walker *et al*., 2005).

Rhipicephalus are medium-sized ticks (3 to 5 mm long including mouthparts). Their integument (cuticle/outer covering) texture has striations. *Rhipicephalus* mouthparts are anterior; the palpi are wider, long and the palp articles are all small. The basis capituli (basal part of the mouthparts, the 'gnathastome') has a hexagonal shape. *Rhipicephalus* legs are slender with pulvilli (pads) and usually do not have pale rings. A scutum ('dorsal shield') is present in the female with a conscutum in the male. *Rhipicephalus* are usually not ornate although four species have enamel. Eyes are present and rather flat (except in *Rhipicephalus evertsi* where they are bulging). Festoons (wrinkles) are present in both sexes, but unclear in fed females. Spiracular plates are large and posterior to legs (Bowman *et al.,* 2008). Ventral plates are present only in males, usually as two pairs.

The anal groove is posterior to the anus. The fourth pairs of coxae 4 are of normal size, whilst the first pair 1 has large and equal paired spurs. There are about 74 known species in the genus *Rhipicephalus*. The dog tick (*R. sanguineus*) has a worldwide distribution, but otherwise species of the genus are restricted to the Old World especially Africa. They mainly occupy savanna, open woodland habitats, and

only feed on mammals (Walker *et al.*, 2005). Most species are three host ticks. The genus *Rhipicephalus* includes important disease vectors. Disease organisms transmitted to domestic animals include *Theileria parva*, the causative agent of East Coast fever (ECF) in cattle, and *Anaplasma* spp., which cause bovine anaplasmosis (Cranefield, 1991).

2.3 *Rhipicephalus appendiculatus* (Brown Ear Tick)

Rhipicephalus appendiculatus is classified into kingdom animalia, phylum arthropoda, class arachnida, order ixodida, Family Ixodidae, Subfamily: Rhipicephalinae, genus *Rhipicephalus* and species *appendiculatus.* It is a three-host tick where all three stages; egg, larva, nymph and adult target different hosts (Figure 2.1). Eggs are laid and hatch on ground (Walker, 2003).

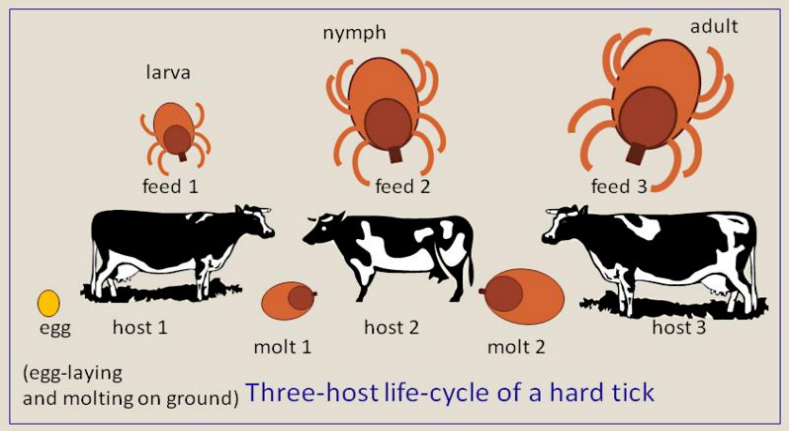


Figure 2.2: Life cycle of hard tick.

Figure obtained from Walker, 2003.

Theileria parva parasite multiplies and undergoes cyclical changes within two developmental stages (nymphs and adult) of the vector found on most animal species like cattle, horses, sheep, goats, antelopes, dogs and rodents (Soulsby, 1982).

Brown ear tick transmits *T. parva*, *Babesia* spp and other protozoan and viral diseases including Nairobi sheep disease and louping illness (De Deken *et al.*, 2007).

Female *R. appendiculatus* have the lateral angles of the basis capituli (basal part of the gnathastome) blunt as shown in plate 2.1. The separation of the porose areas is broad and the palp pedicels are short. The cervical fields have wrinkled areas and are large and curved. The scutum is dark with the posterior margin distinctly sinuous. The eyes are slightly convex and the genital aperture has posterior lips forming a broad V shape. Male *R. appendiculatus* have small to medium interstitial punctuation. The anterior spurs on the first pair of coxae are prominent and visible dorsally, a character present in only a few *Rhipicephalus* species (*R. punctatus, R. pravus, R. zambeziensis* and *R. duttoni*). The cervical fields' depression is apparent and wrinkled. The conscutum colour is dark. Posterior grooves are distinct but shallow with wrinkled texture. The adanal plates are narrow and trapezoid and the accessory adanal plates are very small (Plate 2.1) (Walker, 2003).

Plate 2.1: Dorsal and ventral view of male *R. appendiculatus.*

Plate: Courtesy of Walker, 2003.

The dorsal view (left above) shows the festoons at the posterior edge of the male conscutum and the distinct posterior grooves. The anterior spurs on the first pair of coxae are just visible. The ventral view (Plate 1) shows clearly the narrow trapezoidal shape of the adanal plates, although the (very small) accessory adanal plates are scarcely visible.

Rhipicephalus appendiculatus is found from southern Sudan, through to the southeastern coast of South Africa. It is not clear how far west it extends, but it has definitely been recorded from Zambia and the Democratic Republic of Congo (Perry *et al.*, 1991). It is characteristic of savanna and temperate climatic regions, ranging from hot coastal areas to cool highland plateau as long as the climate is humid.

Brown ear ticks feed on cattle, goats, and a variety of larger antelopes as well as dogs and sheep. All stages may feed on cattle although immature ticks can feed on smaller antelopes and scrub hares. The immature stages attach mainly on the neck and dewlap, the cheeks, eyelids, muzzle and ears. The preferred feeding site of the adults is the pinna of the ear (Plate 2.2); with very few in the ear canal, but in heavy infestations adults are also found around the eyelids and horns, on the upper neck, in the tail-brush and around the anus (Walker, 2003).

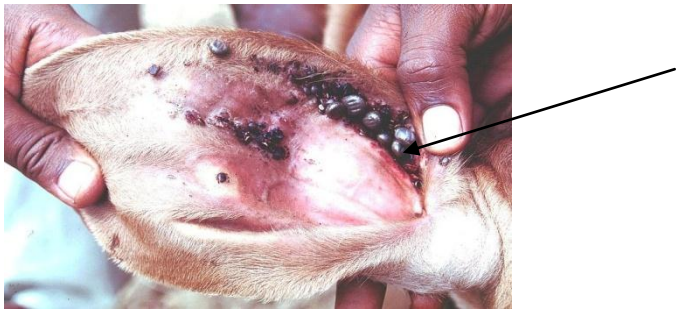


Plate 2.2: *R. appendiculatus* adults feeding at their favorite site the pinna of the ear.

Photo: Courtesy of KEPDA and ILRAD

The tick is a three-host tick which has been extensively studied (Perry *et al.*, 1991.; Randolph *et al.*, 1994). Under the most favorable conditions the whole life cycle can be completed in three months, but occurrence becomes seasonal where there is a pronounced dry season. The pattern of seasonal occurrence is regulated by the unfed adults, which enter diapause and do not engage in host-seeking until the rains start. Where rainfall is more evenly spread through the year, several overlapping generations are completed annually, and no clear pattern of seasonal abundance is evident (Randolph *et al.*, 1994).

In addition to ticks transmitting many diseases, there is also evidence of loss of potential growth of cattle even without any disease transmission. Each engorging female reduces calf weight gain by 4g (Zaman *et al*., 2012). As a result, efforts are usually made to control tick populations. The simplest (albeit most time consuming) method is to simply pick the ticks off from the host. Whilst hand-picking is commonly done for very young animals, acaricides are widely used for older animals. Traditionally they have been applied using dips for large numbers of animals and sprayers for smaller numbers. More recently, pour-on formulations of synthetic pyrethroids have become popular (Di Giulio *et al.*, 2009).

Whilst frequent and sustained tick control can certainly reduce the incidence of ECF, it is expensive and does commit livestock keepers to sustained and sometimes increasing levels of insecticide application. Cessation of treatment can lead to high cattle mortality because of the disruption of enzootic stability. The current recommendation is therefore that an integrated approach is adopted with acaricides

to reduce tick numbers, treatment of clinical cases and vaccination using the infection-and-treatment method recently registered in several East African countries (Di Giulio, 2009).

2.4 On-host behavior of *Rhipicephalus*

As documented by (Wanzala *et al.*, 2002) on-host observations of the ticks showed a typical sequence of different behaviour after the ticks were placed in six different positions on the host cattle. Interestingly, following initial random movements most of the respondents at each release point oriented toward their respective feeding sites (Figure. 2.3) although during the observation period, some appeared subsequently to lose their way. Wanzala *et al.* (2002) showed relatively high rates of successful orientation of the ticks to their respective feeding sites and this suggested the mediation of specific stimuli in the process. They first hypothesized that gradients of volatile odours from these sites could provide the appropriate orientation signals. These volatiles could be produced by microorganisms which reside inside the cattle ear, and this work was conceptualized to test this hypothesis and characterize the microbes involved.

2.5 Behavioral responses

On-host behavior of adults of the brown ear tick *R. appendiculatus* and the red-legged tick *R. evertsi* showed that they prefer to feed mainly inside the ears and the anal regions of bovids respectively (Wanzala *et al.*, 2002). They found that both species were relatively successful in orienting towards the ear and anal region as they located their respective feeding sites from different parts of the host body. According to Wanzala *et al.*, (2002) observations suggested the operation of both

avoidance and (closer to the feeding site of the other) and attraction (closer to its own feeding site) responses of the ticks. In the laboratory, odour trapped from cattle ears attracted *R. appendiculatus* but repelled *R. evertsi,* whereas that from the anal region had the opposite effect. This odour-based 'push-pull' pair of stimuli could largely account for efficient orientation behavior of the two tick species to their respective feeding sites. This concurrent deployment of repulsive and attractive cues may be quite widespread among arthropods and related organisms that specialize on specific hosts or microenvironments in response to their biological functions.

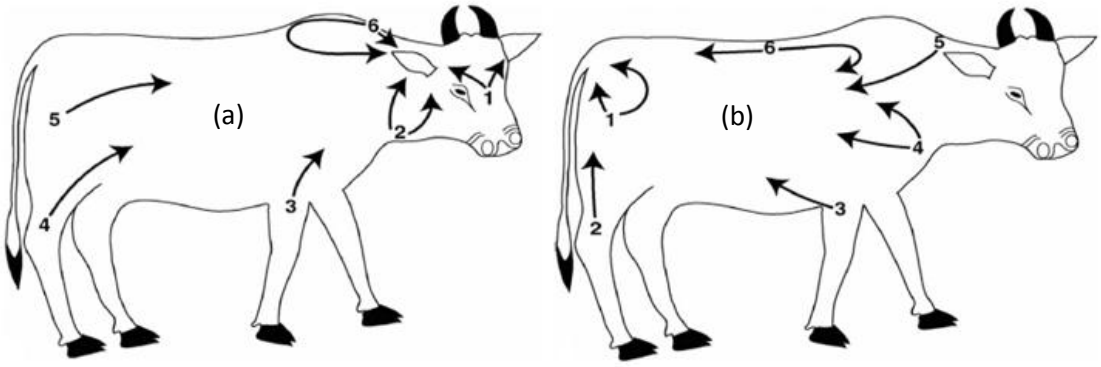


Figure 2.3: General orientations of (a) *R. appendiculatus* and (b) R. evertsi that initiated movements from different release points (1-6).

Figure from Wanzala *et al*. (2002)

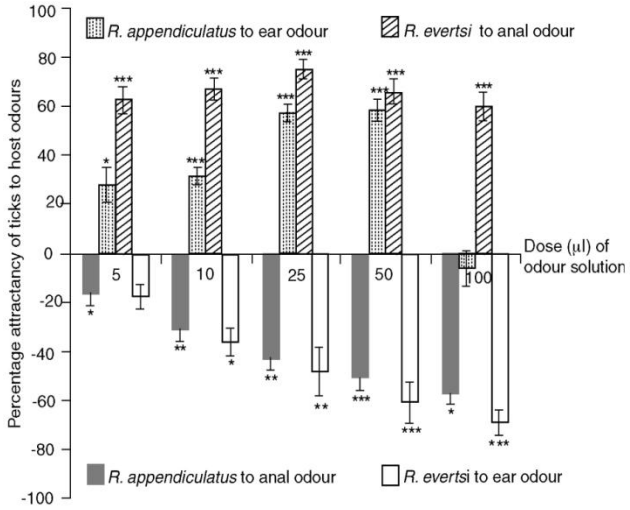


Figure 2.4: Mean percentage attractancy or repellency of ear and anal volatiles to *R. appendiculatus* and *R. evertsi* in climbing bio assays (one star, two stars and three stars indicate statistical significance at P = 0.05, 0.01 and 0.001, respectively).

Figure from Wanzala *et al*. (2002).

To verify this, Wanzala *et al.* (2002) also studied the responses of the ticks in the laboratory to odour collections from the ear and anal region of the steers, respectively. The bioassay design exploited the well-known predisposition of the ticks to climb up and aggregate on grass stems to await a passing host. According to him, a choice of two glass covered rods, one with a vertical concentration gradient of the test odour and the other with clean air was offered to groups of ticks. *R. appendiculatus* was attracted to the ear volatiles but repelled by anal volatiles. On the other hand, *R. evertsi* was repelled by the ear volatiles, but attracted to the anal volatiles. Thus, the odour collections from the two sites have opposite effects on the two tick species and support the hypothesis on the presence of different micro flora which produce different volatiles which have repellent and attractant effects in the feeding site location behaviors of these ticks, and the proposed work was also to determine if the different micro-flora could be culturable.

2.6 Antibiotics

The broad-spectrum antibiotic spray (Vetmycin) contains Oxytetracycline. Oxytetracycline stops the spread of the infection and the remaining bacteria are killed by the immune system or eventually die (Connell *et al.*, 2003).

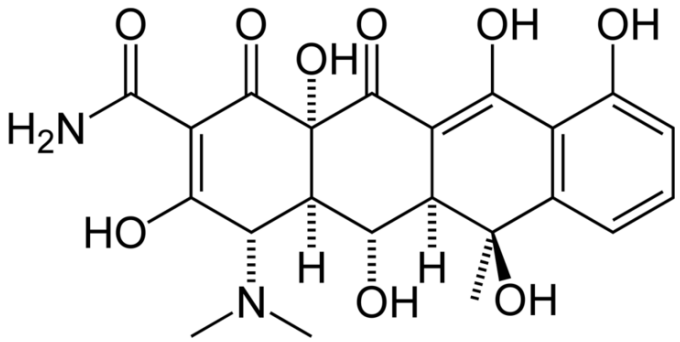


Figure 2.5: Oxytetracycline-2D-skeletal structure.

Oxytetracycline belongs to a group of antibiotics called Tetracyclines which binds to the 30S subunit of microbial ribosomes (Figure 2.5). It inhibits protein synthesis by blocking the attachment of charged aminoacyl-tRNA to the A site on the ribosome. Thus, it prevents introduction of new amino acids to the nascent peptide chain (Akul, 2012). The action is usually inhibitory and reversible upon withdrawal of the drug. Mammalian cells are less vulnerable to the effect of tetracycline, despite the fact that tetracycline binds to the small ribosomal subunit of both prokaryotes and eukaryotes (30S and 40S respectively). This is because bacteria actively pump tetracycline into their cytoplasm, even against a concentration gradient, whereas mammalian cells do not. This accounts for the relatively small off-site effect of tetracycline on human cells (Todar, 2008).

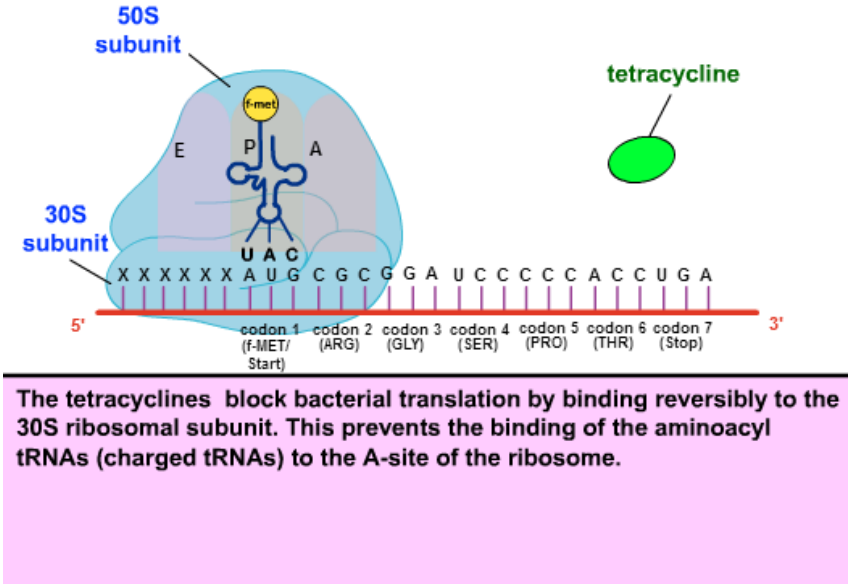


Figure 2.6: Mechanism of action of tetracyclines.

Figure supplied by Dr. Gary E. Kaiser from the Community College of Baltimore County)

The Tetracycline (tetracycline, doxycycline, demeclocycline, minocycline) block bacterial translation by binding reversibly to the 30S subunit and distorting it in such a way that the anticodons of the charged tRNAs cannot align properly with the codons of the mRNA (Connell *et al.,* 2003).

2.6.1 Antibiotics sensitivity tests

In the United States, the total amount of antibiotics used in food-producing animals rose by 16% between 2009 and 2012, to 14.61 million kilograms per year, and there is a great deal of overlap between the drugs used in animals and those used in humans. The most recent data on human use in the United States, from 2011, shows that Americans used 3.5 million kilograms of antibiotics that year (FDA report 2012). Antibiotics thereby can still be used in reduction of normal flora on the ear; this can go ahead in number of microbes in production of ear volatiles.

Exposed skin outer surface tissue provides a favorable substratum for a wide variety of microorganisms to colonize in the cattle ear. Several factors affect the microbiome available in the cattle ear for example host specific factors like age, location and sex, contribute to the variability seen in the microbial flora of the skin. Age has a great effect on the microenvironment of the skin and, thus, on the colonizing microbiota (leyden *et al*., 1975).

Environmental factors specific to the host, such as occupation, clothing choice and antibiotic usage, may modulate colonization by the skin microbiota. The skin is an intricate habitat for many bacteria. A sterile milieu prenatally, skin soon becomes host to resident bacteria after birth. The type and density of bacteria are determined by anatomic location, local humidity, the amount of sebum and sweat production, and the host's hormonal status and age (Aly *et al.,* 1991). Bacterial skin flora is commensal, symbiotic, or parasitic relative to the host; although alterations in host immune status are known to have a significant impact, the type of relationship established is often inherent to the bacteria. Persistent colonization is the result of the ability of bacteria to adhere to skin epithelium, grow in a relatively dry and acidic milieu, and rapidly re-adhere during the normal process of desquamation (Feingold, 1986).

