PROFILES AND ANTIMICROBIAL RESPONSE PATTERNS OF HUMAN FEET BACTERIAL ISOLATES OF INDIVIDUALS WITH DIFFERENTIAL ATTRACTION TO *ANOPHELES GAMBIAE* IN KILIFI, KENYA

JAMES MUHUNYU GATHIRU (B.Sc.)

I56/28267/2014

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE (MICROBIOLOGY) IN THE SCHOOL OF PURE AND APPLIED SCIENCES OF KENYATTA UNIVERSITY

MAY 2019

DECLARATION

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

Signature..... Date.....16/6/2019.....

James Muhunyu Gathiru

I56/28267/2014

Department of Biochemistry, Microbiology and Biotechnology

APPROVAL BY THE SUPERVISORS

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

Dr James Nonoh Signature ..... Date 18/06/2019

Department of Biochemistry, Microbiology and Biotechnology

Kenyatta University, Kenya

Prof Ahmed Hassanali Signature ..... Date 18/06/2019

Department of Chemistry

Kenyatta University, Kenya

DEDICATION

I dedicate this work to Almighty God for making this entire process a success. I also dedicate this work to my caring family and friends for their encouragement and support during the whole study.

ACKNOWLEDGEMENTS

I would like to extend my sincere gratitude and appreciation to my supervisors' Dr James Nonoh and Professor Ahmed Hassanali for their excellent supervisory role, their insightful guidance and advice during the whole study. I also acknowledge Dr David Mburu, Pwani University for his relentless support and guidance through the research process. I acknowledge all the technicians in the Department of Microbiology, Kenyatta University and the Department of Biology, Pwani University for all the support they gave me during the research period. I would also like to extend my special gratitude to Mr Morris Muthini for his technical support and guidance.

I acknowledge and appreciate the Kenya National Research Fund for the research grant awarded which was of major financial support to this study. I acknowledge Mr Shida, KEMRI Kilifi for his assistance in the study. I extend my special appreciation to my dad, Mr Josephat Gathiru Muhunyu and my uncle Mr John Mwangi Muhunyu for the unconditional support they gave me in the entirety of this research. Finally, a special acknowledgement goes to my colleagues and specifically, Simon Mburu, Gilbert Koskey, Domitilla Muendo, Hibert Opinde and Harry Musonye for the encouragement, support and teamwork.

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ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of variance
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
CO1	Cytochrome c oxidase subunit 1
CFU	Colony forming unit
DNA	Deoxyribonucleic acid
DNTPs	Deoxy-nucleotide triphosphate
DDT	Dichlorodiphenyltrichloroethane
EDTA	Ethylene diamine tetra-acetic acid
ESR	Evolved Spatial Repellents
GC-MS	Gas chromatography- mass spectrometry
GSM	Genetic System Mechanisms
H_2S	Hydrogen sulphide
HRM	High Resolution Melting
HSD	Honest significant difference
IRS	Indoor residual spraying
ITN	Insecticide treated mosquito nets
KEMRI	Kenya Medical Research Institute
MBC	Minimum Bactericidal Concentration
MEGA7	Molecular Evolutionary Genetics Analysis version 7.0
MIC	Minimum Inhibitory Concentration
MR-VP	Methyl Red- Vogues Proskauer

NCBI	National Centre for Biotechnology Information
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
RIDL	Release of Insect carrying a Dominant Lethal gene
rRNA	ribosomal ribonucleic acid
SDS	Sodium Dodecyl Sulphate
SIM	Sulphur Indole Motility
SIT	Sterile Insect Technique
SSA	Sub Saharan Africa
SSU	Small Sub Unit
TSI	Triple Sugar Iron
VOCs	Volatile Organic Compounds
WHO	World Health Organization

ABSTRACT

Malaria is a mosquito-borne illness caused by *Plasmodium* parasites carried by certain species of mosquitoes. It is currently a global problem where more than 2 billion people in 110 countries at risk, with an estimated 2.7 million deaths per year of which the majority are children. In Kenya, *Anopheles gambiae s.s* is the most effective vector in human malaria transmission. Studies have indicated that some of the chemical cues in the feet odour that mediate attraction of *An. gambiae s.s* to their preferred feeding site may be of microbial origin. However, the profiles and phylogenetic affiliations of the microbes have not been fully characterized. The objectives of this study were to determine the different levels of attraction of female *Anopheles gambiae* s.s to the feet odours, to isolate and characterize the bacterial isolates, and to assess their antimicrobial response patterns. Ten volunteer male adult participants in Kilifi, Kenya, were recruited for the differential attraction experiment. Swab samples from the most to the least attractive individuals were used to isolate feet derived bacterial isolates. The isolates were then characterized based on cultural, morphological and molecular techniques. Specifically, the isolates were characterized based on 16S rRNA gene-based Sanger sequencing using 27F and 1492R bacterial primer pair. The phylogenetic analyses were then done on MEGA7 together with its available software tools. The antimicrobial patterns of the bacterial isolates were characterized using commercially available antibiotics and antiseptics. Results indicate that, of the ten (10) participants recruited in the study, participant two (2) was the most attractive while participant six (6) was the least attractive to the female *An. gambiae* s.s mosquitoes. There was a significant ($p=0.001$) variation in the attraction of the female mosquitoes to the feet sites (front and back), the front site being more attractive. On the other hand, the attraction of the mosquitoes to the left and right legs did not differ significantly ($p=0.274$). Nineteen (19) bacterial morpho-groups were obtained from the samples with a majority of them fourteen (14) being mainly present on the front part of the feet. Phylogenetic analysis revealed diverse bacterial communities belonging to different genera. Based on phylogeny, participant two (2) was mainly dominated by isolates belonging to *Staphylococcus capitis* and *Staphylococcus simulans*, both are associated with the production of lipase enzyme that breaks down lipids in sweat leading to production of foot odour. Isolates belonging to *Staphylococcus xylosus* and *Bacillus pumilus* were shared among participant two (2) and participant six (6). Two novel isolates, PTXV and PTXVI, could not be identified based on phylogeny. Antibiotic response patterns revealed that isolates belonging to *Bacillus safensis*, *Bacillus pumilus* and a potentially novel isolate PTXV were susceptible to the six antibiotics. Ciprofloxacin was the most effective antibiotic while antiseptic A was the most effective antiseptic against the bacterial isolates. The results show that the differential attraction of *Anopheles gambiae s.s* females to individuals in malaria-endemic regions is evident and mediated by differing bacterial numbers among the participants.

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

Malaria is a mosquito-borne disease caused by plasmodium parasites that infect certain types of mosquitoes that feed on humans (Smith *et al.,* 2014). Symptoms of malaria usually appear 10 to 15 days after being bitten by a mosquito. People who are infected with malaria are typically very sick and have symptoms such as high fevers, headaches, shaking chills and flu-like illnesses. If not treated, malaria infections can quickly become life-threatening by disrupting the blood supply to the organs (Rénia *et al*., 2012).

From a global perspective, an estimated 2.5 billion people worldwide face the risk of getting infected with malaria (Majeed, 2013). Apart from the above-estimated numbers, 1.2 billion people are at a high risk with >1 in 1000 chance of developing malarial infections in a year. In view of the latest estimates, 198 million cases of malaria occurred globally in 2013 with an uncertainty range of 124 to 283 million cases. The disease led to 584,000 deaths with an uncertainty range of between 367,000 to 755,000 death cases (Gore-Langton *et al*., 2015). The burden brought about by malarial infections is highly evident in the African region where an estimated 90 % of all malarial death do occur (Hemingway, 2014). This is especially evident among populations living tin the Sub-Saharan Africa. In Kenya there are about 25 million people who are at a risk of malaria. There are four malaria zones; endemic, epidemic , seasonal and low-risk regions in Kenya (Control and Macro, 2010).

Transmission of malaria in humans is by female mosquitoes belonging to the genus *Anopheles* (Cox, 2010). The female mosquitoes take blood meals for egg production (Omolo *et al*., 2013). These blood meals act as a link between the human hosts and the mosquito in its parasitic life cycle, from the gametocyte to the sporozoite stage (Bousema *et al.,* 2011). This is dependent on several factors, the most important being ambient temperature and humidity, higher temperatures accelerating the growth of the parasite in the mosquito (Paaijmans *et al*., 2010). Transmission of the *Plasmodium* by the mosquito vectors is also dependent on different behavioral adaptations. Some of these adaptations include resting patterns, feeding behaviors, mating, parasite influence and host preferences (Batool *et al.*, 2016).