The skin is a barrier that limits invasion and growth of pathogenic bacteria. The cutaneous antimicrobial defense mechanisms include the mechanical rigidity of the stratum corneum and its low moisture content, stratum corneum lipids, production of lysozyme, acidity (pH 5), and defensins (Harder *et al.,* 1997). Specifically, most areas of skin are dry, creating an unfavorable environment for bacterial replication.

Dead keratinocytes slough and physically remove colonizing bacteria. Skin is cooler than normal body temperature and slightly acidic; most bacteria grow best at a neutral pH and at 37°C. If organisms can evade cutaneous host defenses, the next line of protection involves the immune system, or skin-associated lymphoid tissue (SALT).

Skin supports the growth of commensal bacteria, which protect the host from pathogenic bacteria both directly and indirectly. Direct effects include bacteriocin production, production of toxic metabolites, induction of a low reduction oxidation potential, depletion of essential nutrients, prevention of adherence of competing bacteria, inhibition of translocation, and degradation of toxins. Commensal bacteria compete for nutrients, niches, and receptors. For example, *Staphylococcus epidermidis* bind keratinocyte receptors and inhibit adherence of virulent *S. aureus* (Bibel *et al.*, 1983a). Commensals can release species-specific antibiotic substances known as bacteriocins. For example, *S. aureus* strain 502A release bacteriocins that inhibit other virulent staphylococcal organisms (Peterson *et al.*, 1976). Indirectly, bacteria can induce the host to enhance antibody production, stimulate phagocytosis and clearance mechanisms, and augment interferon and cytokine production. For example, Propionibacterium acnes release fatty acids from lipid breakdown, acidifying the milieu and inhibiting growth of Streptococcus pyogenes (Hentges, 1993).

2.7 Molecular analysis of cattle ear microbiota

Genomic approaches to characterize skin bacteria have revealed a much greater diversity of organisms than that revealed by culture based methods, (Fierer *et al*.,

2008; Costello *et al*., 2009; Grice *et al*., 2009). As defined by 16S ribosomal RNA metagenomic sequencing, most skin bacteria fall into four different phyla: Actinobacteria, Firmicutes, Bacteroidetes and Proteobacteria. These four dominant phyla also constitute the microbiota that is found on the inner mucosal surfaces (Eckburg *et al*., 2005; Bik *et al*., 2006; Dewhirst *et al*., 2010; Bik *et al*., 2010)

However, the proportions differ vastly: whereas Actinobacteria members are more abundant on skin, Firmicutes and Bacteroidetes members are more abundant in the gastrointestinal tract. A common feature of gut and skin microbial communities seems to be low diversity at the phylum level, but high diversity at the species level.

2.7.1 Variation of microbes by skin site

Molecular approaches examining bacterial diversity have underlined the concept that the skin microbiota is dependent on the body site and that caution should be taken when selecting and comparing sites for skin microbiome studies. Our group and others have demonstrated that colonization of bacteria is dependent on the physiology of the skin site, with specific bacteria being associated with moist, dry and sebaceous microenvironments. In general, bacterial diversity seems to be lowest in sebaceous sites, suggesting that there is selection for specific subsets of organisms that can tolerate conditions in these areas. Sebaceous sites that contain low phylotype richness include the forehead (Costello *et al*., 2009), the retroauricular crease (behind the ear) (Grice *et al*., 2009), the back (Grice *et al*., 2009) and the side of the nostril (Grice *et al*., 2009). The dominant organisms are Propionibacterium spp. and also in other sebaceous areas, which confirms classical microbiological studies that describe Propionibacterium spp. as lipophilic residents of the

pilosebaceous unit. Microbial transplant experiments suggest that the microenvironment of sebaceous areas (such as the forehead) is a stronger force in determining microbial colonization than the microenvironment of dry areas such as the forearm, (Costello *et al*., 2009).

Cattle also have different microbes at various parts of their bodies (Russell, 1988). There is need therefore to analyze microbes in the cattle ears and get to know the particular microbe which reside in the cattle ears that could be producing the volatiles (Lozupone *et al.,* 2008).

CHAPTER THREE

MATERIALS AND METHODS

All procedures requiring experimental animals were approved by ILRI's Institution Review Board and Kenyatta University Animal Care and Use. They were performed in compliance with guidelines published by Kenya Veterinary Association and Kenya Laboratory Animal Technician Association.

3.1 Study Site

The experiment in the field was carried out at Lenana School dairy farm because it is within the Eastern Africa and mostly because it was nearer to the lab facilities at ILRI the Pictures of the dairy site (Plate 2.1). The collected swab samples were transported in a cool box to ILRI (point A in Figure 3.1) where all other laboratory experiments were done.

Plate 3.1: Picture of the tick experiment field site showing a: cows that were used for the tick experiment; b: experimental animals one day prior the experiment start; c: picture of site where the animals was restrained while ticks were placed on the cow; d: feeding and site in the dairy.

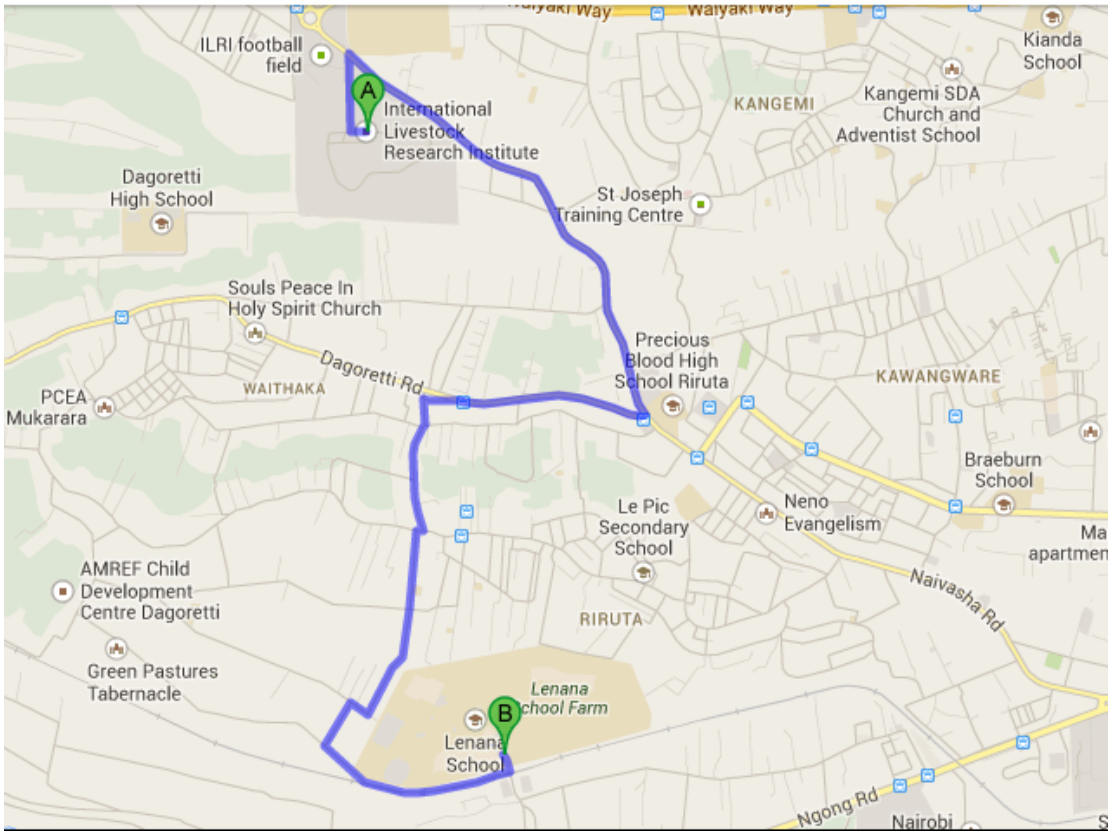


Figure 3.1: Map of Nairobi showing the areas where the swab sample was collected: Lenana School Farm point B.

3.2 Study design

The study was designed to follow a particular schematic flow to facilitate a good workflow of the activities during the research experiment. Figure 3.2 shows the design work plan of the entire experimental process.

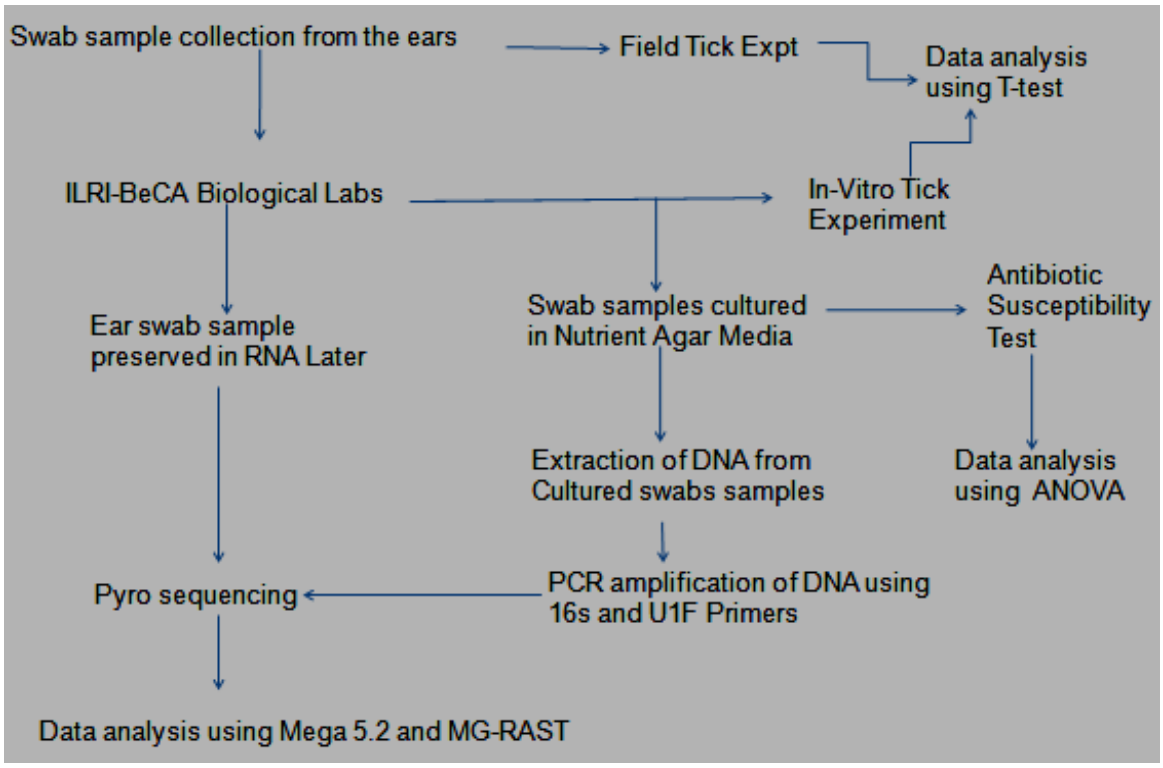


Figure 3.2: Study design

3.2.1 Cattle

A total of twelve cows from Lenana school farm were used which were born at the same site and were confined in zero grazing units in the same location where the experiments were done. Since the cattle were on regular treatment of acaracide (sprayed once a week using Dominex 100 EC in the ratio of 1ml of acaricide to 2000 ml of water and 8000 ml dilution for every cattle), this routine was not interfered with as the experiment was done a day before the actual day of acaracide treatment.

Their feeding regime remained the same during the experiment and comprised of a mixed blend of processed dairy feed. Each cow was fed with 40 kg of feed and 50L of water per day. Cattle were fed twice at 0600 h and at 1800 h. The zero grazing units had

enough space dimensions with a Crush length of 1.4m at the skulling gate, width of 650 mm – 700 mm standard and a height 1.4m above floor level. These studies were carried out using twelve cows of known tick exposure history and acaricidal treatment history, thuscattle had not been infested with ticks before since they were normally sprayed routinely in case stray dogs could come around and infest them with some ticks.

3.2.2 Ticks

Adult *R. appendiculatus* ticks were obtained from colonies at ILRI tick unit. Rearing conditions and management are as described previously (Bailey, 1960; Irvin *et al.,* 1972).

These ticks were of the Muguga line, which was originally isolated from the central highlands of Kenya in the 1960s and thereafter maintained at the East African Veterinary Research Organization and ILRI laboratories. Ticks were reared in a biological oxygen demand (BOD) incubator at 28ºC with 80% relative humidity (Bailey 1960; Irvin *et al.,* 1970).

3.3 Pre-experimental on-host observation studies

Twelve cows were kept in zero grazing units at Lenana School dairy farm. They were held in a tick free zero grazing units which were sprayed with the acaricide on a weekly basis. The animals were monitored for a period of two months before the start of the experiment to confirm absence of any tick infestations. No acaricide was sprayed a week before on-host experiments with ticks.

3.3.1 Broad-spectrum antibiotic for the study

Vetmycin, a broad-spectrum antibiotic aerosol spray containing oxytetracycline HCL BP (Vet) 2% w/w and Gentain Violet 0.18% w/w (Cosmos Ltd), was used in the ear treatment. The broadspectrum antibiotic (Vetmycin) are mostly used in by the farmers to treat livestock wounds against microbial infection in kenyan farms. The storage conditions of the spray was in a dry place below 25ºC and well protected from sunlight.

3.3.2 Treatment of cattle ear with broad-spectrum antibiotic in the field

Twelve cattle were used for the tick behavior experiment. A week before tick behavior experiments, ears of the animals were swabbed using sterlin swab, (Table 3.1).

Table 3.1: Number of left and right ears used in the experiment

Count of ear			
	Treated Ears	**Untreated Ears**	**Grand Total**
Left ear	6	6	12
Right ear	6	6	12
Grand Total	**12**	**12**	**24**

Vetmycin antibiotic treatment was undertaken a day before the tick experiment on ears that were randomly selected. Selected ears were sprayed three times within the 24 h to ensure that there was a significant reduction on the microbial community on the ears. The spray bottle was shaken well before administering. Each ear was sprayed continuosly for five seconds with the spraying can held at a distance of 15 – 20 cm from the area to be treated.

Twenty four hours later, randomized ticks of mixed sex adult *R. appendiculatus* were singly placed on six bilateral sites (Figure 3.3 A) of the cow by use of tweezers.

Two ticks were placed on each side of the six parts at a reasonable distance of 10 cm apart to avoid them following each other due to their odour. Tick movements were observed for 28 h as described by Wanzala *et al.* (2002).

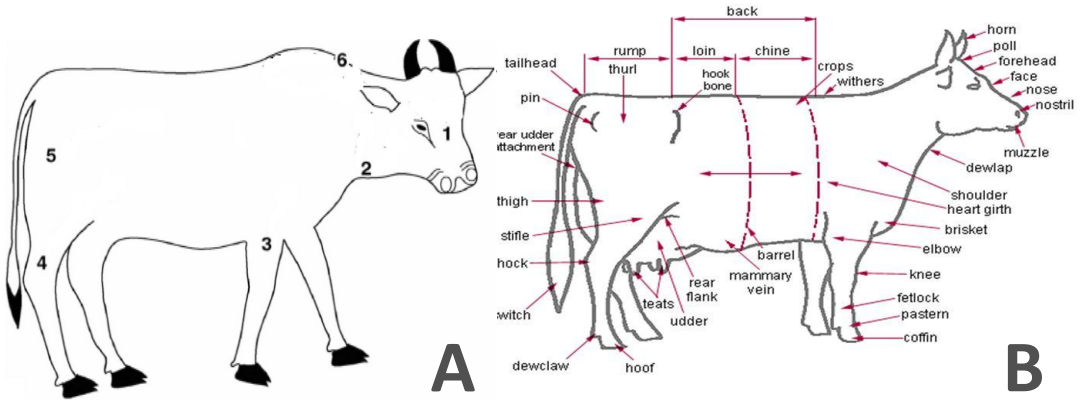


Figure 3.3: (A) and (B): Locations for tick placement (1 face, 2 dewlap, 3 elbow, 4 hock, 5 thigh and 6 withers.

All tick movements were recorderd including those that arrived at the left and right ears during the first 11 h of obeservation. After 28 h of observation, all ticks were removed and disposed.

All ticks used in the experiment were accounted for (number of ticks put in each cow were the same number of ticks removed after the experiment) and disposed by dropping into a small jar containing isopropyl alcohol 70%. Any tick that had fallen off the animal during the experiment was disposed off immediately into 70% alcohol jar.

During this observation time, the cattle were fed with their normal diet and the environmental conditions recorded. After the experiment all the animals were cleaned with 70% alcohol and rinsed with distilled water, cleaning of the cattle was later done.

3.4 Swab sample collection

The activity was carried out using sterile swabs: (Fischer chemicals UK). The swabs had colour coded caps for ease of identification and supplied in a tube for easy of handling, sterilized by irradiation and individually packed (Plate 3.2). Similar to the prototype described previously in Medical Device Directive 93/42/EEC. The bar-coded swabs were rolled on the inner side of ear and the outer region for 5 min. The swab from each ear were transferred into its own cap and put in a cold box then transported immediately to the labs for culture, thus transport time was less than an hour.

Two samples for each cattle ear were collected using a sterile cotton swab (one for in-vitro tick experiment and the other for culturing to extract the DNA). Individual knobs were placed in cool box with ice bags and transported to the ILRI-BecA lab (Veterinary Genetics Laboratory, 2012).

3.4.1 Aseptic measures taken during collection of the ear swab samples

Hands were washed, dried and gloves worn to minimize the risk of transmitting infectious agents between animals and contaminating the samples. Thereafter cotton swabs were peeled opened in a sterile pouch at the point as described in the manufacturer's instruction manual.

The animal head was restrained to prevent movement. Movement of the head can cause the swab to break off. The cap was then twisted to remove the swab from the transport tube and swabbing done as already described

The swabs were placed back into the transport tube (labeled). And the end of the swab was pushed firmly to ensure that the swab is inserted into the end of the transport tube. The transport tube cap was then secured with lids.

Plate 3.2: Photographs of sealed and opened ear swabs used for collection of swabs from the ears of experimental cows

3.4.2 Culturing of swab samples

Culturing of microbes was done in order to obtain cultivable cultures for DNA extraction and for sensitivity tests to different antibiotics. All swabs were inoculated by spreading the swab on nutrient agar plates immediately on arrival in the lab and incubation done at 37ºC for 24 h. The microbial growth on the agar plates was monitored after 24 h.