The genus *Anopheles* has been identified as the most important genera when it comes to transmission of malaria as it has the greatest potential in terms of *Plasmodium* parasite transmission. In tropical Africa, there are two mosquito species that are considered to be the most effective vectors in relation to human malaria. These are; the *Anopheles funestus* and *Anopheles gambiae s.s sensu lato* (Mukabana *et al*., 2012). They have characteristic bionomics that guarantee a strong mosquito-host interaction that is favorable for transmission of malaria (Smallegange *et al*., 2011). In Kenya, the most abundant species of malaria vectors are *Anopheles funestus, Anopheles gambiae s.s* and *Anopheles arabiensis*. Effectiveness of the vector depends on how fast it can locate the host. Various chemicals have been shown to play a role in host location by the vector. However, the source of such chemicals remains largely unclear. Previous work by Omolo *et al*. (2013) has shown that mosquitoes are attracted to the human foot. They made further observation that recently worn socks placed next to a blood-feeding membrane device did enhance the feeding of mosquitoes (Omolo *et al*., 2013).

With advances in research, mosquitoes have been shown to use their olfactory senses when selecting human hosts for feeding. There is a two-step process in the orientation behaviour of *An. gambiae s.s,* the first being location of the hosts from long distance mediated by odour mainly from the breath. The second step is influenced by close range odours mediated by site specific volatile blends (Omolo *et al*., 2013). The attraction of malaria vectors to their preferred hosts is usually mediated by several determinants which enable the mosquitoes to have higher chances of finding the target hosts for feeding. These determinants include body mass, odorants, relative humidity, gender and physiology of the mosquitoes (Takken *et al*., 2013). The role of odorants in mediating attraction of the vector to the host has been investigated. Several compounds have been demonstrated on how they do attract the mosquitoes. These compounds include carbon dioxide, lactic acid, nonal and octenol. As a control strategy, these compounds have been used as lures designed to trap the mosquitoes and in turn, reduces the transmission of the disease (Poulin *et al*., 2017).

Several vector control strategies have been put in place in an effort to cut the link between the vector and the human host. These strategies include insecticide-treated materials such as bed nets, indoor residual spraying, use of repellents, larvicidal and biological control, environmental management and population suppression measures. All these approaches are geared towards reducing the transmission of the disease, which in turn has a positive impact on both the social and economic pillars of the society.

Bacteria that are present on the human skin have been implicated in the production of odours which in turn play a great role in the close-range attraction of mosquitoes to their preferred feeding sites (Verhulst *et al.*, 2011). These bacteria can be located around skin glands where they breakdown the glandular secretions to produce the odour. In humans,

there is a correlation between the intensity of odour produced by the individuals and the number of skin microbiota present (Smallegange *et al*., 2011). As shown by (Bovell, 2015), human feet do contain high numbers of eccrine glands which produce sweat that is metabolized by bacteria present to release chemical cues which may act as attractants to the female *Anopheles* mosquitoes. Attraction of mosquitoes to the human foot odours can be attributed to the variations of the volatile chemical compositions produced from the feet (Omolo *et al*., 2013). There is a very significant decrease in biting episodes by mosquitoes when the feet are washed with antibacterial soaps (Chaudhari, 2016). In order to devise an effective control strategy based on microbial-derived chemical attractants, it is imperative that the identities of such foot based microbial communities should be well investigated. Scientific knowledge on this attraction should be enhanced to try and find out if microbes play a role in this phenomenon. With advances in science and technology, techniques have been developed that enable researchers to define the profiles of the microbial communities inhabiting the human skin. Such techniques include Sanger sequencing and Illumina sequencing which give researchers and scientists a deep insight into the occurrence of the microbial communities on the skin (Jo *et al.*, 2016).

1.2 Statement of the problem

Malaria is currently a major global problem affecting ~40 % percent of the world's population in 100 countries (Majeed, 2013). There are about 350 to 500 million cases of malaria each year with an estimated 2.7 million people dying (Kioko *et al*., 2013). High-risk groups of people vulnerable to malaria infections include pregnant women, non-immune travelers, refugees, and workers entering into malaria-prone areas (Adebayo *et al*., 2013). Tropical Africa bears the biggest brunt of malaria with more than 90 % of the 300

million annual cases of malaria (Hemingway, 2014), costing more than 12 billion dollars annually (Reilly, 2014).

As malaria increases so does mortality and morbidity rates, and with these come socio-economic losses (Asenso-Okyere *et al*., 2011). Age distribution of the affected population has an effect on the burden of malaria in highly endemic areas where the older populace develops some collective immunity hence suffer less as compared to children less than 5 years. In Africa, most rural set ups do not have access to proper healthcare facilities (Mupela *et al*., 2011). Drug-resistant malaria is now common, and anti-malarial drugs are becoming less and less effective as the *Plasmodium* parasite develops resistance to affordable medications (Petersen *et al*., 2011). This, in turn, poses serious challenges to clinical control and treatment of malaria. There is lack of knowledge with regard to the identities and antimicrobial response patterns of human foot derived bacterial communities that mediate the attraction of female *An. gambiae s.s* to their preferred human hosts.

1.3 Justification

The high levels of infections and deaths brought about by malaria require a more extensive scope of remedial measures. This makes research and development aimed at reducing the vector host interaction very vital. In the recent past, several control measures have been put in place to try and curb the spread of malaria. These include insecticide-treated mosquito nets (ITNs) and indoor residual spraying (IRS) (Russell *et al*., 2013)

Currently, there are efforts being put in place to minimize the contact between humans and mosquitoes (Omolo *et al*., 2013). Such strategies have revealed that attractive odours could be used to draw mosquitoes away from humans and into traps (Jawara *et al*., 2011). The discovery that feet odour attracts mosquitoes (Omolo *et al*., 2013) has opened up

several potential tactics of controlling mosquitoes. Various chemicals have been implicated in this attraction but the source remains unclear. Foot based microbial communities have been implicated in the production of chemical cues potentially playing a role in mosquito attraction to the feet, but the hypothesis has not been well investigated (James *et al.*, 2013). Moreover, there is very limited data on morphological and phylogenetic profiles of such microbial communities producing vector attractive odours. Isolation and characterization of such foot microbiota may give a lead on their role in vector attraction to the human foot and open up novel strategies for minimising host-vector interactions.

1.4 Hypotheses

i. There are no different levels of attraction of female *An. gambiae s.s* mosquitoes to foot odour.

ii. The feet of individuals with differential attraction to *An. gambiae s.s* do not harbour culturable and diverse bacterial isolates with varied morphological, biochemical and phylogenetic characteristics.

iii. Human feet bacterial isolates from the feet of individuals attractive to *An. gambiae s.s* do not exhibit varied response patterns to commonly used antibiotics and antiseptics.

1.5 Objectives of the study

1.5.1 General objective

To assess the profiles of human feet derived bacterial isolates of individuals with differential attraction to *An. gambiae s.s.*

1.5.2 Specific objectives

i. To determine the different levels of attraction of *An. gambiae s.s* mosquitoes to foot odour.

ii. To culture and characterize bacterial isolates in feet samples of individuals with differential attraction to *An. gambiae s.s*.

iii. To determine the antibiotic and antiseptic response patterns of human feet derived bacterial isolates.

1.6 Significance of the study

Identification of the specific bacterial communities in human feet that potentially mediate the attraction of mosquitoes to the preferred feeding site in the host will help in elucidating the phenomenon of human host- mosquito vector interactions. This will then pave the way for gradual investigations to find out the specific compounds produced by the specific foot-based bacterial communities that can then be utilized in designing effective strategies for minimizing host-vector interactions and thus minimizing malaria incidences, especially in endemic regions. Such efforts will eventually help communities most affected by the malaria scourge.

CHAPTER TWO

LITERATURE REVIEW

2.1 Current malaria situation

Malaria is a mosquito-borne disease that is caused by *Plasmodium* parasites transmitted by female *Anopheles* mosquitoes that feed on humans (Neafsey *et al*., 2014). Transmission of malaria occurs in all the six regions under WHO. Globally, there are an estimated 3.3 billion people present in 97 countries that are at risk of getting infected with malaria and developing the disease. Of these 3.3 billion people, 1.2 billion are at a higher risk, that is, >1 in every 1000 chance of getting infected with malaria in a year. In 2013, there were an estimated 198 million cases of malaria globally with an uncertainty range of between 124-283 million cases. The disease led to the death of 584,000 people with an uncertainty range of between 367,000-755,000 deaths (Caragata and Moreira, 2017).

The burden of malaria is most heavily felt in the African WHO regions in which an estimated 90 % of all the malaria deaths occur. Populations living in Sub-Saharan Africa (SSA) do have the highest risk of getting infected with malaria. Among the 216 million cases of malaria reported in 2010, approximately 174 million cases or 84 % were reported from the African region. There were an estimated 655,000 deaths resulting from malaria in 2010, of which, 91 % were from Africa. Malarial deaths of children aged below five years do account for 78% of all deaths (Region *et al*., 2015).

In Kenya, 25 million people are at risk of getting malaria. Malaria accounts for 30-50 % of all outpatient attendances and 20 % of all the hospitalizations in health facilities. Malaria is also estimated to be responsible for 20 % of all deaths in children under five years of age.

Malaria is also the cause of significant adult mortality and is also the leading cause of workdays lost due to illnesses (Kioko *et al.*, 2013).

2.2 Epidemiology of malaria in Kenya

Evidence increasingly indicates that the risk and epidemiology of malaria has declined from the year 1998 to 2009 (Control *et al.*, 2010). Comparisons between previous malaria data and recently updated ones on the prevalence of malaria show a shrinking of malaria endemic zones and an expansion of the low transmission zones. An estimated 60-70 % of the Kenyan land mass has a parasite prevalence of less than 5 % where 78 % of the Kenyan populace lives (Control *et al*., 2010). With regard to the malaria risk map and the eco-epidemiology of malaria in Kenya, there are four malarial zones: Endemic lake and coastal regions; Epidemic prone highland regions; seasonal transmission risk regions and low-risk regions.

2.2.1 Endemic lake and coastal regions

These are regions of stable malaria infections having altitudes that range from 0 to 1,300 meters around the Lake Victoria and the Coastal regions. Temperature, humidity, and rainfall are the main determinants of malarial transmissions. The life cycle of the vector is short with high survival rates attributed to the suitable climatic conditions. There is intense transmission throughout the year with high entomological inoculation rates annually (Control *et al*., 2010).

2.2.2 Epidemic prone highland regions

There is seasonal transmission of malaria in the western highlands of Kenya, accompanied with considerable year to year variation. Epidemicity is experienced when the climatic conditions do favour the sustainability of minimum temperatures that are around 18 °C.

The increase in minimum temperatures during the long rains favours the breeding of vectors. This then results in the increased prevalence of malaria transmission. The population living in these epidemic regions are prone to malarial infections and fatality rates that are up to ten times greater than those experienced in regions with regular malaria incidences (Control *et al*., 2010).

2.2.3 Seasonal transmission risk regions

These regions comprise of the arid and semi-arid areas present in the northern and southern parts of the country. These regions experience short periods of intense transmission of malaria during the rainy season. Breeding sites for vectors are promoted by high temperatures and water pools created during the rainy season. Extreme climatic phenomenon such as the *El nino* leads to flooding in these regions which results in epidemic outbreaks which is accompanied by high morbidity rates. This is because of the low immune status among the populace (Control *et al*., 2010).

2.2.4 Low risk regions

These regions comprise of the central highlands of Kenya inclusive of Nairobi. The temperature of these regions is usually too low to allow for the completion of the sporogonic cycle of the malarial parasites present in the vector. Changes in the hydrological cycle and higher temperatures are associated with increase in areas favorable for breeding of malaria vectors. This leads to the introduction of malaria transmission in regions which were never affected before (Control *et al*., 2010).

2.3 Malaria vector taxonomy

Mosquitoes belong to the Phylum Arthropoda, Order: Diptera, Sub-Order: Nematocera and Family: Culicidae. There are more than 3,500 species of mosquitoes that have a worldwide

distribution. Most species of the malaria vector that are described fall in the genera *Aedes*, *Anopheles* and *Culex.* In terms of malaria transmission, the most important genera is the Anopheles. The classification of *Anopheles* species is dated back to the year 1818, when the genus *Anopheles* was put into perspective by Meigen (Harbach, 2013).

The *Anopheles* carries the greatest potential amongst the mosquitoes in terms of transmitting the *Plasmodium* parasite to the human host. Currently there are 422 known species of the anophelines in the world of which around seventy species are said to be very competent vectors in relation to the human disease. In Africa, the two main vectors responsible for *Plasmodium* transmission are the *Anopheles gambiae* and *Anopheles funestus*. *Anopheles gambiae sensu lato* comprises of seven species that are morphologically indistinguishable but are largely isolated in terms of genetic relation. Of these seven, *Anopheles gambiae s.s* and *Anopheles arabiensis* are the two most significant vectors. The most common species of malaria vectors in Kenya include; *Anopheles gambiae* Giles *S.s, Anopheles arabiensis Patton* and *Anopheles funestus Giles* (Kweka *et al.*, 2015).

2.4 Mosquito behavior

Different behavioral adaptations influence how mosquitoes transmit the *Plasmodium* parasites to the preferred host ending up in disease transmission. These behavioral adaptations include; parasite influences, mating, feeding behaviors, host preference and resting behaviors (Batool *et al.*, 2016)..

2.4.1 Parasite influences

It has been shown that malarial parasites do alter the behaviour of the mosquitoes with the effects varying upon the parasite's life stage (Cator *et al.*, 2014). Upon ingestion of the

malaria parasites from a human host, the mosquito cannot transmit the parasites immediately to a new host. This can be attributed to the fact that the parasites must first go via a number of developmental stages before it can be infectious. During the pre-infectious period, female mosquitoes become less attracted to human hosts combined by their less persistence in feeding habits (Pollitt, 2011). Infected female mosquitoes feed less frequently coupled up with shorter feeding durations as compared to uninfected female mosquitoes (Cator *et al*., 2014).

Following development in the mid-gut of the mosquito, which lasts typically ten to fourteen days in a high transmission settings, the parasites move into the hemolymph and ends up in the salivary glands (Boissière *et al*., 2012). It is at this stage that the female mosquito is able to infect a new host. The infectious female mosquitoes are reported to be more attracted to the preferred host, more persistent in their feeding attempts, do feed on more hosts per their feeding attempts and they probe more frequently. At this stage the infected female mosquitoes suffer a lot more mortality associated with feeding when compared to the uninfected females. These types of behavioural changes linked to parasitic infections appears to likely increase the transmission of malarial parasites and interpreted as an adaptive manipulation of the parasite on the host's behaviour (Lefevre *et al*., 2018).

2.4.2 Mating

In terms of the behaviours that critically characterize the life strategy of mosquitoes, mating is one behaviour that is least understood and which is mostly understudied (Takken *et al*., 2006). Mosquitoes do depend on sexual reproduction for maintaining their species. This could be a new avenue which could be undertaken in coming up with new control strategies in trying to minimize the spread of the mosquito-borne disease. Newly emerged

male mosquitoes are usually unfit for coupling with a female mosquito. This is because the external genitalia of the male mosquitoes require a morphological change. This is usually attained by the inversion of the terminalia within the first 24 hours following their emergence (Takken *et al*., 2006).

Like many species of mosquitoes, *Anopheles gambiae* mates in flight (Diabate and Tripet, 2015). The males gather on swarm markers, which are landmarks that have mating sites. There are various markers that have been found and they include; wells, trash piles, grasses, wood piles and intersection of paths. The swarms are made up of males and females that are typically entering or leaving a swarm. The mating sites do not contain any necessary resources and the female mosquitoes only visit these sites for copulation. Female *Anopheles gambiae* mosquitoes, as in other mosquitoes, only mate once in their lifetime (Diabate and Tripet, 2015). The mating system of *Anopheles gambiae* has been described as lek-like, it incorporates scramble mating competition traits. The hotspot model has been described as the best model in reflecting on the swarming of *Anopheles gambiae*. In this model the male mosquitoes initiate the mating process. The mate set themselves in places that increase the probability of them coming into contact with receptive female mosquitoes (Diabate and Tripet, 2015).

For many species of mosquitoes, the male accessory glands mature during the first few days of their adult life. This is usually a requirement before the successful transfer of sperms to the female mosquitoes. Males of numerous mosquito species do require several days to mature to adult life. Sterilisation of males at this stage, before they mate, plays a great role in reducing mosquito populations. In *Anopheles gambiae* Giles *s.s*, mating does occur within 2-day old male mosquitoes (Sawadogo *et al*., 2013).