3.4.3 Inhibitory effect of antibiotics on microbial culture from the host ear

Antibiotics (n=13) gentamicin (G), ciprofloxacin (CF), chloramphenicol (C), tetracycline (T) and ampicillin (AX), were evaluated on their inhibitory effects on the community of microbial culture. The experiment was repeated ten times. These was done using disk diffusion method (Jorgensen *et al.,* 2007)

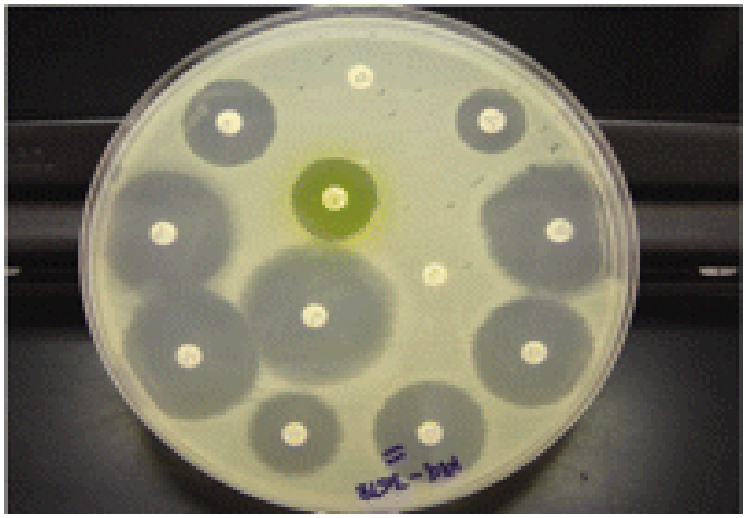


Plate 3.3: Discs of various antibiotics showing their inhibition level.

The test was performed by applying a community of inoculums culture of approximately 1:1 ratio of scrapped cultures to liquid broth to the surface of nutrient agar plate. Up to 13 commercially prepared discs with fixed antibiotic concentrations were placed on the inoculated agar surface and plates were incubated for 24 h at 37ºC.

The zones of growth inhibition around each of the antibiotic disks were measured to the nearest millimeter with a Vanier caliper. The zone diameters of each drug was interpreted using the criteria published by the Clinical and Laboratory Standards

Institute (CLSI, formerly the National Committee for Clinical Laboratory Standards or NCCLS) (Wayne, 2009), or those included in the US Food and Drug Administration (FDA)-approved product inserts for the disks. The results of the disk diffusion test was "qualitative," in that a category of susceptibility (thus, susceptible, intermediate, or resistant) was derived from the test rather than an MIC.

3.4.4 *In vitro* tick experiment on cattle ear microbes

This was done to confirm the attractive activity of volatiles associated with the cultured microbes to the ticks. Inoculation of the swabs from the cow ears on nutrient agar media in sterile tubes was done (Figure 3.3), thus from twelve cows both right and left ear while putting equal number of tubes with nutrient agar but without an inoculum control. Then the inoculated nutrient agar tubes were laid down in a flat surface in an incubator.

The tubes with green colour were inoculated with the swabs from the cattle ear (Treatment) while the tubes with the blue colour were cattle ear inoculants free (Control). The control had a pure colony of *E. coli* and human ear swabs cultured in Nutrient Agar tubes and tubes without any microbial culture. This was done to remove any doubts that other microbes apart from the cattle ear microbes could be responsible in producing the attractive volatiles. The tubes were positioned in a way to form a circle (Figure 14) around the centre where the ticks were placed. Twenty four ticks were placed at the centre in each test and their movement patterns monitored.

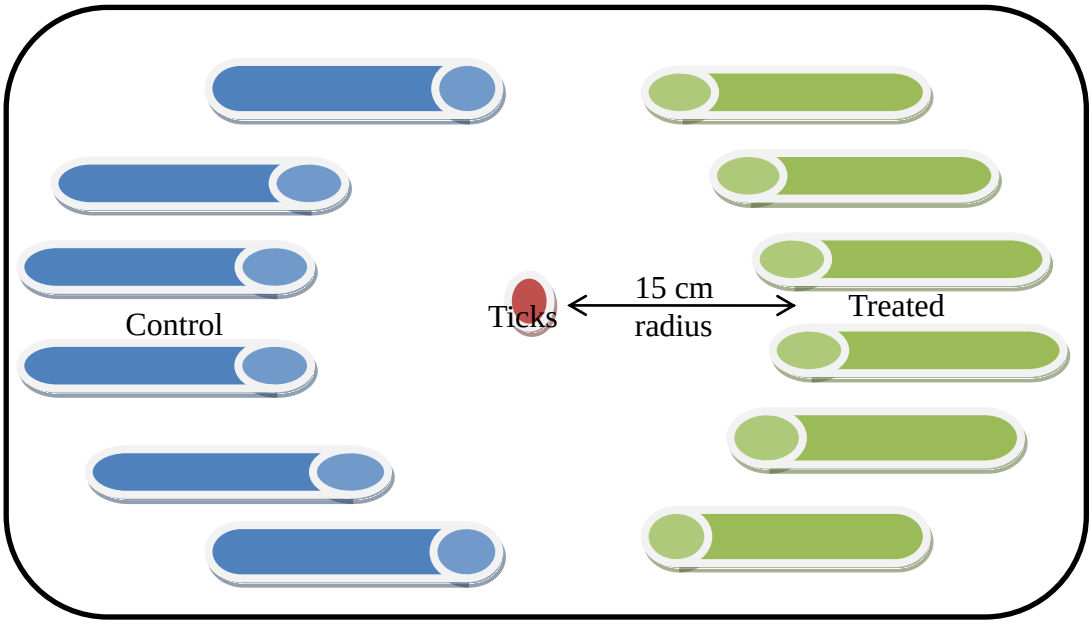


Figure 3.4: Design showing *in-vitro* lab experiment to confirm the effect of microbial culture on behavior of ticks

3.5 Genomic DNA extraction by use of Kit

This was done using two types of kits; Invitrogen Pure Link™ Genomic DNA Kits Catalog No's. K1820-02- 250 preps and ZR Fungal/Bacterial DNA Mini Prep™ D 6005.

3.5.1 Extraction of genomic DNA from cattle ear culture using Invitrogen Pure Link kit

Two water baths were set at 55ºC and 37ºC and thereafter organisms which had been scrubbed from the incubated plates were harvested by centrifugation at 14000 rpm for 1 min in an Eppendorf tube. The pellets were re-suspended in 180 µl Lysozyme Digestion Buffer (it was ensured that the pellet was completely re-suspended in the buffer mix). Incubation for 30 mins at 37ºC was done with occasional vortexing. Proteinase K (20

µl) was added to the tubes cell suspension and mixed well by vortexing. PureLink™ Genomic Lysis/Binding Buffer (200 µl) was added and mixed well by vortexing to obtain homogenous solution. Incubation was again done at 55°C for 30 min. Absolute ethanol (200 µl) was thereafter added to the lysate and mixed well by vortexing for 5 secs to yield a homogenous solution. Note: (Due to multiple samples, a master mix of the buffer and ethanol were mixed in equal volumes of each relative to the number of samples). The lysates were then spinned at 13000 rpm for 2 min at room temperature to remove any particulate materials.

Lysate measuring ~640µl was transferred to the PureLink™ in Spin Column already in a collection tube. The columns were centrifuged at 13,000 rpm in a microcentrifuge for 1min at room temperature. The collection tubes containing the flow through were discarded and the spin column placed into a clean PureLink™ collection tube supplied with the kit. Wash Buffer 1 measuring 500 µl (already prepared with ethanol by adding 30 ml alcohol) was added to the column, and spinned at 13,000 rpm at room temperature for 1 min. The collection tubes were discarded and spin column placed into a clean PureLink™ collection tube supplied with the kit. Wash Buffer 2 measuring 500 µl of (already prepared with ethanol) was again added to the column and spinned at 13,000 rpm for 1 min. The flow through was discarded and the columns spinned again in the collection tubes for another 1 min at 13,000 rpm (this step was necessary to remove excess ethanol from the column. Any remnant ethanol inhibits downstream DNA manipulations). The columns were transferred to a sterile 1.5 ml Eppendorf tube

and 50 µl MilliQ Water (pre-warmed at 55 ºC before) added to the center of the column and left at room temperature for 2 min. The columns were again spinned at 14,000 rpm for 1 min to elute the DNA. (The column was not discarded, as the second elution was done into a separate tube with 25µl MilliQ Water). One micro-litre of the DNA was quantified on the Nanodrop and the rest stored at -20ºC to await purification and subsequent PCR amplifications. (The entire steps were performed according to manufacturer's directions). http://tools.lifetechnologies.com/content/sfs/manuals/purelink_genomic_man.pdf.

3.5.2 Extraction of genomic DNA from the cultures using ZR Fungal/Bacterial DNA MiniPrep™ Kit

This kit is designed for the simple, rapid isolation of DNA from tough-to-lyse fungi, including *A. fumigatus*, *C. albicans*, *N. crassa*, *S. cerevisiae*, *S. pombe*, also from mycelium and Gram positive and Gram negative bacteria. Microbial cultures were added directly to ZR bashingBead™ Lysis tube and rapidly and efficiently lysed by bead beating without using any organic denaturants or proteinases. The DNA was then isolated and purified using fast-spin column technology which is ideal for downstream molecular technology including PCR array, summary of schematic ZR Fungal/Bacterial DNA MiniPrep™ procedure (Figure 3.4). The DNA isolation steps were followed according to; http://www.zymoresearch.com/downloads/dl/file/id/88/d6005i.pdf.

For better performance, beta-mercaptoethanol was added to the Fungal/bacteria DNA Binding Buffer to a final dilution of 0.5 % (v/v). Microbial culture cells were

resuspended in 200 µl of liquid media (liquid broth) and added to ZR Bashing Bead™ lysis tube and 750 µl of Lysis solution was then added. Thereafter they were secured in a bead beater fitted with a 2 ml tube holder assembly (Disruptor Genie™) and processed at a maximum speed of 1400 rpm for 5 min. The ZR Bashing Bead™ Lysis Tube was put in a microcentrifuge and centrifuged at 10,000 rpm for 1 minute. Transferred up to 400 µl supernatant to a Zymo-Spin™ IV spin filter (orange top) in a collection tube and centrifuged at 7,000 rpm for 1 min. (The base of Zymo-Spin™ IV Spin Filter was snapped of prior start). Approximately 1,200 µl of fugal/bacterial DNA binding buffer was added to the filtrate in the collection tube from the previous step and 800 µl of the mixture was transferred to a Zymo-Spin™ IIC Column in a collection tube and centrifuged at 10,000 rpm for 1 min.

The flow through was discarded from the collection tube and centrifugation at 10,000 rpm for 1 min was done. DNA Pre-Wash Buffer (200 µl) was added to the Zymo-Spin™ IIC Column in a new collection tube and centrifuged at 10,000 rpm for 1 min. Fungal/Bacterial DNA Wash Buffer (500 µl) was also added to the Zymo-Spin™ IIC Column and centrifuged at 10,000 rpm for 1 min. Zymo-Spin™ IIC Column was transferred to a clean 1.5 ml microcentrifuge tube and 50 µl of pre-warmed nuclease free water was added (instead of DNA Elution Buffer) directly to the column matrix. The column was centrifuged at 10,000 rpm for 30 secs to elute DNA. The DNA was quantified and quality checked on a Nanodrop spectrophotometer and concentration recorded. The DNA was stored at -20°C for use in subsequent experiments.

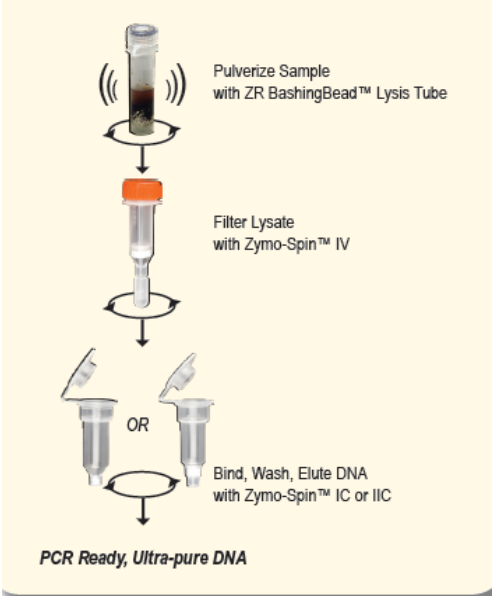


Figure 3.5: ZR Fungal/Bacterial DNA MiniPrep™ procedure

3.5.3 DNA Normalization

All DNA from the samples were normalized to obtain one common working concentration, this was done using the formulae $C_1V_1=C_2V_2$, Where; C_1 is the concentration of initial nucleotides, V_1 is volume of the initial DNA, C_2 is the working concentration and V_2 is volume of water added to normalize the DNA.

3.5.4 DNA amplification by PCR

Pioneer *AccuPower*® PCR Premix was used, it is a ready to use PCR reagent optimized for more accurate PCR amplification. The PCR master mix for 27F 1492R primer was composed of 0.5 µl 27F primer, 0.5 µl 1492R primer, 19 µl nuclease free water, 1 µl template DNA for every single reaction and the total volume totaled to 20 µl. While for U1F U1R primer was composed of 0.25 µl Forward primer, 0.25 µl Reverse primer,

18.5 µl nuclease free water, 1 µl DNA template for every single reaction, all volume totaled to 20 µl.

Distilled water was added to *AccuPower*® PCR tubes to a total volume of 20 µl and the lyophilized blue pellet was dissolved by vortexing and briefly spinning down. Sample genomes were amplified by PCR reaction.

3.5.5 The PCR reaction

The PCR reaction was performed in a thermo-cycler (Figure 3.6) and involved an initial DNA denaturation, followed by a number of cycles of denaturation, primer annealing and product extension. A final DNA extension step completed the reaction.

Figure 3.6: A Thermo-cycler used in PCR reaction

Initial denaturation was done at 94ºC for 5 minutes in 27F/1492R primer set and at 94ºC for 3 minutes in U1F/U1R primer set, this made all potential primer binding sites available. In the cycles, denaturation was done at 94ºC for 30 seconds in 27F/1492R primer set and at 94ºC for 45 seconds in U1F/U1R primer set which denatured the entire DNA in the first amplification cycle. The annealing step lasted for 30 seconds in 27F/1492R primer set and 45 seconds in U1F/U1R primer set, this allowed enough time

for the primers to locate their complement and anneal to the template DNA. The annealing temperatures were 57ºC and 56ºC respectively; this is usually 5 ºC lower than the melting temperature of the primer-template duplex. Extension was done at 68ºC(27F/1492R primer) and 72 ºC (U1F/U1R primer) both used duration of1 minute. The cycle numbers for both were 25 cycles this was because the DNA template was higher. The final extension incubation was done at 68ºC for 7 minutes in 27F/1492R primer set and at 72ºC for 6 minutes in U1F/U1R primer set, this made sure that the protruding ends of newly synthesized PCR products were filled. After this step the PCR products were purified and electrophoresed then stored at -20 ºC.

3.5.6 PCR product purification

Purification of the DNA products was done using QIAquick® PCR Purification kit (cat. No 28104 and 28106) which had been stored at room temperature (15-25 ºc). All the DNA products amplified using the pioneer kits were purified to remove dimmers, dyes and salts phenols which could hinder the sequencing process. The resulting cleaned product was then quantified using a Thermo Scientific nanodropTM 1000 spectrophotometer. The minimum concentration accepted for downstream processing was 15 ng/µl. In cases where the concentrations did not reach the minimum required concentrations, the sample amplification was repeated.

Five volumes Buffer PB was added to 1 volume of PCR reaction and mixed, no colour change was observed and hence 10 µl 3M sodium acetate was not added. To bind the DNA, the sample was applied to QIAquick column and centrifuged for 1 min. The flow through was then discarded and QIAquick column placed back into the same tube. Washed by adding 750 µl of Buffer PE to the QIAquick column and centrifuged for 1 min. The flow through was discarded again and placed back into the QIAquick column in the same tube. The QIAquick column was centrifuged again in a 2 ml collection tube for 1 min to remove the remaining wash buffer. Each QIAquick column was then placed in a clean 1.5 ml microcentrifuge tube. Nuclease free water (50 µl) was added to the center of the QIAquick membrane and centrifuged the column for 1 min at 1,200 rpm. The purified DNA was now ready to be analyzed on a gel. http://www.qiagen.com/handbooks

3.5.7 DNA analysis by Gel electrophoresis

Analysis of quality and quantity in all amplified DNA were done on a gel. One volume of loading dye was added to 5 volumes of purified DNA and mixed by pipetting up and down before loading in a 1 % agarose gel. The gels were run for 50 min at 100 volts.

3.6 Pyro-sequencing of 16S RNA sequence

This included DNA amplification with specific primers, the DNA was amplified using universal primers U1F/U1R designed because of conserved ribosomal sequences of prokaryotic and eukaryotic microorganisms (Rivas *et al.,* 2004). DNA was also amplified using 27F/1492R which amplifies universal region of 16S RNA in all

bacteria. Amplicons and cDNA libraries were prepared for sequencing on Roche/454 sequencer (Daigle *et al.*, 2011). Amplicon sequences identified bacterial and fungal species simultaneously while cDNA sequences provided genes expression.

Genomic DNA was prepared according to the manufacturer's protocol and the libraries prepared for 454 sequencing. The primers were short and target (U1F, U1R primer) Target size 512, 492 base pairs and (27F, 1492R primer) Target size 1492 base pair 1492 genomic regions.

3.6.1 Pyro-sequencing procedures

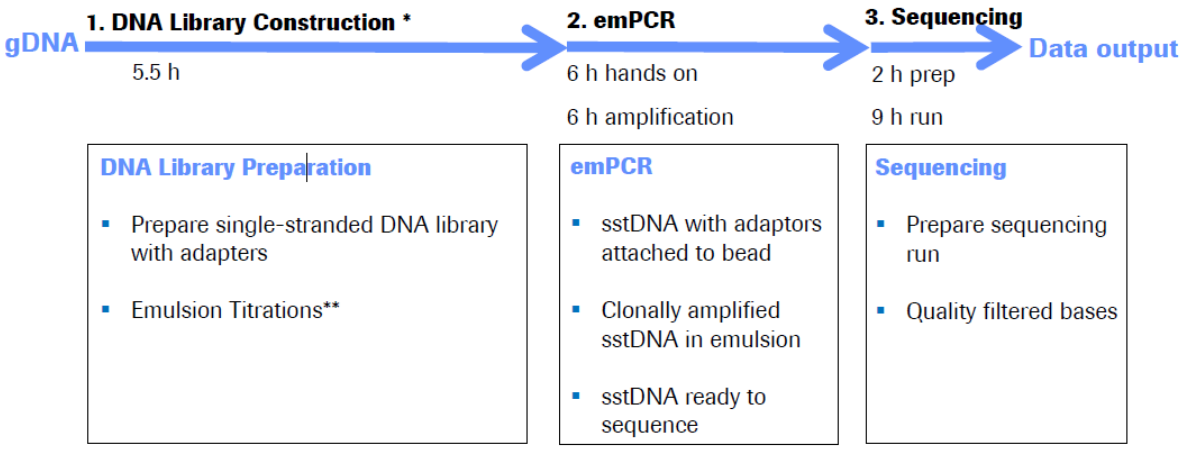


Figure 3.7: Summary showing 454 sequencing procedures.