Female mosquitoes mate before taking a blood meal, however, in several anophelines, a large section of virgins may take a blood meal before they do mate (Takken *et al*., 2006). This kind of blood meal is usually essential for developing a metabolic energy reservoir. Many female mosquitoes may carry out nectar imbibition or any other source of carbohydrates prior to their mating. This might help in accumulating energy prior to flying and finding mates. As an example of behavioral change brought about by mating, in *Aedes aegypti*, behavioral change is brought about by the transfer of matrone, which is a male hormone that makes the female refractory to successive mating episodes and also induces the host-seeking behavior. However, in *Anopheles gambiae s.s*, there are no occurrences of such behavioral physiology as the male accessory gland substances do not induce any change in the female behaviors (Takken *et al*., 2006).

2.4.3 Host preferences

Host preferences can be defined as the characteristic preferential selection of certain host species above other species. In insects, the blood-feeding trait is thought to have evolved when the plant-sucking insects accidentally bit vertebrate hosts (Takken and Verhulst, 2011). This then led to them developing a digestive physiology that enabled them to take up and metabolize nutrients rich in proteins. Host preference can be expounded as the adaptive characteristic that ultimately culminates to optimal reproduction in the vector. Some of the mosquito species do express an opportunistic feeding trait, feeding on preferable hosts indiscriminately (Takken and Verhulst, 2013). Other mosquito species are specialists, in that, they only feed on selected hosts (Chaves *et al.*, 2010). The selective

behavior exhibited by mosquitoes in turn has a great influence on the transmission of the disease.

An. gambiae s.s have been shown to enter homes in the early evening hours, feed in the late evening hours and finally exit in the early morning hours. Several studies have been carried out to compare *Anopheles gambiae* indoor versus outdoor biting behavioral adaptations in various countries and have shown between 18 % to 100 % endophagy. In Cameroon as reported by Wanji *et al*., 2003 and Reddy *et al*., 2011) it was found that only 29 % to 35 % of *Anopheles gambiae* do bite indoors, with no significant difference between the wet and dry seasons. However, in Ghana, it was indicated that *Anopheles gambiae* is more endophagic during the dry season than in the wet season (Reddy *et al*., 2011). This characteristic is dependent on seasons and location or both. In multiple regions *Anopheles gambiae* usually exhibits an extremely heightened degree of anthropophagy. Although *Anopheles gambiae* has this distinct behavioral host preference, in the absence of the human host, they usually feed on cows and dogs (Lyimoand Ferguson, 2009).

2.4.4 Resting behavior

In mosquitoes, resting behaviors can be broken down into two categories, that is, endophilicity and exophilicity (Paaijmans and Thomas, 2011). Endophilicity is the tendency of mosquitoes to rest indoors, that is, in a human dwelling (Lindh, 2007). These mosquitoes rest in these dwelling places during their period between the end of blood-feeding and the beginning of the search for a site to carry out ovipositioning. In the sub-Saharan Africa region, the key malaria vectors such as *Anopheles gambiae s.s* are considered endophilic mosquitoes spending most of their time indoors (Paaijmans and Thomas, 2011). *Anopheles funestus* is also considered to be an endophilic mosquito

spending some considerate time indoors. Indoor temperatures do strongly depend on factors such as altitude, season, and nature of the building structure, number of occupants and also the surroundings of the structure. There are several records showing exactly where the African mosquitoes do rest within an indoor setup. In Burkina Faso, for example, 95% of the *Anopheles gambiae* and *Anopheles funestus* were found to be resting within the ceiling (Paaijmans and Thomas, 2011). In Brazil, *Anopheles darlingi* 59% were collected from the ceiling, 4% resting on the floor and 37% were found resting on the wall (Paaijmans and Thomas, 2011).

Exophilicity is the resting behavioural adaptation of mosquitoes where the mosquitoes tend to spend their resting periods outside the human dwellings (Paaijmans and Thomas, 2011). *Anopheles arabiensis* is a good example of an exophilic mosquito but does exhibit some endophilic behavior. Mosquitoes that rest outside tend to seek shelter in a variety of environments. These include; in dry pots, in tree holes, undersides of bridges, under rocks, canal water pipes and crevices in brick pits. These sites are most likely shaded and do have their own microclimate. Resting behavior among mosquitoes is relatively plastic with some variations between and within the different species. With adequate knowledge on the resting behavior of mosquitoes, more interventions can be put in place in regards to the fight against malaria (Singh *et al.*, 2014).

2.5 Malaria vector control strategies

Vector control strategies should be geared towards taking advantage of the survival requirements and specific characteristics of the different malarial vectors. Most of the strategies target the period of larval development or they make use of the feeding and resting habits of the adult mosquitoes (Beier *et al*., 2008). The different control strategies

are geared towards reducing or eliminating vector reproduction, reducing the lifespan of the adult female mosquitoes, reduction of adult vector populations and also preventing the contact between the vectors the human hosts.

2.5.1 Indoor residual spraying

During the 1950s and 1960s, success in malaria control was mainly attributed to indoor spraying with DDT (Sadasivaiah *et al.*, 2007). It is during this period that malaria was eradicated from many parts of the world. When applied under the right circumstances, house spraying remains a valuable strategy in the fight against malaria. Large-scale and continued insecticide application has shown not to be sustainable as there are various operational and financial problems. Development of resistance to the insecticides by the vectors is also a technical problem that may affect the sustainability of such an approach. For indoor residual spraying to be considered effective, several conditions have to be met. These include the vector being mainly endophilic, the vector being susceptible to the insecticide and the structures to be sprayed having surfaces that can be adequately sprayed with the insecticides (Enayati and Hemingway, 2010).

If the above conditions are met then indoor residual spraying can end up reducing the life span of the vectors, reduce the population of the vectors, reduce the number of human hosts bitten and ultimately reduce the transmission of malaria. The efficacy of the insecticides used in residual spraying does vary with the type of surfaces to be sprayed. These would include, wood palm leaves, mud or thatch. Effectiveness of the residual spraying can also be attained by making sure that the equipment used are well maintained and making sure that that the spraying personnel are well trained. When selecting for an effective insecticide several factors have to be considered. These would include; safety,

residual effectiveness, vector susceptibility, costs, insecticide specifications and the management of resistance to the insecticide (Guyatt *et al.*, 2002).

2.5.2 Insecticide-treated materials

These would include; insecticide-treated mosquito nets, hammocks, curtains and papyrus mats which are utilized as barriers or repellents to minimize vector-host contacts. These barriers or repellents do then fall under personal protection measures. The efficacy of these measures are mainly determined by the feeding and resting habits of the vectors. These measures are mostly effective when the vectors are mainly endophilic and are less effective when the vectors are exophilic and also when the human hosts are not protected when the vectors are active. Treatment of bed nets with the insecticides is mainly geared at improving the protection provided by the nets by preventing the mosquitoes from biting via the holes, entering through the holes and kill any mosquitoes that do come into contact with the net (Enayati and Hemingway, 2010).

People who sleep outdoors either on hammocks or on mats can use the insecticide-treated bed nets to reduce contact with the malaria vectors. Promotion of these personal protection measures can be achieved via two approaches. The first approach is using the insecticide-treated materials as a means of protecting the community and this is promoted via community sensitization and education (Beier *et al*., 2008). This can be achieved via motivating the community individuals to purchase the bed nets. The second approach is to make the personal protection measures to be integrated into malaria control strategies that are aimed at higher levels of coverage for a given population. This can be supported fully or partially via use of public funds (Singh *et al*., 2014).

2.5.3 Repellents and domestic insecticides

In combination with insecticide-treated bed nets, repellents can be applied to the skin so as to increase the host's personal protection from the mosquitoes. Repellents are mainly utilized to prevent the vectors from biting the human host during the early evening hours before they go to bed and in the early morning hours when the human hosts are not fully protected by the bed nets. Potential intervention strategies have been brought up that encompass the evolution of the malaria vector in improving rather than reducing disease control. The combination of the limited repellents, that were initially used, together with toxic insecticides having the potential for selective effective repellence that can lead to sustainable disease control by using evolved spatial repellents (ESR's) (Lynch and Boots, 2016). Insecticides used in vector control programmes have three modes of action, namely, toxicity, contact-irritancy and spatial repellence. Contact-irritancy entails the repellence of the mosquitoes after they come into contact with the insecticide, toxicity is the ability of the insecticide to generate mortality once the vector comes into contact with while spatial repellence impacts from a distance by deflecting the vectors before they come into contact with a treated surface. Repellence that acts by preventing the malaria vector from gaining entry into treated areas is of more importance than repellence that is localised in a treated area. Spatial repellents can then be used as a tool for maintaining vector free homes which in turn is a great avenue for reducing the transmission of malaria (Lynch and Boots, 2016).

2.5.4 Larvicidal and biological control

Larval control, either biological or with chemical agents is very relevant as the main method for vector control only if there is a high proportion of the breeding sites that are within mosquito range of the community to be protected and can be easily located. The control of vector larva can be used in addition to other control measures (Thiyagarajan *et*

al., 2014). Temephos is a safe compound used for killing the malaria vectors but it also eliminates mosquito predators. Bacterial toxins, for example, those produced by *Bacillus thuringeinsis* serotype H-14 and insect growth hormones are more larvicidal to mosquito larvae (Mehrabi *et al.*, 2015). There has been the use of floating layers of expanded beads made from polystyrene that prevent mosquitoes from breeding, when used appropriately in enclosed sites such as water tanks (Yapabandara and Curtis, 2002).

2.5.5 Environmental management

Environmental management is aimed at making modifications to the environment so as to deprive the targeted vector population of its survival requirements that is, for breeding, feeding and resting (Lindsay *et al*., 2004). This in turn reduces the contact between the humans and vectors and in turn this makes the conditions for disease transmission, less conducive. Environmental management strategies include the installation and maintenance of drains, removal of stagnant water pools, vegetation management and the altering of rivers to enhance the faster flow of water (Kibret *et al.*, 2018). Environmental management in Ethiopia has brought about a reduction in the mosquito breeding grounds which has then led to a significant reduction of mosquito abundance. Environmental management practices carried out at the community level are more beneficial and pair up well with other vector control methods such as indoor residual spraying (IRS) and insecticide-treated nets (ITS) (Bekele *etal.*, 2012).

2.5.6 Population suppression: Sterile Insect Technique (SIT)

This is a species-specific, environmentally benign design geared towards insect population control. SIT is usually based on mass rearing, radiation mediated sterilization ending with release of a large number of male mosquitoes into a given target area (Wilke and Marrelli,

2012). Successful mating that may take place with the sterile male mosquitoes will result in no offspring. If enough sterile male mosquitoes are released, then the population will decline. This reduction in the vector population will tend to reduce the transmission rate of the vector-borne diseases. Genetic System Mechanisms (GSM) has also been developed to complement the Sterile Insect Technique (SIT) (Wilke and Marrelli, 2012). One of such an approach is the Release of Insect carrying a Dominant Lethal Gene (RIDL) (Wilke and Marrelli, 2012). It consists of introducing a lethal dominant gene that could be under control of a female-specific promoter, such as that of the vitellogenin gene (Wilke and Marrelli, 2012). Expression of the lethal gene could be inactivated by treatment with tetracycline, allowing a colony to be maintained. When male and female mosquito separation is required, tetracycline is removed from the system causing the death of all females (Wilke and Marrelli, 2012).

2.6 Compounds that attract mosquitoes

Mosquitoes use their highly sensitive olfactory organs to select for more attractive human hosts over the less attractive ones (Mukabana *et al.*, 2012). They do so by identifying specific chemicals present in sweat, breath and any other emanations from the skin of the hosts. These evolutionary preferences by the host benefit the mosquitoes by enabling them to identify hosts with more nutritive blood or hosts that have less defence against mosquito bites. Several compounds have been investigated in their ability to attract mosquitoes. They include; nonanal, carbon dioxide, L-lactic acid, Octenol (1-octyn-3-ol), and synthetic mosquito lures (Cilek *et al.*, 2011).

2.6.1 Nonanal

Nonanal is an alkyl aldehyde that is produced by the human body (Irish *et al.*, 2014). It is shown that nonanal is highly synergistic when combined with carbon dioxide and it results in significant increment rates of over 50% in the capture of mosquitoes.

2.6.2 Carbon dioxide

Mosquitoes have been shown to look for increased concentrations of carbon dioxide in air to locate their prey. Studies carried out by (Smallegange *et al.*, 2010) showed that carbon dioxide is an effective attractant for the *Anopheles gambiae s.s* species. Additionally, (Smallegange *et al.*, 2010) also found out that the amount of carbon dioxide affects the effectiveness of the traps where taps with increased amount of carbon dioxide caught more mosquitoes. Effectiveness of the mosquito traps peaks at 100 ml of carbon dioxide per minute.

2.6.3 L-lactic acid

Lactic acid, which is also known as milk is a chemical compound that plays various roles in several biochemical processes. Lactic acid is present among other sources human sweat. The human mouth has lactic acid bacteria where it converts sugars into lactic acid. Lactic acid has significant effects when it comes to attracting mosquitoes when combined with other substances. Combinations of carbon dioxide, ammonia and lactic acid increases the effectiveness of traps (Logita and Yewhalaw, 2016).

2.6.4 Octenol (1-octyn-3-ol)

Octenol is similar to lactic acid and is also contained in human breath and sweat. Octenol attracts mosquitoes but it differs greatly in its effectiveness for attracting different species of mosquitoes (Grant and Dickens, 2011).

2.6.5 Synthetic mosquito lures

There have been efforts by entomologists in trying to develop an alternative mosquito lure that is more attractive and effective than humans. Development of synthetic mosquito lures has been carried out by Okumu *et al.,* (2010). These lures consist of carbon dioxide, ammonia, L-lactic acid, propionic acid, butanoic acid, pentanoic acid, 3-methyl butanoic acid, heptanoic acid, octanoic acid and tetradecanoic acid (Okumu *et al.*, 2010). 2-butanone has been identified as a potential replacement for carbon dioxide in synthetic blends for mosquito lures. It induces a close-dependent activation of the cleavage product A carbon dioxide receptor in *Anopheles gambiae* mosquitoes (Mburu *et al*., 2017).

2.7 Compounds identified in skin emanations from the human foot

There have been studies that seek to characterize the compounds present in human foot skin emanations. One such study is that by (Verhulst *et al.*, 2016) did show there indeed are compounds present in the skin emanations. These included; hexadecanoic acid, tetradecanoic acid, dodecanoic acid, geranylacetone, octanoic acid, 2-ethylhexanoic acid, heptanoic acid, hexanoic acid and lactic acid (Verhulst *et al.*, 2016).

2.8 Determinants of mosquitos' attraction to human hosts

Differential attraction of mosquitoes to the human hosts is influenced by several determinants that can be classified under two broad categories, that is, extrinsic and intrinsic determinants.

2.8.1 Extrinsic determinants

Extrinsic determinants affect the attractiveness of the host to the vector. For example, when the host species preferred by the vectors are not available and when there are severe

weather conditions that prevent the vectors from venturing out from the residential set ups (Takken and Verhulst, 2013).

2.8.1.1 Odorants

Attractiveness of a host to mosquitoes is mainly influenced by olfaction which is the main way that the mosquitoes do detect the human hosts. Carbon dioxide is a general attraction mode for the mosquitoes to the hosts and it acts as a signal to the presence of the preferred host (Takken and Verhulst, 2013). This has been exhibited by *Aedes aegypti* which is attracted to the head region of the host due to carbon dioxide emanations (Majeed, 2013). Contrastingly, *Anopheles gambiae s.s* is mainly attracted to the feet of the human host for feeding, mediated by volatiles enhanced by carbon dioxide (Verhulst, Mukabana, et al., 2011). Emanation coming from the skin do have host specific cues that in turn play a role in attracting the mosquitoes to the preferred human hosts (Verhulst *et al.*, 2013).

2.8.1.2 Body heat

Humans as with other mammals do emanate heat resulting from metabolic activity. The resulting body heat then creates convention currents that do affect the spread of semiochemicals which in turn alter the attraction of the malaria vectors to the preferred human hosts (Takken *et al*., 2013).

2.8.1.3 Body mass

The size of the human host may determine the attraction of mosquitoes to them. This is because the larger the host the higher the quantity of olfactory cues produced (Takken *et al*., 2013). Production of metabolic carbon is directly associated with body size and it affects the range of attraction for the mosquitoes. Adult humans are bitten more than children who are influenced by body mass.