3.6.2 DNA Library preparation (I)

The aim of this step I was to end up with a single-stranded DNA library from a double-stranded genomic DNA. The following steps were followed in the GS FLX Titanium protocol during library preparation by Roche. The DNA first undergoes Nebulization

which is shearing DNA into appropriate size fragments by use of nitrogen, the fragments are there after are isolated by either gel isolation or SPRI based removal of fragments smaller than 400 base pairs. The third step is DNA end repair which involves making the ends of DNA blunt and phosphorylated. This step is preceded by adaptor ligation, after the ligation the small fragments are removed by SPRI based which removes fragments lesser than 250 base pairs. The fragments are the immobilized and bound on a solid support. The strands are displaced to make them double stranded in a step called (nick repair). The strands that remain single stranded are isolated and number of molecules recovered estimated (quantization).

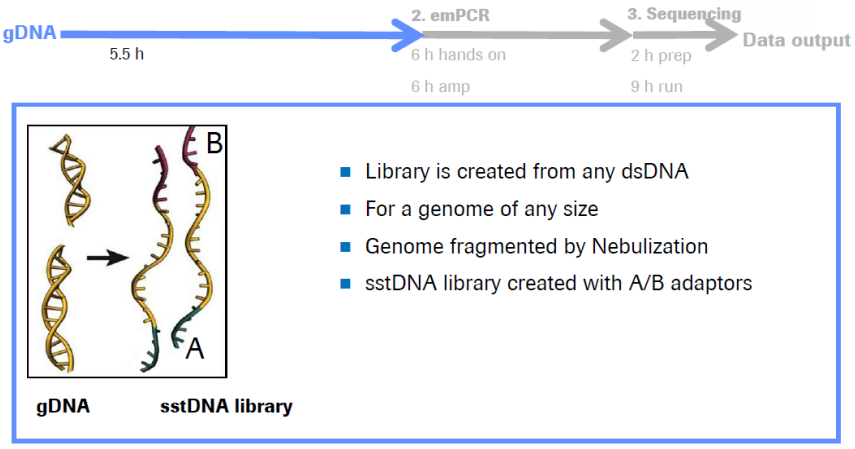


Figure 3.8: Preparation of single-stranded DNA library from genomic DNA.

3.6.3 Emulsion PCR (II)

This was the second step in the protocol which was aimed at obtaining clonally amplified single-stranded DNA attached to the bead from the end product of the first

step (Figure 3.9). The 454 life sciences emulsion PCR used is based on methods by Margulies et al. (2005).

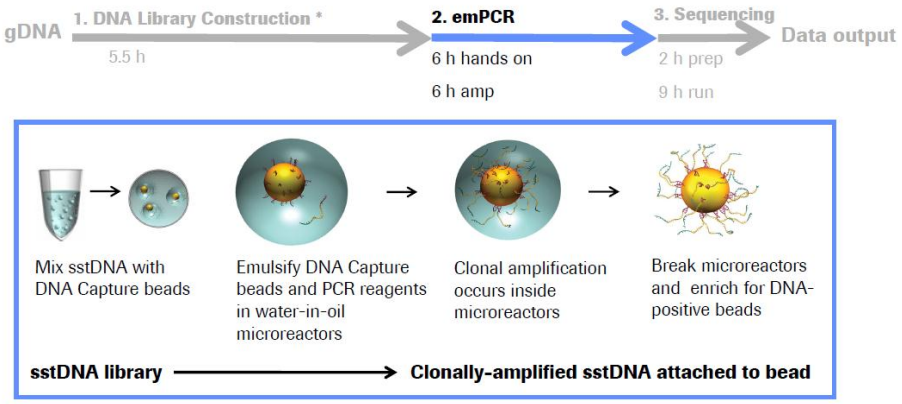


Figure 3.9: Emulsion PCR procedure shows what took place during step II

During this process of clonal amplification the single-stranded library DNA molecules were annealed to capture beads. Thereafter distributed millions of beads in thermo-stable water-in-oil emulsion, in the micro reactors there were complete amplification mix. This was followed by Thermo-cycling then isolation and purification of the beads only with amplified DNA. The titration process involved performing emPCR amplifications on various amounts of library DNA and determining the percentage of enriched beads where a range of between 5%- 20% was recommended in Roche sequencing method manual (http://www.highthoughputsequencing.com/manuals_roche/jan2010/GSFLXTitanium_Sequencing_Method_Manual_RevJan2010.pdf).

3.6.4 Bead deposition in to Pico Titer Plate (PTP)

This step was done in order to obtain PTP ready for sequencing from amplified ssDNA library beads (Figure 3.10).

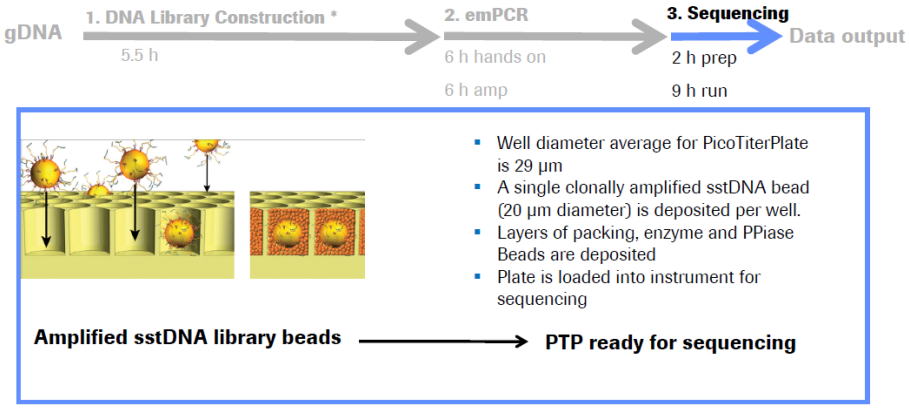


Figure 3.10: Bead deposition into a Pico Titer Plate shows how a single clonally amplified ssDNA bead is deposited per well.

3.6.5 Pyro-sequencing of 16S RNA sequence

Four nucleotides, (TACG) flowed through 200 cycles generating chemiluminescent signals which determine base sequence and quality score. Primer sequences that are highly conserved for all forms of bacteria were considered to be ideal for this type of work.DNA was amplified using 27F/1492R which amplifies universal region of 16S RNA in all bacteria.

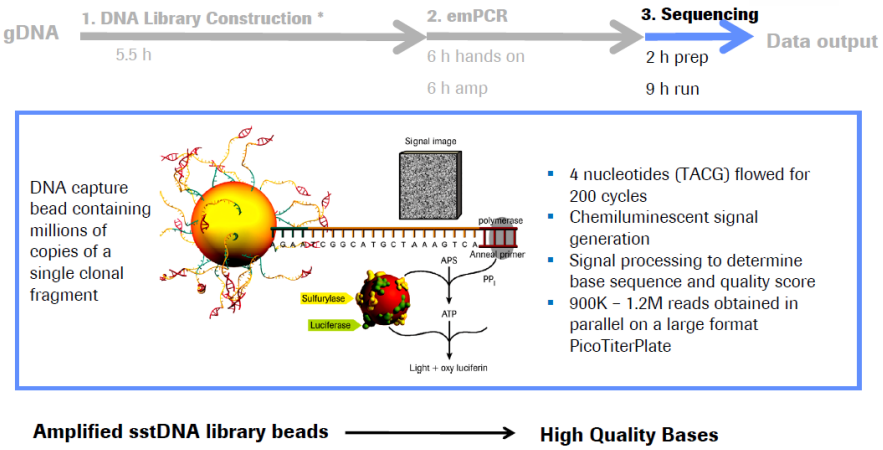


Figure 3.11: Sequencing process

3.6.6 Pyro-sequencing data output

Involved a series of events from image capture, image processing, and signal processing which went through the GS Reference Mapper, GS De Novo assembly and finally Contig generation (Figure 3.12).

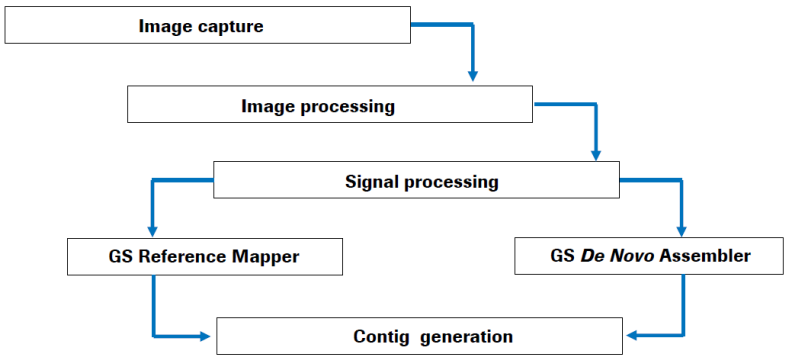


Figure 3.12: Data output process

3.6.7 Pyro-sequencing data processing

Raw data was processed from a series of individual images then each well's data was extracted, quantified, and normalized. Raw signals were extracted corresponding to all nucleotide flows, output compressed well files (cwf).

Analysis of the signal data for each flow for all active wells was done by filtering. DNA was used as an internal control of the sequencing performance and the output was cwf files and standard flowgram files (sff) (Figure 3.13). Sequence fasta files (sff) were split to individual samples by MIDs using custom scripts and sff tools from Roche. Split files were uploaded to metagenomic analysis server (MG-RAST) and analyzed using heatmap/dendogram tool (Meyer *et al.*, 2008). Phylogenetic analysis of sequences was done using MEGA 5 (Tamura *et al.*, 2011) and CLC bioworkbench 5.5.1. All sequences obtained were submitted to genebank.

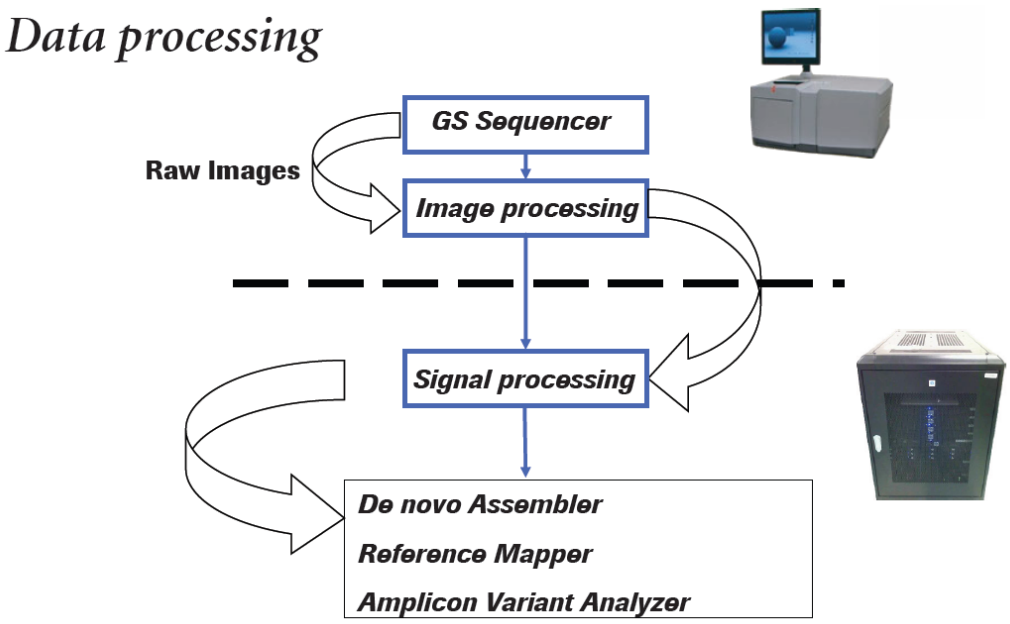


Figure 3.13: Data processing flow chart

3.7 Data analyses

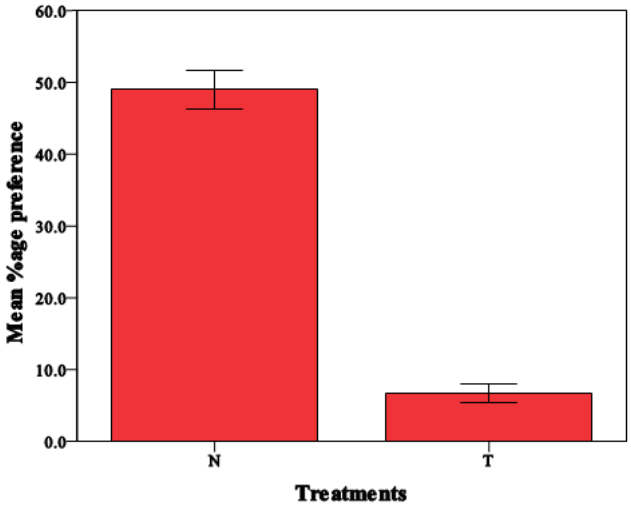
Bioinformatics software (MGRAST and CLC genomics work bench) was used to determine the expression profiles of swab microbes from the ear. To determine the preference of ticks (*R. appendiculatus*) to their preferred feeding site (ear), descriptive statistics was used. Student *t*-test was used to determine if there was significant difference in the number of ticks from each cattle site locating the treated and untreated ear at 95% confidence interval. For the data which was generated during an *in-vitro* tick experiment Student *t*-test and Chi-square were used to analyze the data. While the results from antibiotic sensitivity test were analyzed using ANOVA.

CHAPTER FOUR

RESULTS

4.1 On-host field tick experiment

The results of the experiment conducted on-host in the field to compare choices made by the ticks in locating their preferred feeding site, after treating one ear with vetmycin antibiotic, showed that most ticks preferred the untreated ears (N) unlike the other ear which had been given a broad-spectrum antibiotic (T) (Figure 4.1). These results indicated a significant difference in ticks locating the antibiotic treated and untreated ears. The mean percentage preference of the ticks that preferred untreated ears (N) were significantly higher than the treated (T) ears at ($P<0.001$, t-test). A similar experiment was mimicked *in-vitro* in the lab and a significant difference was observed on mean Percentage preference of ticks on treated and control tubes, treated tubes had significantly high preference (Figure 4.2, $P<0.001$, t-test). These results show that microbes have a role in *R. appendiculatus* locating the cattle ear where it prefers to feed.



Treatment	Mean± SE
Normal	49.0±2.8
Treated	6.6±1.3
p-value	<0.001

Figure 4.1: Mean percentage preference of ticks to antibiotic treated (T) and untreated (N) ears, untreated ears had significantly higher percentage preference (P<0.001, t-test).

4.2 Cultures from the ear swab

Inoculations from the ear swabs which were done on the nutrient agar plate for 24 hours in an incubator at 37ºc showed growth of microbes covering the entire plate as shown (Plate 4.1). This validated that there is diverse microbial growth as it is evident by clear lines of different microbial community growth.

Plate 4.1: Microbial growth on nutrient agar plates

4.3 *In vitro*tick experiment on cattle ear microbes

In the *in-vitro* lab experiment, 49.0% of ticks moved/oriented to the tubes which had cultures from the cattle ear and 6.6% of ticks were observed in the negative control or toward tubes treated with pure cultures from *E. coli*. While the other percentage remained dormant within the region they had been placed.

From the data in figure 4.2 Mean Percentage preference of ticks on treated and control tubes in an in-vitro experiment, treated tubes had significantly high preference ($P<0.001$, t-test), there is a significant difference between the percentage of ticks that reached the positive control and the percentage of the ticks that reached negative control.

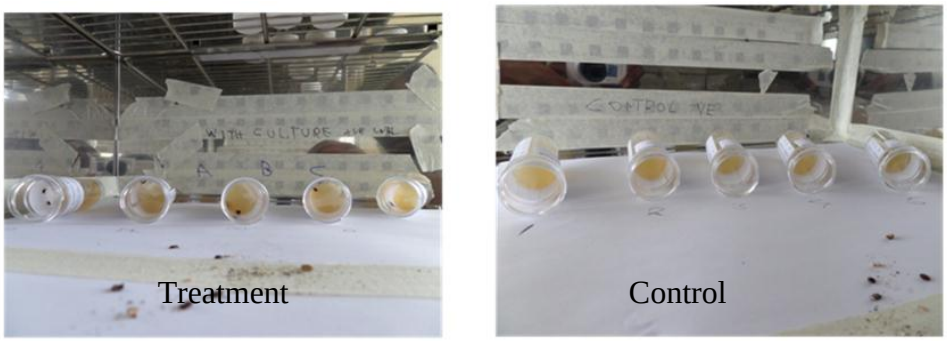
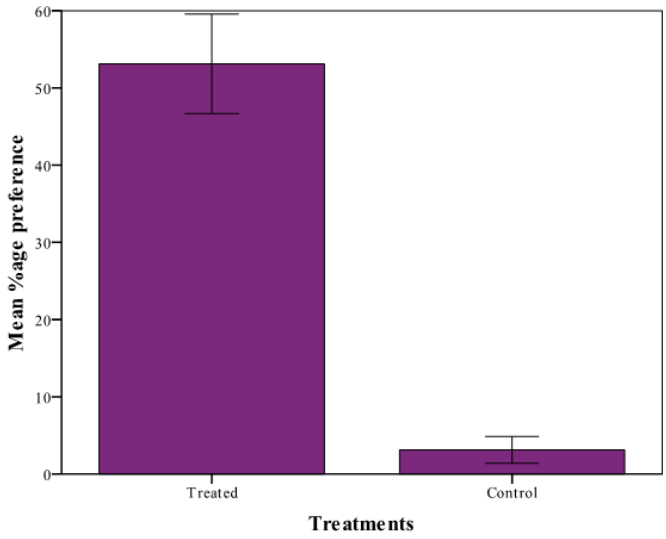


Plate 4.2:Treatment (tubes with ear culture inoculum) and Control tubes without ear culture inoculum) in an *in-vitro* experiment



Treatments	Mean±SE
Treated (cattle ear swab inoculum)	53.1±6.4
Control	3.1±1.7
P-value	<0.001

Figure 4.2: Mean Percentage preference of ticks on tubes treated with ear culture inoculum and control (tubes without ear culture inoculum) in an in-vitro experiment, treated tubes has significantly high preference (P<0.001, t-test).

4.4 Antibiotic susceptibility test

The zones of inhibition of the 13 antibiotics used were measured in millimeters using venire caliper and analysis done to find out if they were resistant, susceptible or

intermediate. Plates showing the zones of inhibition of the 13 antibiotics used (Plate 4.3).