2.8.1.4 Relative humidity

Mosquitoes do have an accurate sense of relative humidity. They are hence able to detect it at a close range (Takken *et al*., 2011). Relative humidity when high tends to increase the odorant cues which in turn enhances the attraction of the mosquitoes to the human hosts.

2.8.1.5 Gender

The attraction of mosquitoes to humans is also influenced by the gender of the humans (Verhulst, 2010). This attraction is presumed to be influenced by odour profiles which are different for males and females (Takken *et al*., 2013). Ansell *et al*., (2002) were able to demonstrate that *Anopheles gambiae s.s* are more attracted to pregnant females than to females that are not pregnant. Differential attraction of mosquitoes to humans can be correlated with the highly variable composition of microbiota on the human skin as well as between males and females (Takken and Verhulst, 2013).

2.8.2 Intrinsic determinants

These are determinants that are from within the mosquitoes which then affect their attraction to the preferred human hosts.

2.8.2.1 Genetics

The choice of a host does not only depend on the innate host preferences for the different mosquito species, but also depends on the tendency of the mosquitoes feeding, whether indoors or outdoors and the feeding time. These behavioural characteristics maybe influenced by selection and could therefore have a genetic background (Takken and Verhulst, 2013). This was demonstrated by Gilles, (1964) in Muheza, Tanzania, who carried out a simple study that was able to reveal that host preferences were based on genetics.

2.8.2.2 Physiology

After male and female mosquitoes emerge from the pupae, they exhibit a very strong behavioural adaptation in response to nectar which is a source for their metabolic energy required for anemotactic characteristics and flight (Takken and Verhulst, 2011). Following twenty-four to forty-eight hours after emergence, the mosquitoes mate and the female mosquitoes change their diet from feeding on sugar to blood. The inherent genetic preference for a specific host is usually established at this stage. *Anopheles gambiae* is shown to have a preference for human volatiles due to its anthropophilic nature. Adaptive learning also influences the attraction of mosquitoes to the preferred host. This is via a memorized host encounter (Takken and Verhulst, 2011).

2.9 Interactions between mosquitoes and human skin microbiota

Human-associated microbiota present on the skin has been extensively studied for their roles as pathogens. This can be exemplified by *Streptococcus pyrogenes* and *Staphylococcus aureus*, which are responsible for causing skin infections and other ailments, and are the two most studied microbiota present on the human skin (Verhulst *et al*., 2010). Bacteria inhabiting the human skin do act as odour producers which plays a major role in the attraction of mosquitoes to the human hosts (Verhulst *et al*., 2010). Mosquitoes apply their olfactory senses in locating their blood hosts (Shen, 2017). Molecular techniques have opened up avenues describing how complex the microbial communities on the human skin are and shows how human do differ strongly based on their skin microbiota profiles (Muthukumar *et al*., 2008).

Human sweat lacks any odour and only acquires its characteristic smell following incubation with bacteria (Verhulst *et al*., 2010). Skin microbiota play a very important role

in the production of human odour (Maraci *et al.*, 2018). The intensity of odour produced is usually correlated to the number of microorganisms present (Verhulst, Takken, *et al*., 2010). Human skin microbiota are mostly present around skin glands where they undertake metabolism of the gland secretions. Human eccrine sweat is only attractive to *Anopheles gambiae* (Frei *et al.*, 2017). Human feet are a major production source of body odour and do contain high numbers of eccrine glands (Ibrahim *et al*., 2009). Increase in foot odour is usually associated with high microbial population densities having proteinase and lipase activity and have been reported to have higher presence of *Bacillus* species (Verhulst *et al*., 2010).

Skin odour is an important cue for host seeking mosquitoes and are derived from volatile organic compounds (VOCs) originating either directly from the skin glands or after conversion by skin bacteria (Busula *et al.*, 2017). These VOCs consist mainly of volatile fatty acids. Different species of bacteria produce different subsets of VOCs. Corynebacteria are responsible for generating volatile fatty acids that are associated with malodour (Verhulst *et al*., 2010). Upon catabolism of skin lipids into long chain fatty acids, only corynebacteria can transform them into short and medium fatty acids which in turn causes malodour (Verhulst, Takken, *et al*., 2010). Via expression in *Escherichia coli*, bacterial enzymes that are involved in production of odour by converting the non-volatile compounds to volatile compounds have been identified (Audrain *et al.*, 2015; Filipiak *et al.*, 2016). Brevibacteria metabolize short and medium chain fatty acids even further. *Staphylococcus* species, which are very abundant on the human skin, convert amino acids to highly volatile short-chain amino acids (Busula *et al.*, 2017). High throughput molecular techniques have shown high bacterial diversities and also indicate that composition of microbiota on the human skin does depend on the characteristics of the skin sites (Oh *et*

al., 2016). When grown *in vitro*, human skin bacteria produce VOCs that are attractive to *Anopheles gambiae* where some bacterial species are more attractive than others. Volatiles from *Corynebacterium minutissimum* are highly attractive whereas volatiles from *Pseudomonas aeruginosa* are poorly attractive to *Anopheles gambiae* (Busula *et al*., 2017). The preferential attraction of *Anopheles gambiae* to volatiles from *Corynebacterium minutissimum* could be explained by the fact that corynebacterium represent the most abundant microbes on the human skin (Busula *et al.*, 2017).

Anopheles gambiae are anthropophilic mosquitoes and accomplish host seeking via odour mediated anemotaxis in which essential cues are provided by human odour (Verhulst *et al*., 2009). Mosquitoes respond to volatiles which are mainly of bacterial origins (Busula *et al*., 2017). When human feet are washed using bacterial soaps, there is significant alteration of the preferred biting sites by the female mosquitoes (Selvamohan and Sandhya, 2012). Human do differ in their attractiveness to malaria vectors and this remains stable over a period of time. There may be a change in the attractiveness of humans to mosquitoes when the individuals are infected with the *Plasmodium* parasites (Verhulst *et al*., 2010). Currently, there is only limited data on microbial profiles in the feet of individuals with differential attraction to malaria vectors. Moreover, the exact link between human foot bacteria and production of chemical cues attractive to the malaria vector requires properly characterized microbial isolates. The differences that are observed in attractiveness of humans and the correlation in composition of microbiota can be explored to mitigate mosquito behaviour as a means of disease control.

2.10 Characterization and profiling of human skin bacteria

Skin bacterial communities were traditionally characterized using culture-based methods (Kong, 2011). These methods select for bacteria that do thrive in artificial growth conditions which in turn underestimates the total diversity of the bacterial communities. This can be exemplified by the genus *Staphylococcus* present on the skin, which is easily cultivated as compared to *Propionibacterium* spp or *Corynebacterium* spp. This bias of culture-based characterization was the overcame by use of sequencing techniques (SenGupta *et al*., 2016). Bacterial sequencing used the 16S ribosomal RNA. Sequencing methods have advanced from Sanger sequencing to 454 pyrosequencing to Illumina sequencing (Heather and Chain, 2016; Levy and Myers, 2016). Illumina sequencing has been regularly used since it accommodates increasing read depths and shorter read lengths (Duan *et al.*, 2014).

In Sanger sequencing, short amplicon lengths (300bp compared to 1,000bp) can enable analysis of 16S rRNA sub regions (Fox *et al.*, 2017). Up to date, the primary pipelines for analysing amplicon data are Mothur and Qiime (García-Rodríguez *et al*., 2018). These two methods employ a read clustering approach by which clustered reads are compared with curated reference databases to classify communities at the genus and species level where possible. Majority of the skin bacterial surveys have employed amplicon sequencing but over the past few years there has been major technological and analytical breakthrough that have enabled studies involving shotgun metagenomics sequencing. Shotgun metagenomics simultaneously captures all genetic materials allowing for relative kingdom abundances to be inferred (Jovel *et al*., 2016). Shotgun metagenomics sequencing has data sets that provide sufficient resolutions to differentiate species and even species within a species (Tessler *et al*., 2017). This method is particularly crucial for identification of

members belonging to the *Staphylococcus genus*, that are predominant on the human skin, which are difficult to classify to the species level using other amplicon sequencing approaches (Barnard *et al*., 2016; Zheng *et al*., 2016).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

The study was set up in Kilifi, a malaria endemic region in the coastal region of Kenya. Kilifi is 56 kilometers Northeast from Mombasa by road and having coordinates of $3^{o}38'S$ $39^{o}51'E$. The weather of Kilifi is generally warm throughout the year with temperatures above 25 °C. There are two seasons of moderate rainfall having about 800-1,000 mm of rain, the long periods of rain do start around March and last into July. The short periods of rain start around October and last till December.

The terrain for Kilifi is generally flat with sandy-loamy soils. Kilifi has mixed ethnic groups where the predominant inhabitants at around 80% are the Mijikenda while other groups include the Arab-Swahili descendants, Somalis, Barawas and the Bajunis. Apart from fishing, cashew nut production and milk processing are some of the economic activities that take place in Kilifi. As stated by Lorenzo *et al*., (2016), in Kilifi, the downward trend of malaria-related hospital admissions reached its lowest point in 2009 after which it started to slowly increase again causing concerns which in turn necessitated the study in this region to come up with effective mitigation measures.

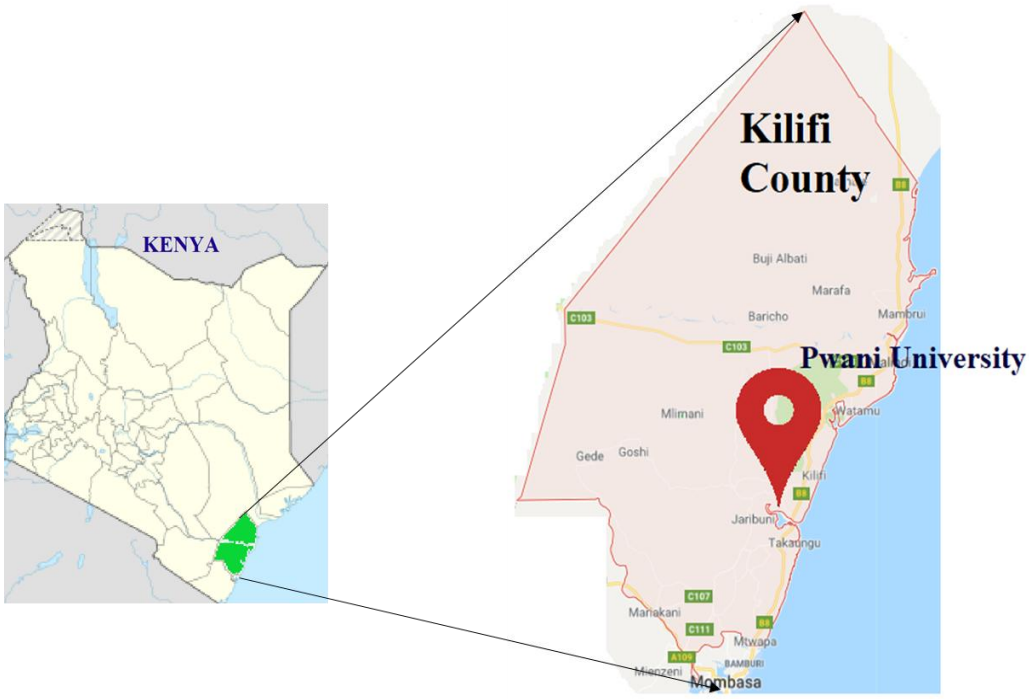

Figure 3.1: Map of Kenya showing Kilifi county (Google maps)

3.2 Study design

The study was based on a case control study design.

3.3 Study criteria

3.3.1 Inclusion criteria

The study only incorporated males between the age of 18 and 35 and who had signed the consent forms. This study also only incorporated males who did not have any form infection on their feet.

3.3.2 Exclusion criteria

The study did not include any males below the ages of 18 years and above the age of 35 years. The study also did not include any males between the age of 18 and 35 who hadn't signed the consent forms. This study did not include participants with any visible form of infections on their feet.

3.4 Sampling and sample population

A purposive sampling design was used in this study. Samples were collected from 10 male volunteers. Only male participants were selected for the study since previous studies have shown that the odour profiles of females do change during the menstrual cycle (Kuukasjärvi *et al*., 2004). The male volunteers were recruited from peri-urban areas within Kilifi. The male volunteers were sampled across the transects. A Homestead was visited along a transect road and a male meeting the inclusion criteria was recruited. To ensure randomization each subsequent recruitment was done after the fourth homestead. For males who did not meet the inclusion criteria, the next homestead was visited. This was to make sure that the volunteers partaking in this study did not have any close family affiliations. The male volunteers were then instructed not to wash their feet with soap prior to commencement of the experiment. They were also instructed not to take any alcohol during the 24-hour period. The feet of the participants were first washed with sterile distilled water. A pair of clean cotton socks were then given to the participants. Sample collection was done after 24 hours, following the male volunteers wearing the socks provided. Using sterile cotton swabs, samples were collected from both feet of the volunteer male adults, that is, from in between the toes and on the sole of the feet. The swabs were then collected in sterile containers and transported to the laboratory within two hours from collection and stored at 4 ℃ until processing of the samples.

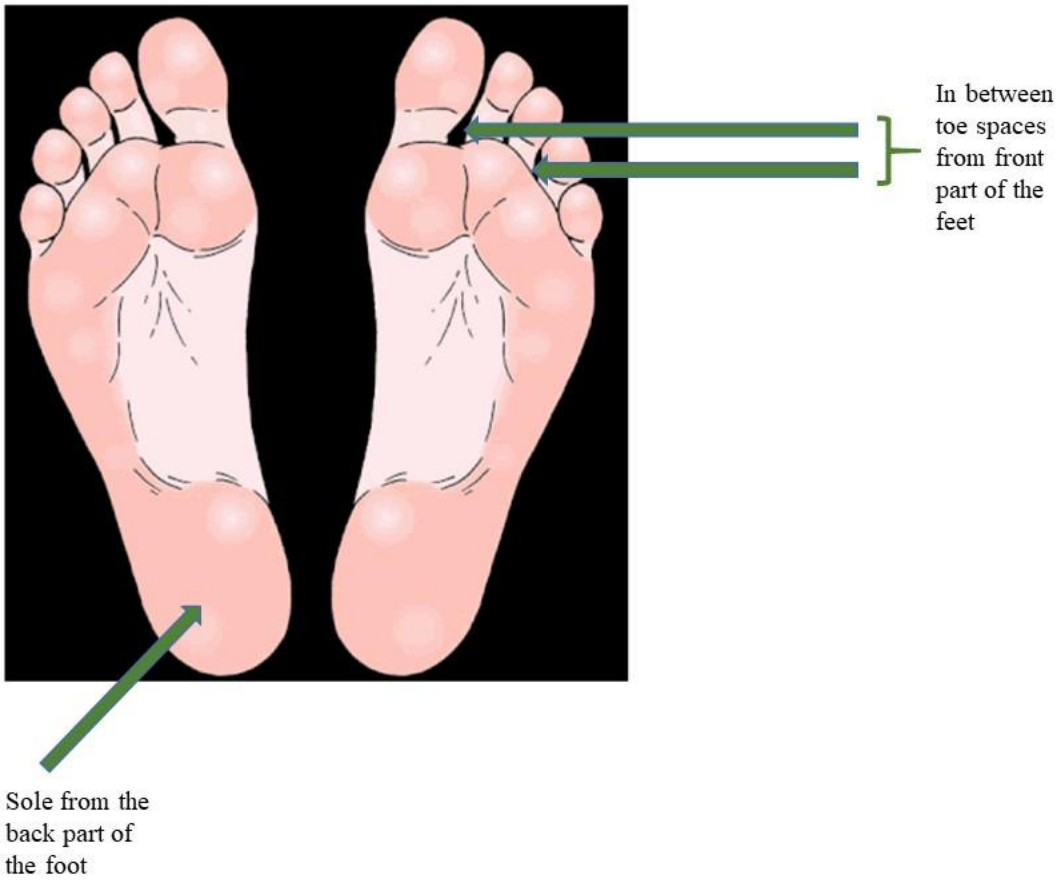

Figure 3.2:Front and back parts of the feet. Source Giphy.com

3.5 Mosquitoes for the bioassay

Anopheles gambiae s.s larvae to be used were collected from their natural breeding sites. Using droppers, the selected larvae were placed in clear zip bags for transportation. Rearing of the mosquito larvae was carried out in Pwani University's biological hut. Clean plastic plates were used for the rearing process of the larvae. 20 litres of distilled water were mixed with sodium chloride in the form of table salt to enhance the development of the larvae and subsequent pupae. The distilled water was poured half full into the plastic plates followed by picking the larvae from the zip bags using droppers and transferring them into the plates. The larvae were equally distributed in the plastic plate to avoid overcrowding. The temperature of the biological hut was regulated to 32 - 34 °C for

optimal development of the mosquito larvae. A relative humidity of 80 % was achieved by placing containers of water in two corners of the biological hut. The mosquito larvae were fed on tetramin on a daily basis, during which clean distilled water was added to clean plastic plates followed by transfer of the mosquito larvae then the feeding. The amount of tetramin used was regulated to make sure that the amount used evenly dispersed on the water for feeding. A mosquito cage was then cleaned for the purposes of holding the pupae that developed from the larvae. A clean glass beaker was placed centrally in the mosquito cage and filled half full with distilled water.