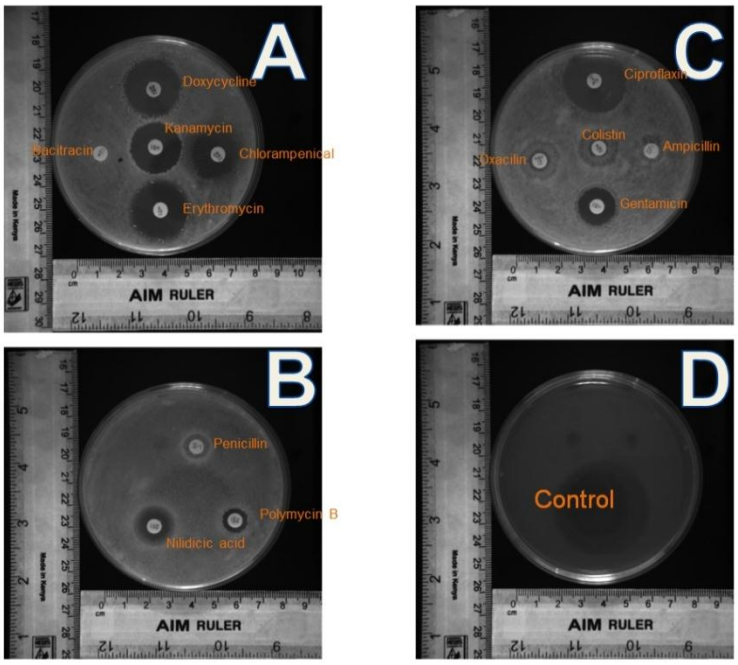


Plate 4.3: Plates showing various antibiotic inhibitions levels of specific antibiotic to microbes from cattle ear, A,B and C are contain different antibiotic while D is the control.

All 13 antibiotics showed different zone level if inhibition (Plate 4.3). Control plate was also was included in the experiment to exclude any chances of contaminations (Plate 4.3 D). The results from the repeated ten experiments were analyzed (Table 4.1). When analysis on percentage susceptibility was done to find out the efficiency of the antibiotic used in the field in accordance to Kirby-Bauer, the results were as shown in (Figure 4.3).

4.4.1 Analysis of antibiotic sensitivity of all antibiotics used

Antibiotic	Inhibitory length (Mean±SD)mm
Ciproflaxin	23.98±0.04[a]
Doxycycline	21.60±0.22[b]
Erythromycin	21.57±0.02[b]
Kanamycin	18.01±0.02[c]
Gentamycin	16.01±0.02[d]
Chlorampenical	13.82±0.01[e]
Nilidixic acid	10.00±0.01[f]
Polymyxib B	8.00±0.01[g]
Oxacilin	7.53±0.02[h]
Colistin	7.20±0.01[i]
Penicilin	4.89±0.01[j]
Ampicilin	5.68±0.02[k]
Bacitracin	3.00±0.00[l]
p-value	<0.001

Table 4.1: Mean values of zones of inhibitions followed by the same small letter do not differ significantly from one another (SNK-test, α=0.05). Superscript a,b,c,d,e,f,g,h,I,j,k,l shows descending order of antibiotic inhibition.

These inhibition results show that there is significant difference in the level of zone inhibition between different antibiotics. In the multiple comparisons between different antibiotics in zonal inhibition it showed that Doxycycline and Erythromycine were having equal inhibition levels.

The percentage sensitivity of various antibiotics according to (Kirby-Bauer antibiotic test interpretation table) plate 4.3 and table 4.1 are showing results from the data collected when multiple antibiotics were used to find out the inhibitory effect of the various antibiotics on the microbial culture from the cattle ear. When analysis was done using one way ANOVA, it showed that there was a significant difference in antibiotic

sensitivity ($p<0.001$, $\alpha=0.05$). The level of sensitivity of Doxycycline and Erythromycin did differ statistically at 95% confidence level($p=0.439$, SNK-test, $\alpha=0.05$).

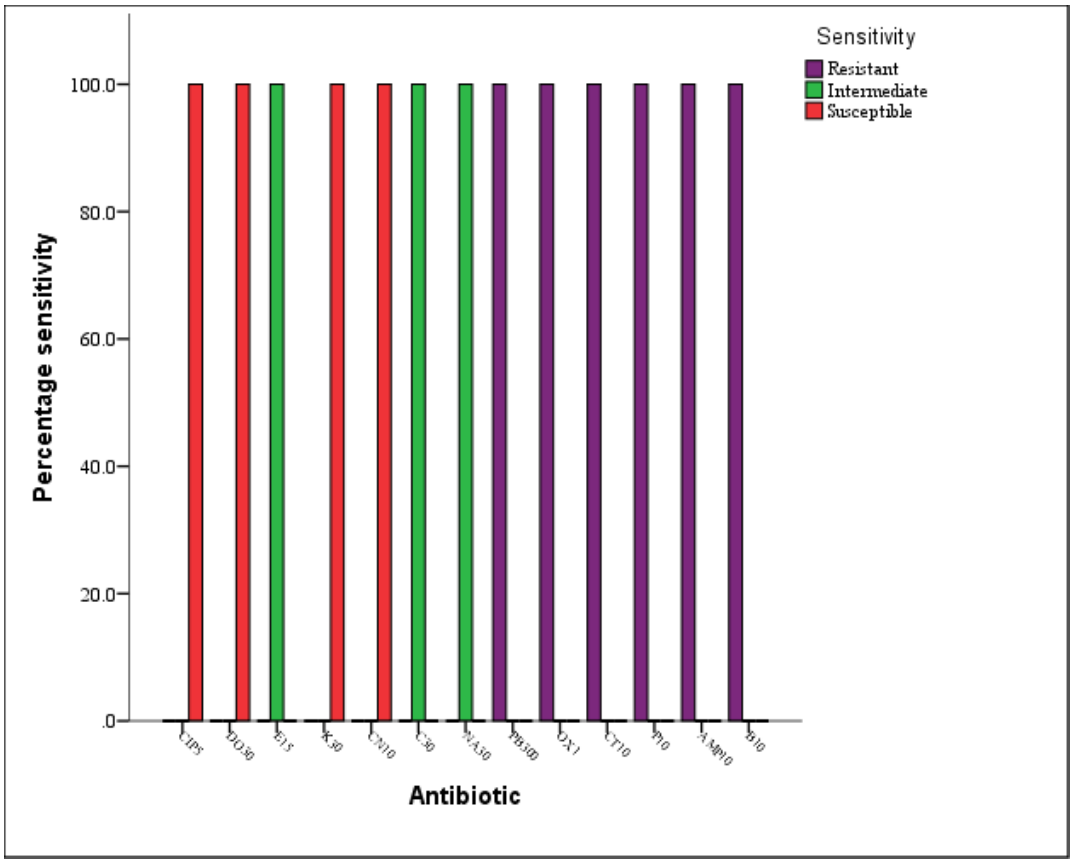


Figure 4.3: Percentage inhibition of various antibiotics, according to (Kirby-Bauer antibiotic test interpretation table)

From the figure 4.3 and table 4.1, the antibiotic used in the field was effective due to its susceptibility to most microbes. The interpretation in figure 4.3 was done in accordance to Kirby-Bauer antibiotic test interpretation table.

4.5 DNA quantity and quality

DNA concentrations ranged from 19 ng/µl to 250 ng/µl. The DNA quality at an absorbance of 260/280 ranged from wavelength of 1.59 nm to 1.94 nm. The DNA quantities and quality were also confirmed from the gel images observed, plate 4.4 and 4.5. The band brightness showed that the DNA was of good quantity and quality.

4.6 Results from the gel products

Amplification of the extracted DNA with U1F and U1R primer showed band lengths of 490bp and 500bp (Plate 4.4). The primers 27F and 1492R DNA gave bands lengths of 1490bp (Plate 4.5). Negative controls were included in all the gels for all the primers (Plates 4.4 and 4.5) to check for any incidence of contaminations during the PCR set-up and amplification.

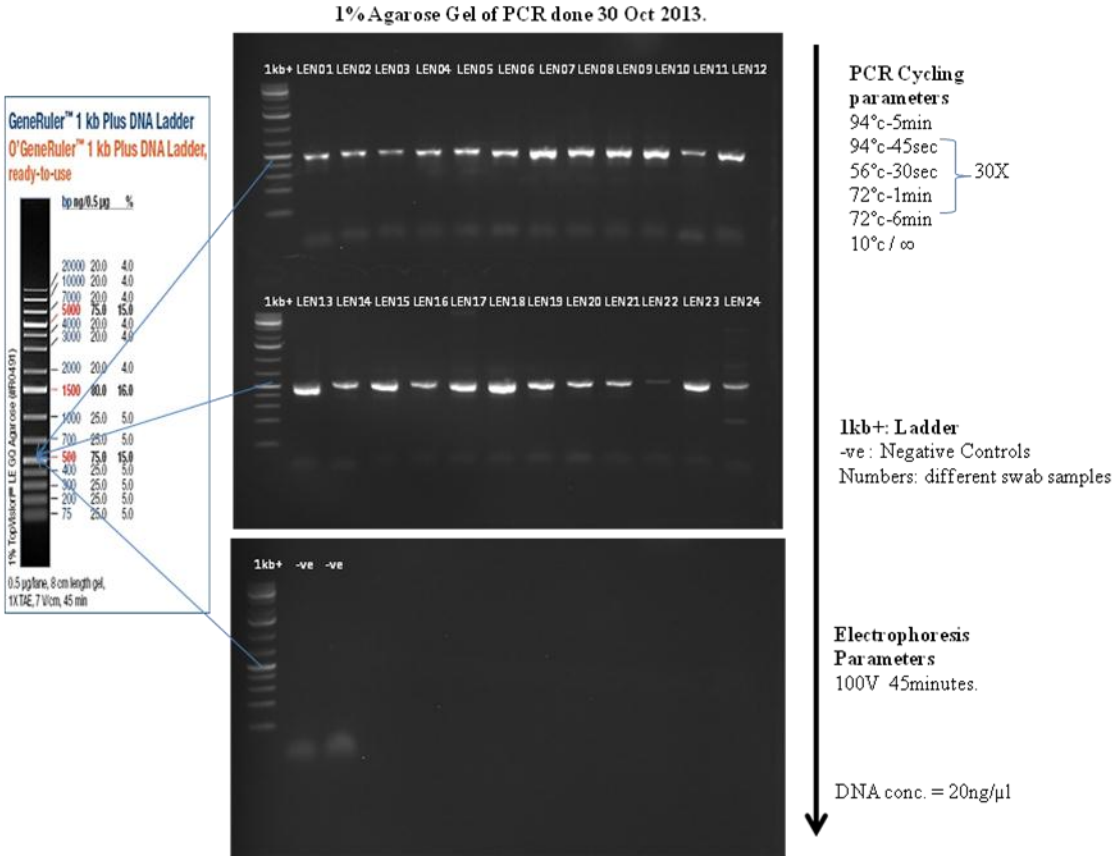


Plate 4.4: Gel image showing the size of PCR product amplified using U1F U1R primer

The 27F 1492R primer is a general primer for bacteria that targets 16S rRNA genes and amplifies at the following sequence 1492R (5′-TACCTTGTTACGACTT) and 27F (5′-AGAGTTTGATCMTGGCTCAG). While the U1F U1R primer is a universal primer which targets 18s with an amplicon size of 512 and 492 bp base pairs (Rivas *et al.*, 2004).

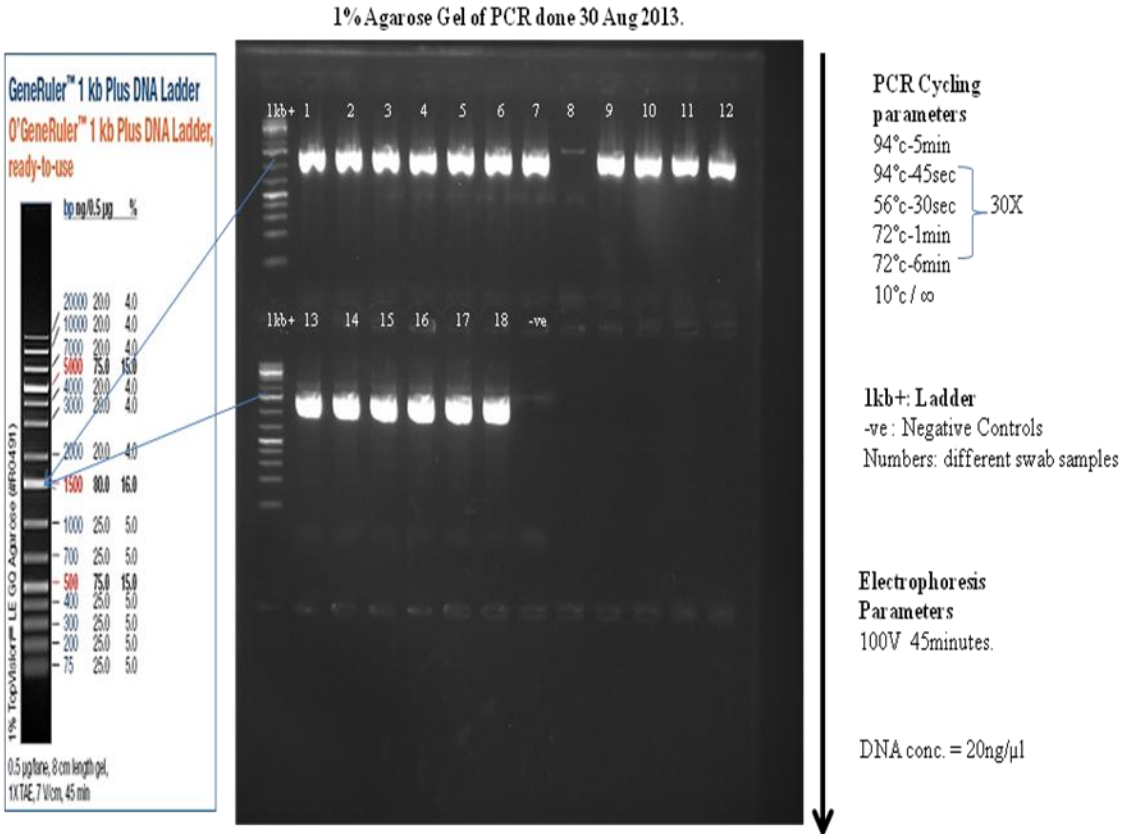


Plate 4.5: Gel image showing the size of PCR product amplified using 27F 1492R primer

4.7 Pyrosequencing data output

The results from Table 4.2, Figure 4.4 and Figure 4.5 (pyrosequencing work) showed that the cattle ears have a community of microbes. The microbes belong to two main domains; Bacteria and Eukaryota. The two domains were observed to have twelve phyla which were; Firmicute, Proteobacteria, Streptophyta, Actinobacteria, Nematode, Chordata, Plantomycetes, Ascomycota, Bacteriodetes, Fusobacteria, Arthropoda and Chloroflexi. These phyla had various orders (Figure 4.4) which also had various classes, families, genera and species.

Bacillus megaterium was the most abundance and *Ciona intestinalis* had the lowest abundance. The average percentage Identity of microbes was high with most values ranging above 80%.

metagenome	source	domain	phylum	class	order	family	genus	species	abundance	avg eValue	avg % ident	avg align ler	# hits
4565865.3	M5NR	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	Bacillus megaterium	1001	-20.68	83.76	59.85	1
4565929.3	M5NR	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	Bacillus megaterium	922	-21.82	83.87	61.66	1
4565865.3	M5NR	Bacteria	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	Staphylococcus aureus	920	-18.42	82.85	56.54	2
4565865.3	M5NR	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	Lactobacillus crispatus	894	-10.73	86.62	41.02	1
4565870.3	M5NR	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	Bacillus megaterium	866	-18.84	85.17	53.19	1
4565929.3	M5NR	Bacteria	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	Staphylococcus aureus	832	-18.86	82.6	57.88	2
4565868.3	M5NR	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	Bacillus megaterium	804	-17.8	82.41	55.26	1
4565870.3	M5NR	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	Lactobacillus crispatus	741	-12.98	82.62	46.76	1
4565868.3	M5NR	Bacteria	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	Staphylococcus aureus	697	-20.75	85.45	59.73	1
4565870.3	M5NR	Bacteria	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	Staphylococcus aureus	688	-23.28	97.39	54.68	1
4565929.3	M5NR	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	Lactobacillus crispatus	652	-10.54	86.18	41.16	1
4565868.3	M5NR	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	Lactobacillus crispatus	585	-11.57	85.72	42.82	1
4565931.3	M5NR	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	Bacillus megaterium	537	-18.67	83.89	55.73	1
4565863.3	M5NR	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	Bacillus megaterium	513	-20.27	84.9	58.35	1
4565931.3	M5NR	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	Lactobacillus crispatus	461	-15.57	84.06	51.42	1
4565934.3	M5NR	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	Bacillus megaterium	448	-21.59	83.3	62.24	1
4565931.3	M5NR	Bacteria	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	Staphylococcus aureus	447	-22.37	85.5	62.42	1
4565863.3	M5NR	Bacteria	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	Staphylococcus aureus	413	-18.88	82.47	57.8	2
4565864.3	M5NR	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	Bacillus megaterium	411	-19.58	84.43	57.09	1
4565934.3	M5NR	Bacteria	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	Staphylococcus aureus	399	-22.06	82.89	64.5	2

Table 4.2: Pyrosequencing results of 16S RNA gene of cattle ear microbes (part of the data output from MGRAST analysis)

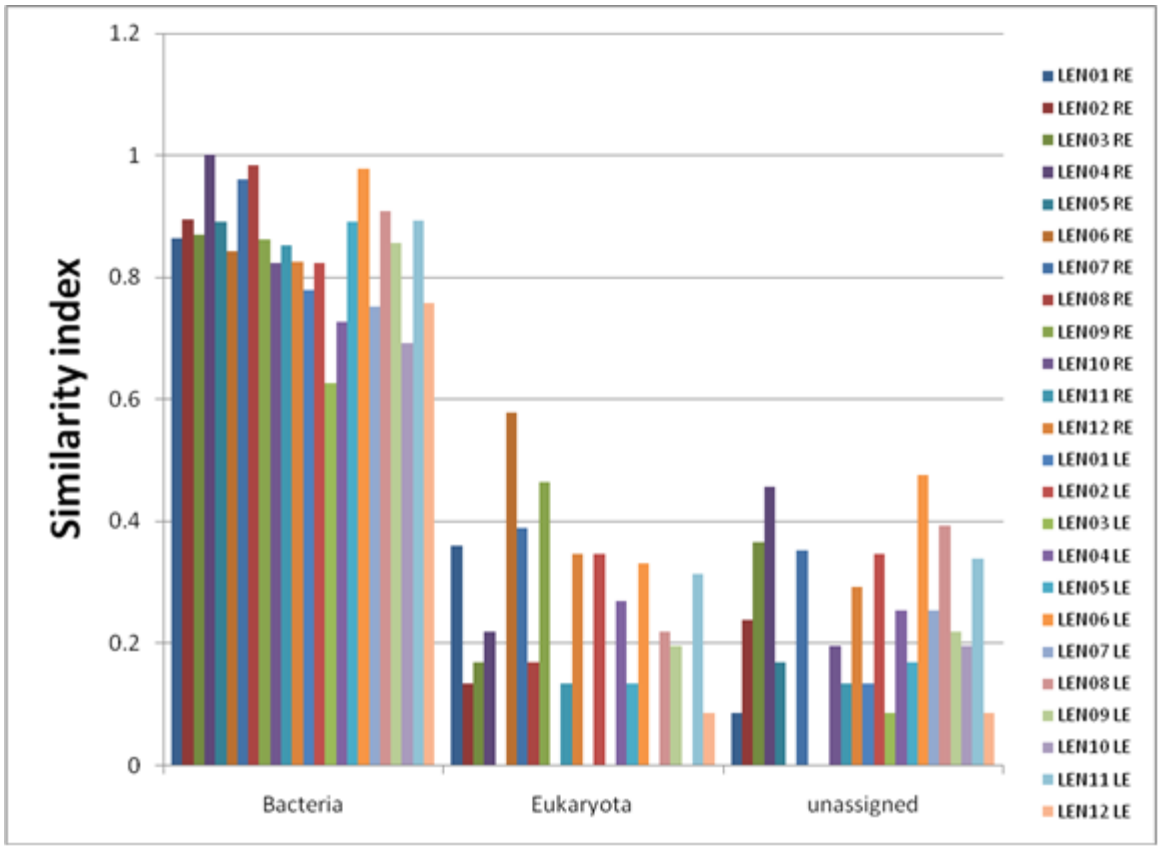


Figure 4.4: Microbial index (LEN 01RE……LEN 12RE- is sample from cow No 1…..12 right ear, while LEN 01LE…….LEN 12LE is sample from cow No1….12; they are identified by different colors on the bar chart)

The heat-map results (Figure 4.5) shows that the most abundant microbes (the one with the green colour) were the bacteria while the less abundant ones (red colour) were the unassigned ones. The names on the left shows the names of various bacterial genus's that were sequenced. The codes on the bottom of the heat map are the codes that were assigned to the samples collected from each cattle ear.

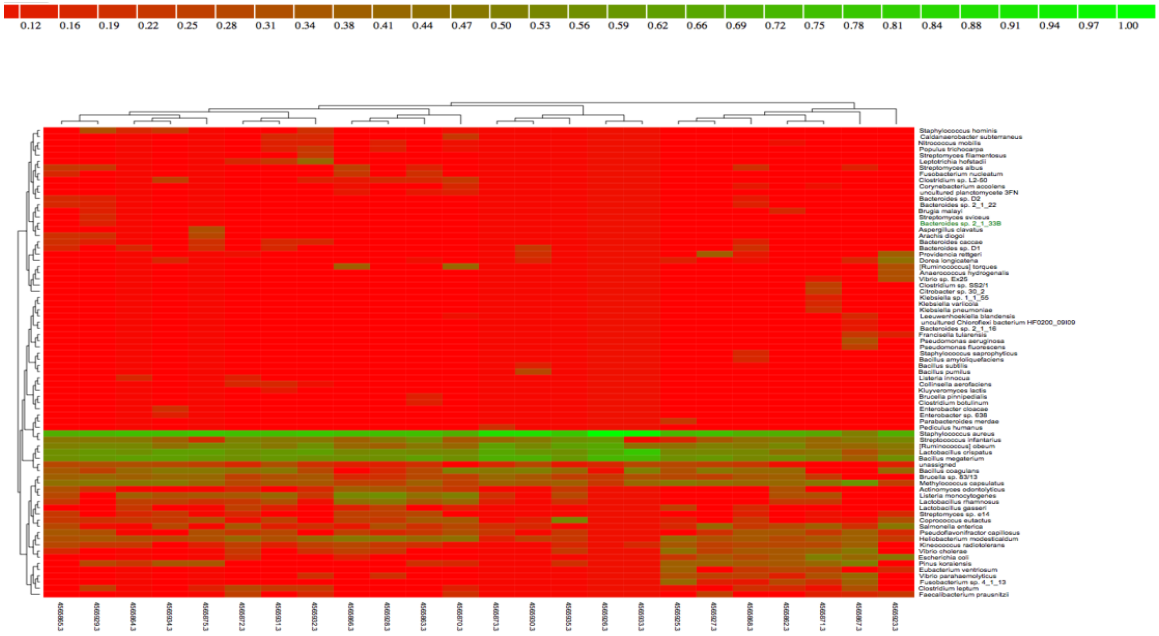


Figure 4.5: A heat-map of diversity of all microbes present in the sequenced ear swab samples

The analyzed results from figure 4.5 and figure 4.6, shows that there is higher Bacterial population in the cattle ear followed by the Eukaryota. The least population was the unassigned organisms that did compare to any other organisms on genebank database.

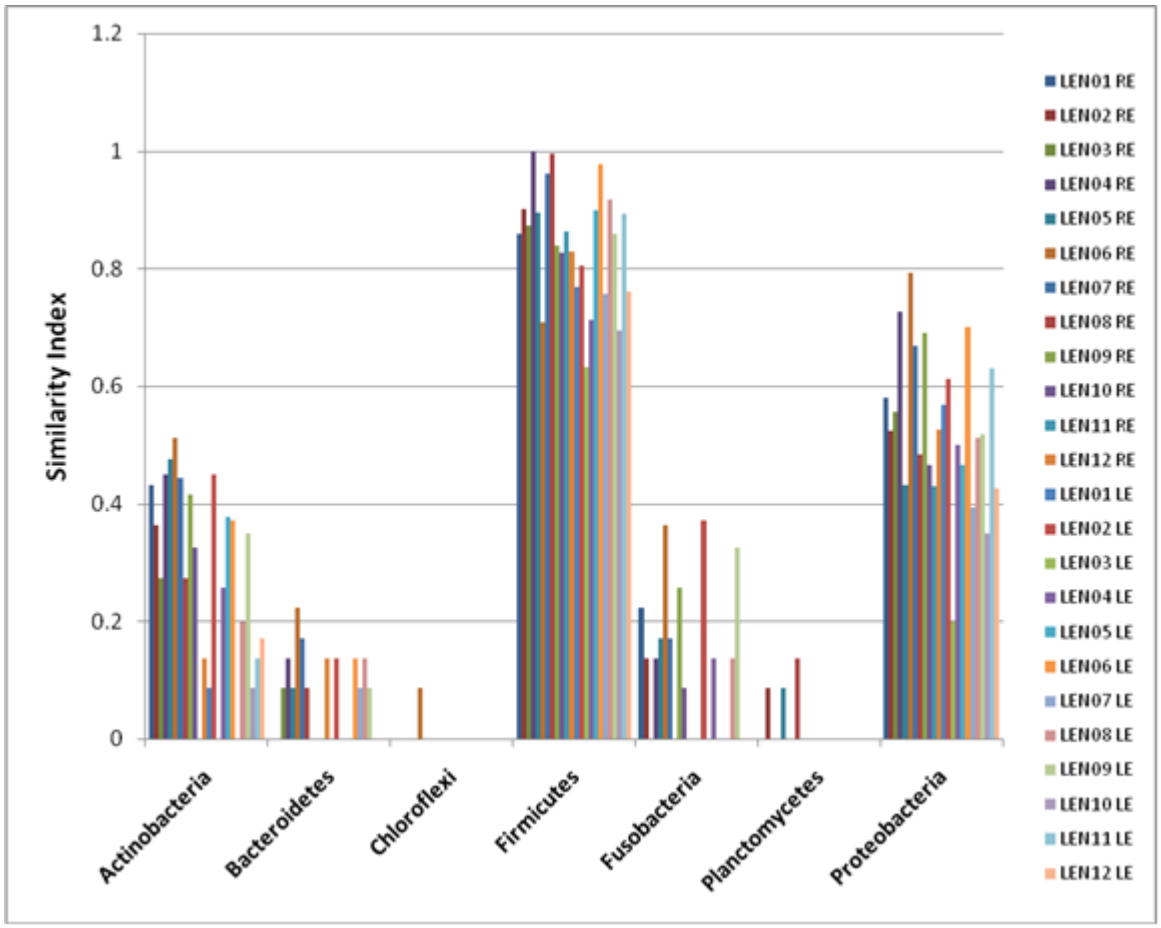


Figure 4.6: Similarity index of Bacteria from the cattle ear

The bacterial community was diverse and this can be seen from the dendogram on figure 4.6. The diverse bacterial population was dominated by organisms from Firmicutes followed by Proteobacteria, the one with the least population was Chloroflexi.

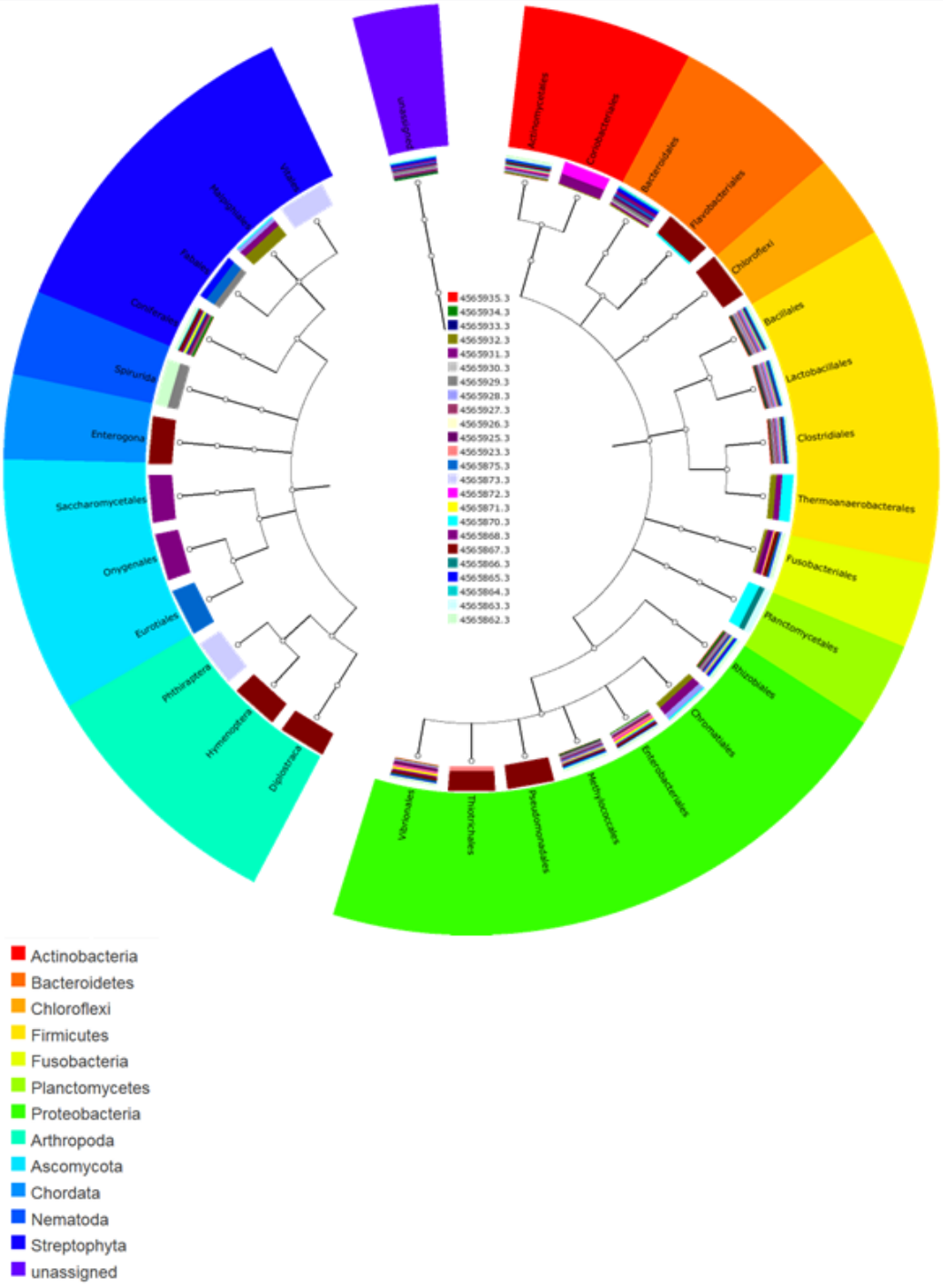


Figure 4.7: Tree diagram Oder level of cattle ear microbes abundance

Figures 4.6 and 4.7 have the same content but have been presented differently so as to give more clarity on the existing population diversity of the microbe.

Bacilli and Clostridia from the samples were highly similar to *Bacilli and Clostridia* sequences in the gene bank (Figure 4.8).

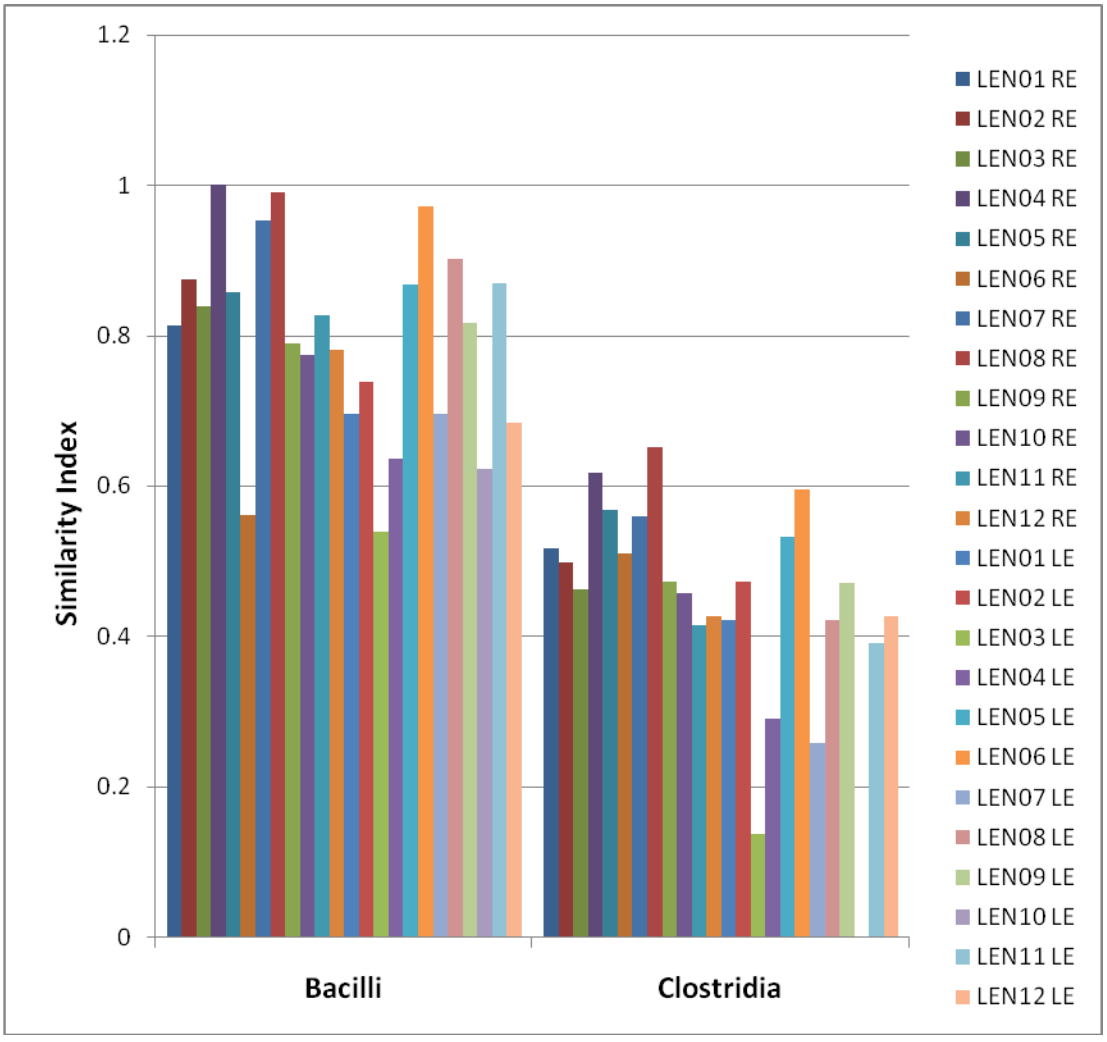


Figure 4.8: Blasts of *Bacilli* and *Clostridia* in the samples

Streptophyta was the dominant in the Eukaryota while Sscomycot, Arthropoda, and Chordata were with very minimal population value (Figure 4.9). The occurrence of the Sscomycot, Arthropoda, and Chordata could be due to host dropping like hair or some insect dropping.

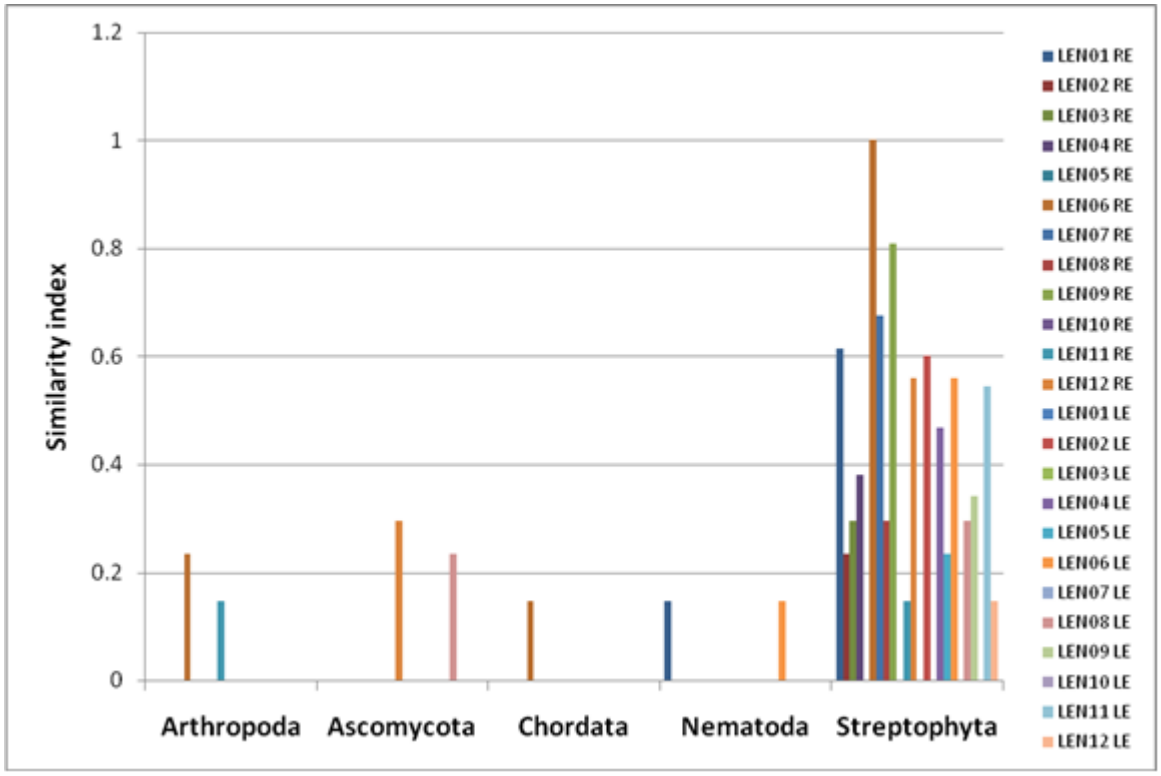


Figure 4.9: Eukaryota blast ressults from the swab samples

Within the Streptophyta, Coniferopsida was the dominant group of organisms which showed similar genes to the one in the gene bank.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATION

5. 1 Discussion

The antibiotic applied on one of the cattle ears reduced the microbial communities which are responsible for production of attractive volatiles to ticks while locating their preferred feeding site. The ear therefore has microbial communities which produce volatiles. These volatiles could be their metabolic wastes that attract the ticks, leading to the significantly high numbers of the ticks in the un-treated ear. Such a trend had been observed by Wanzala *et al.* (2002) where he found that brown ear tick prefers feeding in the ear region due to attractive volatile produced from the ear region. The ear of the cattle is plenty of wax, these wax could be the primary food for the microbial community hence high abundance of bacteria as shown in pyro-sequencing data.

This experiment has shown that it is possible to mask the attraction of the ear to the brown ear tick. A large proportion of the ticks that were released on different regions of cattle failed to locate their feeding site and eventually dropped off when the ear was treated with a broad-spectrum antibiotic (Figure 4.1 and 4.2).

This finding are in agreement with the studies dine on tsetse flies (vectors of animal and human sleeping sickness) which showed a gradation of feeding preference on different vertebrate animals and appeared to use push–pull semiochemistry actively to avoid some hosts and to locate those which are preferred (Gikonyo *et al*., 2000). Other studies in agreement with this finding is the identification of a series of kairomones for

savannah tsetse from preferred hosts (Hall *et al*., 1984) which facilitated the development of baited traps and targets (the latter impregnated with insecticides) effective in large-scale suppression of these species (Brightwell *et al*., 1991). Several synthetic and natural repellents, including a constituent of bovid odours, 2-methoxyphenol, have been evaluated but were found not to be sufficiently effective in protecting cattle in the field (Torr *et al*., 1996). However, with this findings and the recent findings on identification of a potent repellent blend from waterbuck, *Kobus defassa* (Gikonyo *et al*., 2002, 2003), which is refractory to tsetse, these compinations may provide much better protection for cattle and an effective push component in the push–pull approach for faster and more effective suppression of ticks and tsetse populations, particularly where cattle are the dominant source of a blood meal for the flies.

According to Wanzala *et al.* (2007) *R. appendiculatus* prefers feeding in the cattle ear; this region is highly supplied with blood vessels which supplies blood to the ear. At the same time ticks get to reach this region due to attractive cues produced in the ear region. The previous data (Figure 4.2) are showing that microbes are responsible for high population of *R. appendiculatus* in the cattle ear. These cues could be traced back to the microbes which are feeding on the plenty of wax in the ear. The microbes also could be deriving some essential live components from *R. appendiculatus.*

This scenario portrays cattle as a natural host behaving like a habitat where two organisms, microbes and ticks have evolved to exist together at the same time and space without competition of space and food which avoids resource depletion and death.

Previous studies have shown that Volatiles produced by soil-borne endophytic bacteria increase plant pathogen resistance and affect tritrophic interactions, revealed by GC-MS analyses (Marco *et al.,* 2014). The capacity of volatile generation in microbes can either enhance symbiotic relationship with the host or trigger parasitism (Marco *et al.,* 2014; Randy *et al.,* 2009; Xing *et al.,* 2008). However, these profiles of microbes were found to trigger parasitism to the host, thus the cow gets more parasites due to the volatiles produced by the microbes. This is in contrast in with the soil-borne endophyitc bacteria that increase pathogen bacteria (Marco *et al.,* 2014).

The microbial growth on culture media of nutrient agar (Plate 4.1) showed that some microbes residing in the cattle ear are culturable on general media. These microbes were of mixed population Figure 4.4 and 4.5. This was shown as different growth zones from the swabs which were inoculated on the nutrient agar plates. The media which is a general purpose media allowing growth of non-fastidious organisms was used to enable the growth of a wider range of microbes Talaro (2005). The microbial cultures were essential to enable extraction of genomic DNA and antibiotic susceptibility test. Fastidious microbes grows well on blood agar due to the enrichment contained in the agar, in this study blood agar was not used as but it could also be an alternative way to get the more selective microbes.

Based on two different mechanism of action, Doxycycline is a member of Tetracycline which inhibits bacterial growth by binding to 30S Ribosomal subunit in the mRNA translation complex while Erythromycin inhibits bacterial growth at higher concentrations by binding to 50S sub unit of the bacterial 70S rRNA complex. The out put from antimicrial scusceptibility were in agreement with the methods stipulated in Antimicrobial susceptibility testing: a review of general principles and contemporary practices (Jorgensen *et al.,* 2009). Results obtained were expressed in qualitative assessments using the categories susceptible, intermediate, or resistant (Figure 4.3). Doxyccline and Ciproflaxin were resistant while Bacitracin was susceptible.

Nutrient agar significantly enhanced the growth of different species of microbes (Park *et al.,* 2014). In order to understand better the diversity of microbial communities in the mixed culture, extracted DNA was investigated using 16S and 18S rRNA gene pyrosequencing. The negative controls included in the PCR set up for the all sets of primers, Plate 4.4 and 4.5 showed that all the procedures during the set up were contaminants free. There were no amplifications for the controls at the target base pair region for the 16S and 18S regions (Frank *et al.,* 2008). The quality and quantity of the DNA were also of high enough for 454 sequencing. The DNA concentration required of any sample for 454 sequencing is 5 ng/µl (minimum value). These figures showed good quality of the DNA as most DNA sample quality had absorbance at 1.80 nm.

The sequencing results from the DNA of mixed microbial population further confirmed that there exists a mixed population of microbes in the cattle ear. This finding is similar

to findings on Multi-locus real-time PCR for quantitation of bacteria in the environment which revealed *Exiguobacterium* to be prevalent in permafrost (Rodrigues *et al.,* 2007). As expected, *bacteria* was found to be dominant in all the twenty four samples after pyro-sequencing (Figure 4.4), but within bacteria, Firmicutes was present in much higher abundance in all the samples (Figure 4.6), this was in tandem with work on microbes from cow teat skin, which is a potential source of diverse Microbial Populations for Cheese Production (Isabelle *et al.,* 2012). The Eukaryota detected in the study could also provide insight on its capability for volatiles generation.

The results from the meta-genomic study revealed that the cattle ears have a diverse community of microbes. Up to twelve phyla were observed (Figure 4.6 and 4.7); Firmicute, Proteobacteria, Streptophyta, Actinobacteria, Nematode, Chordata, Plantomycetes, Ascomycota, Bacteriodetes, Fusobacteria, Arthropoda and Chloroflexi. These microbes as it had been observed in an *in-vitro* and field tick experiments are responsible in producing volatile constituents that attract brown ear tick to the cattle ears. The Bacilli was found to be more abundant within the Firmicutes (Figure 4.8). They could be working synergistically as a community or as an individual in production of the attractive volatile constituents. Some of the microbes could also not be producing any volatile but could be essential for the existence of others.The microbes *Bacillus megaterium,Staphylococcus aureus, Lactobacillus crispatus* and *Methylococcus capsulatus* were found to be most abundant. With exception of *Methylococcus*

capsulatus which belongs to the class Gamma proteobacteria, the other two three microbes belong to the same class (Bacilli) but are of different genera.

This study can open up more studies on some specific microbes which were found to reside in the cattle ear whose full genome sequence have been done and the literature on the microbes can help to find out their possible role of these microbes in the cattle ear. For example there is a microbe which oxidizes methane (methanophiles) thereby reducing greenhouse gases. Cattle are well known in production of methane (greenhouse gas) but in addition this study has found out that cattle also have evolved to harbor *M. capsulatus* which oxidizes the methane produced by the very cows. These methanophiles are found in cow dung which might have reached the cattle ear while the animal is in the dairy.

Methylococcus capsulatus is an obligate methanotropic Gram-negative, non-motile coccoid bacterium (Soehngen, 1906). In a study by (Ward *et al.*, 2004), phylogenomic analysis, gene order information, and comparative analysis with the partially sequenced methylotroph *Methylobacterium extorquens* was done to detect genes of unknown function likely to be involved in methanotrophy and methylotrophy.

5.2 Conclusion

The results from field tick experiment and the *in-vitro* lab experiment showed that ticks respond to cues produced by the microbes in the cattle ear. These cues guide the Brown Ear Tick to their preferred feeding site (cattle ear). The microbes could also be producing metabolites of which are the one responsible in attracting the ticks. These microbes might be predominantly be feeding on the wax which is in plenty at the cattle ear. In addition the study also demonstrated that broad-spectrum antibiotics (oxytetracycline) can significantly inhibit cultivable aerobic microbes in the cattle ear. Majority of the microbes were scuceptible to the broad- spectrum atibiotic used which showed a lager zone of inhibiton in an antibiotic scusceptibility test.

This study further demonstrated that cattle ears harbor a community of microbes which are responsible for the production of a chemical blend which attract Brown Ear Tick to the ear, the preferred feeding site for the tick species. The microbial population in the ear is diverse; these various types of microbes which inhabit the ear of the cattle could be working in synergy or one particular species could be responsible for production of volatiles which attract Brown Ear Tick to the cattle ears. These microbes have evolved to inhabit the cattle ear to feed on the ear wax. The microbes therefore benefit from the feeding on cattle ear wax and the cattle benefit on reduction of wax from its ear at the same time if suffers from the ticks which suck blood at an expense of ticks finding their way easily to the ear and getting the food in terms of blood. These relationships range therefore from symbiotic to parasitism.

5.3 Recommendations

The development of push and pull in the animal kingdom makes the utilization of repellants and attractants in the reduction of ticks to manageable levels in the grazing fields. Characterization of the attractant semiochemicals may allow the development of a push–pull tactic that combines the use of a source of a synthetic or botanical tick repellent at the ear and an attractant-baited trap treated with fungal pathogen or acaricide located on the back of the animal. The media which was used to culture the ear swabs microbes was a general media, if blood agar is used as alternative media in the next study it would generate a lot of microbes which will in turn give more data when microbial DNA is sequenced by pyro-sequencing. This could be much essential as more data can be analyzed to give more information. This study has demonstrated a strong potential of discovering the genes which code for the enzymes that produce these volatiles. This can be done by studying the genomes of the microbes through functional genomics and proteomics so as to discover the enzymes which produce the attractive volatiles. Thereafter baits which have attractants and repellants are developed and placed in the grazing fields to reduce ticks in the field to manageable levels. The microbes also could be deriving essential live requirement for example food from the ticks, if done in the next study this will be an interesting experiment.

REFERENCES

Bailey, KP. (1960). Notes on the rearing of *Rhipicephalus appendiculatus*and their infection with Theileria parva for experimental transmission.*Bull Epiz Dis Afr* 8: 33–43.

Bibel DJ, R Aly, C Bayles, WG Strauss, HR Shinefield, and HI Maibach. (1983). "Competitive adherence as a mechanism of bacterial interference". *Canadian Journal of Microbiology.* 29: 700-703.

Bowman, A. S., and Nuttall, P. A. (2008). *Ticks: Biology, disease and control*. Cambridge, UK: Cambridge University Press.

Brightwell, R., Dransfield, R. D., and Kyorku, C. (1991). Development of a low-cost tsetse trap and odour baits for Glossina pallidipes and G.longipennis in Kenya. *Medical and Veterinary Entomology*, 2: 153-164.

Connell, Sean R., Tracz, Dobryan M., Nierhaus, Knud H., and Taylor, Diane E. (2003). Ribosomal Protection Proteins and Their Mechanism of Tetracycline Resistance. *American Society for Microbiology.*12: 3675-3681.

Costello, E. K., Knight, R., Lauber, C. L., Fierer, N., Hamady, M., and Gordon, J. I. (2009). Bacterial community variation in human body habitats across space and time. *Science*, 326: 1694-1697.

Cranefield, P. F. (1991). *Science and empire: East Coast fever in Rhodesia and Transvaal*. Cambridge: Cambridge University Press.

D'Alessandro, M., Erb, M., Ton, J., Brandenburg, A., Karlen, D., Zopfi, J., and Turlings, T. C. J. (2014). Volatiles produced by soil-borne endophytic bacteria increase plant pathogen resistance and affect tritrophic interactions. *Plant, Cell and Environment*, 37: 813–826.

Daigle, S. R., Olhava, E. J., Therkelsen, C. A., Majer, C. R., Sneeringer, C. J., Song, J., Johnston, L. D., ... Pollock, R. M. (2011). Selective killing of mixed lineage leukemia cells by a potent small-molecule DOT1L inhibitor. *Cancer Cell*, 20: 53-65.

De Deken, R., Martin, V., Saido, A., Madder, M., Brandt, J., and Geysen, D. (2007). *An outbreak of East Coast Fever on the Comoros; a consequence of the import of immunised cattle from Tanzania?* Veterinary Parasitology, Elsevier 5: 245-253.

Dewhirst, F. E., Chen, T., Izard, J., Paster, B. J., Tanner, A. C. R., Yu, W.-H., Lakshmanan, A., Wade, W. G. (2010). The human oral microbiome. *Journal of Bacteriology*, 19: 5002-5017.

Di Giulio, Godelieve Lynen, Subhash Morzaria and Chris Oura(2009). Live immunization against East Coast fever - current status. *Trends in Parasitology* 25: 85-92.

Eckburg, P. B. (2005). Diversity of the Human Intestinal Microbial Flora.*Science*, 308: 1635-1638.

Erasmus, B. F. N., Van, J. A. S., Chown, S. L., Kshatriya, M., and Wessels, K. J. (2002). Vulnerability of South African animal taxa to climate change.*Global Change Biology*, 7: 679-693.

Feingold, D. S. (1986).Bacterial adherence, colonization, and pathogenicity.*Archives of Dermatology*, 122: 161.

Fierer, N., Hamady, M., Lauber, C. L., and Knight, R. (2008). The influence of sex, handedness, and washing on the diversity of hand surface bacteria.*Proceedings of the National Academy of Sciences of the United States of America*, 105: 17994-17999.

Frank, J. A., Reich, C. I., Sharma, S., Weisbaum, J. S., Wilson, B. A., and Olsen, G. J. (2008). Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. *Applied and Environmental Microbiology*, 74: 2461-2470.

Fuglie, K. O., Rada, N., and United States.(2013). *Resources, policies, and agricultural productivity in Sub-Saharan Africa*. Washington, D.C.: U.S. Dept. of Agriculture, Economic Research Service.

Gachohi, J. M., Ngumi, P. N., Kitala, P. M., and Skilton, R. A. (2010). Estimating seroprevalence and variation to four tick-borne infections and determination of associated risk factors in cattle under traditional mixed farming system in Mbeere District, Kenya. *Preventive Veterinary Medicine*, 95: 208.

Gachohi, John, Skilton, Rob, Hansen, Frank, Ngumi, Priscilla, and Kitala, Philip. (2012). *Epidemiology of East Coast fever (Theileria parva infection) in Kenya: past, present and the future*. BioMed Central.

Gardner, M. J., Bishop, R., Shah, T., de, V. E. P., Carlton, J. M., Hall, N., Ren, Q., Nene, V. (2005).Genome sequence of Theileria parva, a bovine pathogen that transforms lymphocytes. *Science (New York)*, 309: 134.

Gikonyo, N. K., Hassanali, A., Njagi, P. G. N., and Saini, R. K. (2000). Behaviour of *Glossina morsitans* Westwood (Diptera: Glossinidae) on waterbuck Kobus defassa Ruppel and feeding membranes smeared with waterbuck sebum indicates the presence of allomones. *Acta Tropica*, 77: 295-303.

Gikonyo, N. K., Hassanali, A., Njagi, P. G. N., and Saini, R. K. (2003). Responses of *Glossina morsitans* to Blends of Electroantennographically Active Compounds in the Odors of Its Preferred (Buffalo and Ox) and Nonpreferred (Waterbuck) Hosts. *Journal of Chemical Ecology*, 29: 2331-2345.

Grice, E. A., Conlan, S., Deming, C. B., Segre, J. A., Kong, H. H., Turner, M. L., Davis, J., ... Murray, P. R. (2009). Topographical and temporal diversity of the human skin microbiome. *Science*, 324: 1190-1192.

Grice, E. A., Segre, J. A., Kong, H. H., Turner, M. L., Renaud, G., Bouffard, G. G., Blakesley, R. W., ... Young, A. C. (2008). A diversity profile of the human skin microbiota. *Genome Research*, 18: 1043-1050.

Hall, M. (1984). *Frontiers: Southern African archaelogy today*. Oxford: BAR.

Harder, J., Bartels, J., Christophers, E., and Schröder, J. M. (1997). A peptide antibiotic from human skin. *Nature*, 387:6636.

Hayashida, K., Abe, T., Weir, W., Nakao, R., Ito, K., Kajino, K., Suzuki, Y., ... Sugimoto, C. (January 01, 2013). Whole-Genome Sequencing of Theileria parva Strains Provides Insight into Parasite Migration and Diversification in the African Continent. *Dna Research*, 20: 209-220.

Hentges, D. J. (1993). The anaerobic microflora of the human body. *Clinical Infectious Diseases: an Official Publication of the Infectious Diseases Society of America*, 16:175-180.

Inter-African Bureau for Animal Resources,. (2011). *The eradication of Rinderpest from Africa: A great milestone*.

Irvin, A. D., and Brocklesby, D. W. (1972). Attempts to transmit Babesia (Nuttallia) microti in laboratory mice by the tick Rhipicephalus appendiculatus.*International Journal for Parasitology*, 2: 99-103.

Jorgensen, J. H., and Ferraro, M. J. (2009). Antimicrobial susceptibility testing: a review of general principles and contemporary practices. *Clinical Infectious Diseases: an Official Publication of the Infectious Diseases Society of America*, 49: 1749-1755.

Kivaria, F. M., and Noordhuizen, J. P. T. M. (2010). Capture and recapture analysis of East Coast fever in smallholder dairy herds in the Dar-es Salaam region of Tanzania. *The Veterinary Journal*, 184: 187-193.

Leyden, J. J., McGinley, K. J., Mills, O. H., and Kligman, A. M. (1975). Age-related changes in the resident bacterial flora of the human face. *The Journal of Investigative Dermatology*, 65:379-381.

Lozupone, C. A., and Knight, R. (2008). Species divergence and the measurement of microbial diversity.*Fems Microbiology Reviews*, 32: 557-578.

Martin, S. A., and Russell, J. B. (1988).Mechanisms of Sugar Transport in the Rumen Bacterium Selenomonas ruminantium.*Microbiology*, 134: 819-827.

Meyer, F., Paarmann, D., D'Souza, M., Olson, R., Glass, E. M., Kubal, M., Paczian, T., Edwards, R. A. (2008).The metagenomics RAST server - a public resource for the automatic phylogenetic and functional analysis of metagenomes. *Bmc Bioinformatics*, 9: 1-8.

Minjauw, B., and McLeod, A. (2003). *Tick-borne diseases and poverty: The impact of ticks and tick-borne diseases on the livelihoods of small-scale and marginal livestock owners in India and eastern and southern Africa*. Roslin, Midlothian: Centre for Tropical Veterinary Medicine.

Mukhebi, A. W., Perry, B. D., and Kruska, R. (1992). Estimated economics of theileriosis control in Africa. *Preventive Veterinary Medicine*, 12:73-85.

Norval, R. A. I., Perry, B. D., and Young, A. S. (1992).*The epidemiology of theileriosis in Africa*. London: Academic Press.

Olwoch, J. M., Van, J. A. S., Scholtz, C. H., and Horak, I. G. (2007). Climate change and the genus Rhipicephalus (Acari : Ixodidae) in Africa. *Onderstepoort Journal of Veterinary Research*, 74: 45-72.

Ortíz-Castro, R., Contreras-Cornejo, H. A., Macías-Rodríguez, L., and López-Bucio, J. (2009). The role of microbial signals in plant growth and development.*Plant Signaling and Behavior*, 4: 701–712.

Pain, A., Renauld, H., Berriman, M., Murphy, L., Yeats, C. A., Weir, W., Kerhornou, A., Hall, N. (2005). Genome of the host-cell transforming parasite Theileria annulata compared with T. parva. *Science (New York)*, 309: 131-140.

Park, T.-J., Ding, W., Cheng, S., Brar, M. S., Ma, A. P. Y., Tun, H. M., and Leung, F. C. (2014). Microbial community in microbial fuel cell (MFC) medium and effluent enriched with purple photosynthetic bacterium (*Rhodopseudomonas sp.*). *AMB Express*, 4: 22.

Perry, B. D., Kruska, R., Lessard, P., Norval, R. A. I., and Kundert, K. (1991).Estimating the distribution and abundance of Rhipicephalus appendiculatus in Africa. *Preventive Veterinary Medicine,* 11:261-268.

Randolph, S. A. R. A. H. E. (1994). Population dynamics and density-dependent seasonal mortality indices of the tick Rhipicephalus appendiculatus in eastern and southern Africa.*Medical and Veterinary Entomology,* 8: 351-368.

Rivas, R., Willems, A., Palomo, J. L., García-Benavides, P., Mateos, P. F., Martínez-Molina, E., Gillis, M., Velázquez, E. (2004). Bradyrhizobium betae sp. nov., isolated from roots of Beta vulgaris affected by tumour-like deformations. *International Journal of Systematic and Evolutionary Microbiology,* 54:1271-1275.

Rodrigues, D. F., and Tiedje, J. M. (2007). Multi-locus real-time PCR for quantitation of bacteria in the environment reveals *Exiguobacterium* to be prevalent in permafrost. *Fems Microbiology Ecology,* 59: 489-499.

Shaw, M. K. (2003). Cell invasion by Theileria sporozoites.*Trends in Parasitology,* 19: 2-6.

Soehngen NL. (1906). Über Bakterien, welche Methan als Kohlenstoffnahrung und Energiequelle gebrauchen. *Zentrabl Bakteriol Parasitenk Infektionskr*. 15:513–517.

Soulsby, E. J. L. (1982). *Helminths, arthropods, and protozoa of domesticated animals*. Philadelphia: Lea and Febiger.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution*, 28: 2731-2739.

Talaro, K. P. (2005). *Foundations in microbiology*. Dubuque, IA: McGraw-Hill.

Todar, K., and Flying Publisher. (2008). *Todar's Online Textbook of Bacteriology*. Place of publication not identified: University of Wisconsin.

Verdier-Metz, I., Gagne, G., Bornes, S., Monsallier, F., Veisseire, P., Delbès-Paus, C., and Montel, M.-C.(2012). Cow Teat Skin, a Potential Source of Diverse Microbial Populations for Cheese Production. *Applied and Environmental Microbiology*, 78: 326–333.

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

Walker, J. B., Keirans, J. E., and Horak, I. (2000).*The genus Rhipicephalus (Acardi, Ixodidae): A guide to the brown ticks of the world*. Cambridge: Cambridge University Press.

Wanzala, W., Sika, N. F. K., Gule, S., and Hassanali, A. (2004). Attractive and repellent host odours guide ticks to their respective feeding sites. *Chemoecology*, 14:229-232.

Ward, Naomi, Larsen, Øivind, Sakwa, James, Bruseth, Live, Khouri, Hoda, Durkin, A. Scott, Dimitrov, George, ... Eisen, Jonathan A. (2004). *Genomic Insights into Methanotrophy: The Complete Genome Sequence of Methylococcus capsulatus (Bath)*. Public Library of Science.

Yacob, H. T., Ataklty, H., and Kumsa, B. (2008).Major ectoparasites of cattle in and around Mekelle, northern Ethiopia.*Entomological Research*, 38: 126-130.

Zaman, M. A., Iqbal, Z., Abbas, R. Z., Khan, M. N., Muhammad, G., Younus, M., and Ahmed, S. (2012). In vitro and in vivo acaricidal activity of a herbal extract. *Veterinary Parasitology*, 186:431-436.

Zaura, E., Keijser, B.J., Huse, S.M., and Crielaard, W. (2009). Defining the healthy "core microbiome" of oral microbial communities.

http://www.google.com/url?q=http%3A%2F%2Fen.wikipedia.org%2Fwiki%2FParasitif

ormesandsa=Dandsntz=1andusg=AFQjCNFc8p7FxpKiUhajuGF9v-bQ5Q1-Vw

http://en.wikipedia.org/wiki/File:Rhipicephalus-appendicula

http://www.qiagen.com/handbooks

Appendix

Appendix 1.1: Data of Preference treated and control tubes in an in-vitro experiment.

Tube ID	**No of ticks that reached Treated tubes**	**No of ticks that reached Control tubes**	**No of ticks in Expt.**
LEN 01R	3	0	4
LEN 01L	2	0	4
LEN 02R	3	0	4
LEN 02L	4	0	4
LEN 03R	1	0	4
LEN 03L	0	0	4
LEN 04R	1	1	4
LEN 04L	3	0	4
LEN 05R	2	0	4
LEN 05L	3	0	4
LEN 06R	4	0	4
LEN 06L	1	0	4
LEN 07R	2	0	4
LEN 07L	1	0	4
LEN 08R	3	0	4
LEN 08L	0	0	4
LEN 09R	3	0	4
LEN 09L	2	1	4
LEN 10R	4	0	4
LEN 10L	1	0	4
LEN 11R	2	0	4
LEN 11L	3	0	4
LEN 12R	0	0	4
LEN 12L	3	1	4
Total No of ticks	**51**	**3**	**96**

Appendix 1.2: Invitrogen Pure Link™ Genomic DNA Kit Components

The Pure Link™ Genomic DNA Kit contents were; Genomic Lysis/Binding Buffer, Genomic Digestion Buffer, Genomic Wash Buffer 1, Genomic Wash Buffer 2, Genomic Elution Buffer (10 mM Tris-HCl, pH 9.0, 0.1 mM EDTA), RNase A (20 mg/ml) in 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, Proteinase K (20 mg/ml) in storage buffer, Spin Columns with Collection Tubes and Collection Tubes (2.0 ml).

Component/reaction size	**20µl reaction**
Top DNA *polymerase*	1 U
Each: dNTP (dATP, dCTP, dGTP, dTTP)	250 µM
Tris-HCL (pH 9.0)	10 mM
KCL	30 mM
$MgCl_2$	1.5 mM
Stabilizer and tracking dye	

Appendix 1.3: PCR master mix for Bacteria DNA using 27F 1492 R primer

PCR master mix for Bacteria DNA using 27F 1492 R primer		
Premix	**X1 Rxn**	**Xn Rxn**
27F	0.5 µl	0.5*n µl
1492R	0.5 µl	0.5*n µl
ddH_2O	19 µl	19*n µl
Template DNA	1 µl	1*n µl
Appox reaction Vol	20 µl	20*n µl

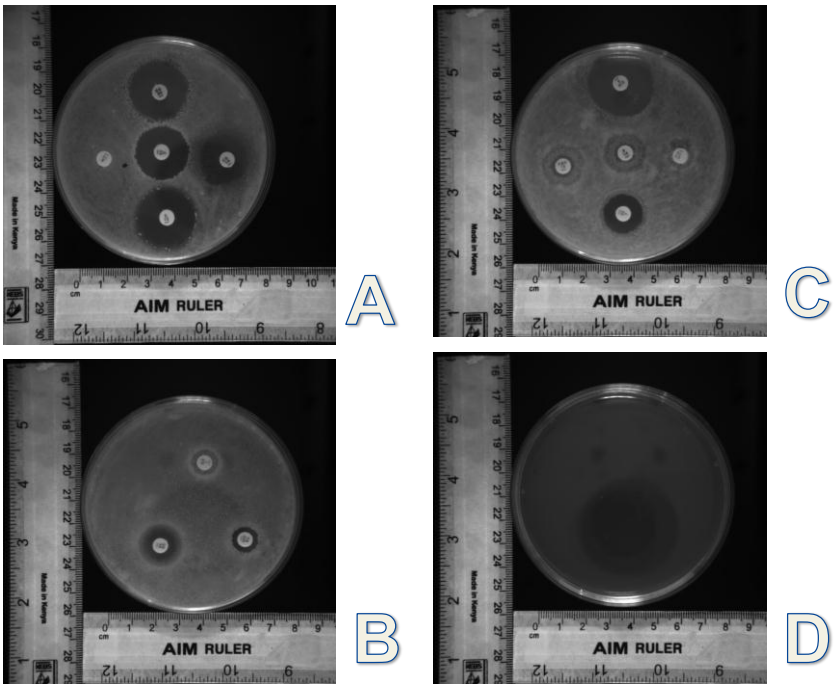
27F/1492R primer PCR Program	
94 ^{0}c	5 min
94 ^{0}c	30 sec
57 ^{0}c	30 sec
68 ^{0}c	1 min
68 ^{0}c	7 min
Holding	**10 ^{0}c**

Appendix 1.4: PCR master mix for Bacteria DNA using U1F/U1R primer

PCR mastermix for Bacteria/Fungi DNA using U1F/U1R primer		
Premix	X1 Rxn	Xn Rxn
U1F	0.25 µl	0.5*n µl
U1R	0.25 µl	0.5*n µl
ddH_2O	18.5 µl	19*n µl
Template DNA	1 µl	1*n µl
Appox reaction Vol	20 µl	20*n µl

U1F/U1R primer PCR Program	
94 ^{0}c	3 min
94 ^{0}c	45 sec
56 ^{0}c	45 sec
72 ^{0}c	1 min
72 ^{0}c	6 min
Hold	**10 ^{0}c**

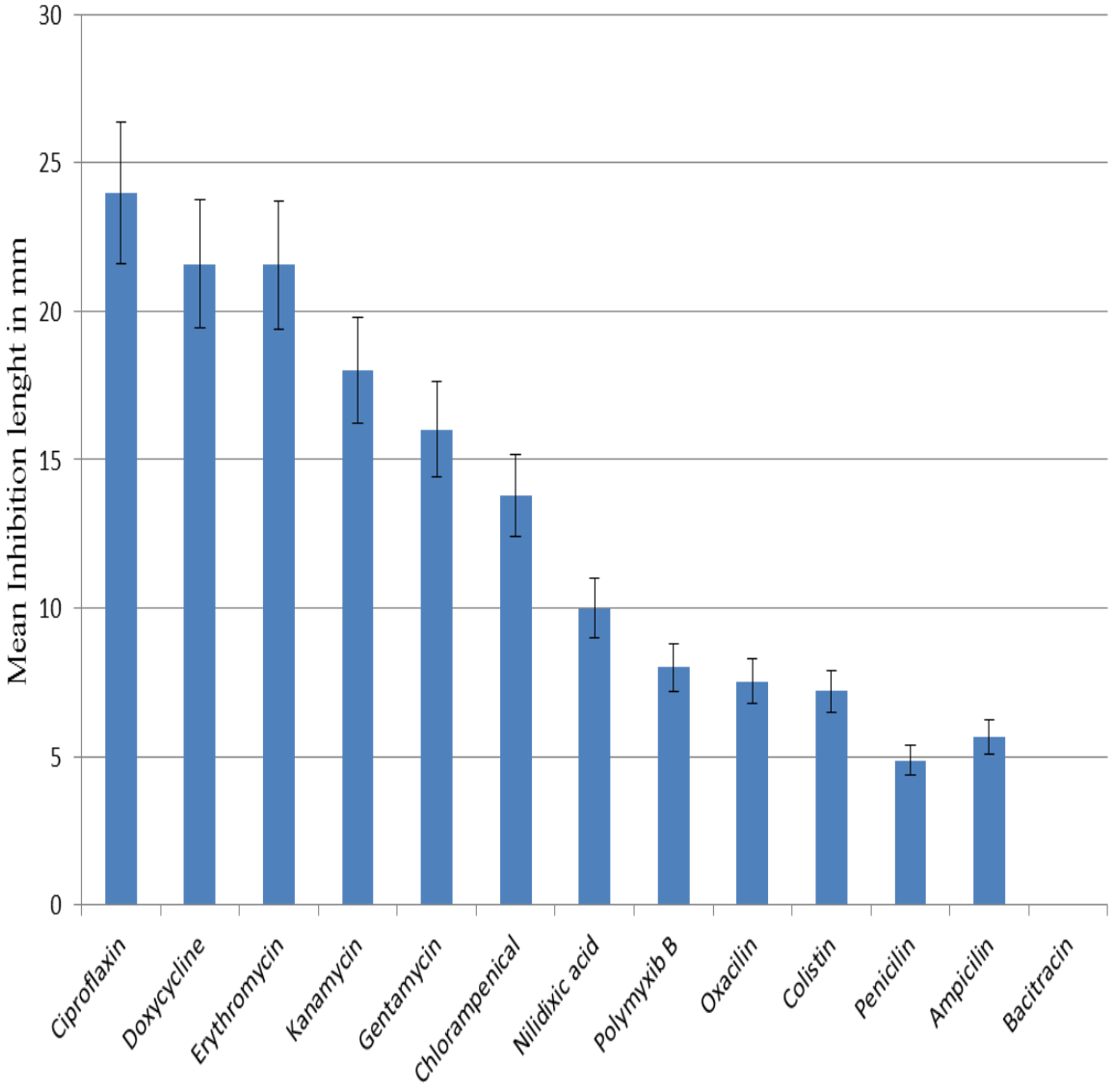
Appendix 1.5: Picture showing various antibiotic inhibitions



Appendix 1.6: Antibiotic inhibition measurements taken from various antibioticcs

Name of antibiotic	**Antibiotic symbol**	**Experiments on antibiotic inhibition measurement in (mm)**					
		Expt1	Expt2	Expt3	Expt4	Expt5	Average
Ciproflaxin	CIP_5	24.00	23.90	24.01	24.00	23.98	24.00
Doxycycline	DO_{30}	21.50	22.00	21.48	21.49	21.51	21.60
Erythromycin	E_{15}	21.56	21.55	21.56	21.57	21.56	21.56
Kanamycin	K_{30}	18.00	18.01	18.00	18.05	18.01	18.00
Gentamycin	CN_{10}	16.01	16.05	16.00	16.01	16.01	16.01
Chlorampenical	C_{30}	13.80	13.83	13.83	13.81	13.82	13.80
Nilidixic acid	NA_{30}	10.00	10.01	10.00	9.98	10.01	10.00
Polymyxib B	PB_{300}	8.00	8.02	8.01	8.00	7.99	8.00
Oxacilin	OX_1	7.54	7.52	7.53	7.55	7.54	7.54
Colistin	CT_{10}	7.20	7.18	7.20	7.19	7.21	7.20
Penicilin	P_{10}	4.88	4.88	4.90	4.89	4.87	4.88
Ampicilin	AMP_{10}	5.68	5.67	5.66	5.69	5.68	5.68
Bacitracin	B_{10}	0.00	0.00	0.00	0.00	0.00	0.00

Appendix 1.7: Average length of each antibiotic inhibition (measurement in mm).



Appendix 1.8: Kirby-Bauer test interpretation table for the antibiotic susceptibility tests

Antibiotic (Antimicrobial Agent)	DISC CODE	Resistant < or = mm	Intermediate mm	Susceptible = or > mm
Amoxicillin (other)	AMC	<13	14-17	>18
Amoxicillin (Staph)	AMC	19		20
Ampicillin (other)	AM	11	12-13	14
Ampicillin (Staph)	AM	28		29
Carbenicillin (other)	CB	17	18-22	23
Carbenicillin (Pseudomonas)	CB	13	14-16	17
Cefoxitin	FOX	14	15-17	18
Cephalothin	CF	14	15-17	18
Chloramphenicol	C	12	13-17	18
Ciprofloxacin	CIP-5	15	16-20	21
Clindamycin	CC-2	14	15-20	21
Enoxacin (Fluoroquinolone, 2nd gen.)	ENX-10	14	15-17	18
Erthromycin	E	13	14-22	23
Gentamycin	GM	12	13-14	15
Kanamycin	K-30	13	14-17	18
Methicillin (Staph)	M(orDP)	9	10-13	14
Oxacillin (Staph)	OX	10	11-12	13
Penicillin G (Enterococcus)	P	14		15
Penicillin G (Staph)	P	28		29
Streptomycin	S-10	14	15-20	21
Sulfamethoxazole-trimethoprim	SXT	10	11-15	16
Tetracycline	Te-30	14	15-18	19
Tobramycin	NN-10	12	13-14	15
Vancomycin	Va-30	9	10-11	12

Appendix 1.9: ANOVA analysis results on antibiotic susceptibility tests

ANOVA

Distance

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3399.650	12	283.304	67428.683	.000
Within Groups	.218	52	.004		
Total	3399.869	64			

Appendix 1. 10: Student Newman Keuls (SNK-test, α=0.05)

Distance

Student-Newman-Keuls[a]

Name of antibiotic	N	Subset for alpha = 0.05											
		1	2	3	4	5	6	7	8	9	10	11	12
Bacitracin	5	.000											
Penicilin	5		4.888										
Ampicilin	5			5.680									
Colistin	5				7.196								
Oxacilin	5					7.528							
Polymyxib B	5						8.004						
Nilidixic acid	5							10.000					
Chlorampenical	5								13.818				
Gentamycin	5									16.014			
Kanamycin	5										18.014		
Erythromycin	5											21.568	
Doxycycline	5											21.600	
Ciproflaxin	5												23.978
Sig.		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.439	1.000

Means for groups in homogeneous subsets are displayed.