After developing into pupae, the pupae were picked using a clean dropper and transferred into the glass beaker in the mosquito cage. The transfer of the pupae was done daily with a change of distilled water till all of the developed pupae were transferred into the mosquito cage. At the start of adult mosquitoes emerging from the pupae, the temperature of the biological hut was regulated to 26 - 28 °C as the optimal temperature for development of adult mosquitoes. Following the emergence of the adult mosquitoes, the glass beaker was removed from the mosquito cage. The adult mosquitoes were fed on a sucrose solution that was soaked in cotton wool and the cotton wool placed a top of the mosquito cage. Prior to feeding the adult mosquitoes to blood, they were starved for 24 hours.

A lab-reared mouse was used for the feeding of the female mosquitoes. The mouse was entrapped in a wire mesh to make sure it wasn't mobile. The mouse was then placed on top of the cage holding the mosquitoes. This was done till mosquitoes were blood gorged. After a period of 24 hours, a half-filled beaker was placed in the cage followed by a filter paper that was placed in the beaker. The female mosquitoes laid eggs onto the filter paper which was then removed from the beaker. A plastic basin was cleaned and filled half full

with the distilled water and strips of water soaking paper placed in a circle at the midpoint of the plastic basin. The filter paper containing the laid eggs was placed centrally into the basin at which point the eggs dispersed to the edges of the basin and attached to the paper strips. The temperature of the room was then adjusted to 32 - 34 ℃. The eggs were allowed to hatch after which they were picked using droppers and transferred into plastic plates containing distilled water. The larvae were fed with tetramin on a daily basis with a change of water before each feeding cycle. The larvae were allowed to develop into pupae and the pupae were transferred into beakers placed in the mosquito cage. Following emergence of the adult mosquitoes, the temperature of the biological hut was regulated to 26 - 28 °C. Another mosquito cage was cleaned and using an aspirator, female mosquitoes were separated from the males and placed in the second cage. To differentiate between the *Anopheles gambiae s.s* and *Anopheles arabiensis patton,* which are morphologically indistinguishable, High- Resolution Melt (HRM) was carried out at KEMRI Kilifi. Thirty female mosquitoes from the first generation of the established mosquito colony were used. DNA was extracted from a single mosquito leg following the hot sodium hydroxide and Tris DNA extraction protocol (Ajamma *et al.,* 2016). Primer set CO1-AnophF\HC O2108R was used to amplify the CO1 mitochondrial gene region. Amplification was done using a single-plex PCR in a Rotor-Gene Q HRM real time PCR thermocycler (QIAGEN Hannover, Germany). Outcomes were then automatically plotted followed by visualization and analysation using the Rotor-Gene Q software v2.1 (Ajamma *et al.*, 2016). HRM curves were then checked for the differentiated mosquito species. The identified female *Anopheles gambiae s.s* were then used to establish a second generation of the mosquitoes that were then used for the differential attraction bioassays.

3.5.1 Differential attraction of female *An. gambiae s.s* to foot odour

On the first day a clean pair of socks (95% cotton) was given to each of the volunteers at intervals of one hour. The socks were then collected after a period of 24 hours. After the lapse of 24 hours, the socks were collected from the 10 volunteers and each sock placed in a different plastic zip bags (Kachraj Jivraj Limited, Kenya) for both the right leg and left leg. For the differential attraction experiment a modified olfactometer made of Plexiglas, the olfactometer was 110 cm in length, 17 cm in width and 15 cm in height. The olfactometer was divided into three chambers, right and left chambers measuring 17cm x 17cm x 15cm with a mid-chamber measuring 76cm x 17cm x 15cm. A wire mesh was used to divide the right and left chambers from the mid chambers. The three chambers had lids fitted with PVC pipes, 15 cm, to facilitate aeration. Prior to the experiment, the olfactometer was wiped both inside and outside using 70% ethanol. Using an aspirator, female mosquitoes were aspirated from the mosquito cage and placed in plastic glasses in groups of ten.

Socks obtained from the first volunteer were cut into four parts, that is, front and back part for the left and right feet. Each piece was placed in its own zip bag. The front part of the left sock was placed in the left chamber of the olfactometer while the front part of the right sock was placed in the right chamber of the olfactometer. 10 female mosquitoes were released into the mid chamber of the olfactometer and timed for 3 minutes. After the lapse of the 3 minutes, female mosquitoes that moved to both the left and right parts of the olfactometer were counted and recorded. The socks were removed followed by aspirating the female mosquitoes into the plastic glasses. The process was repeated using two different sets of female mosquitoes. The front parts of the socks were replaced with the hind parts of the socks and subjected to the same process with the number of female

mosquitoes attracted to the left and right counted and recorded. The entire set up was repeated for all the ten volunteers. A second pair of socks was given to the ten volunteers and the differential attraction experiment run again.

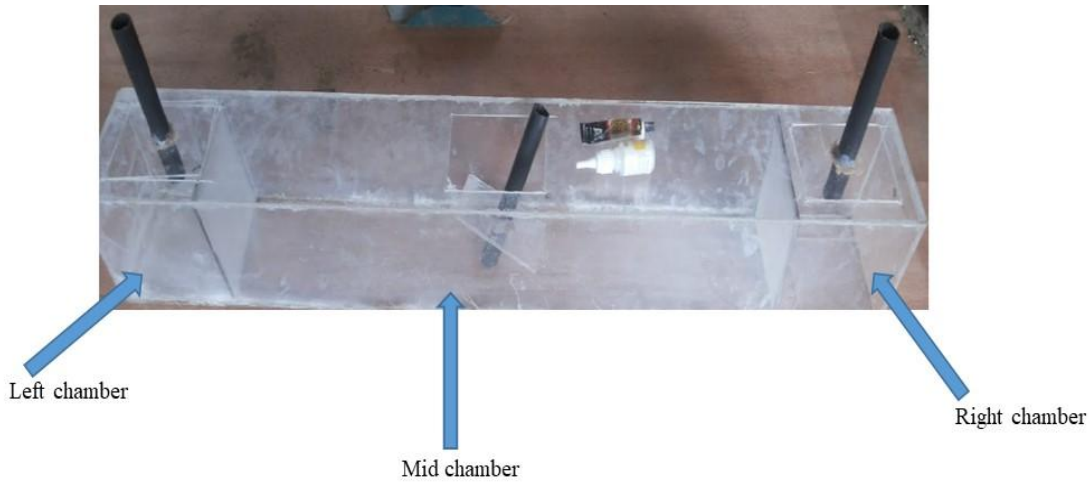

Figure 3.3: Modified olfactometer

3.6 Isolation of bacteria

Isolation of bacteria was done by adding 0.05 mg/l of fluconazole to sterile nutrient agar medium. This was for inhibiting the growth of moulds and yeast. The fluconazole and nutrient agar medium (HI Media, India) were mixed uniformly and 15 ml of the mixture was poured into 20 culture plates (Greiner bio-one, Austria, 90 x 15mm). The medium was then allowed to cool and solidify. Swabs from the 10 adult males, both from the front part and back part, were then inoculated onto the culture medium. The culture plates were then incubated in a biochemical incubator for 24 hours at 37 °C (Li *et al*., 2011).

3.6.1 Purification of isolates

In regards to the different morphology of the colonies, the single colonies of the uniquely appearing bacteria were picked and inoculated onto fresh nutrient agar (HI Media, India) plates. Purified colonies were obtained by repeated streaking each single colony obtained

onto fresh agar plates. Resulting morphologies were then recorded on the basis of their classification (Li *et al*., 2011).

3.8 Morphological characterization

Morphological characterization of each of the bacterial isolates was carried out using the basic nutrient agar media. This included colour, shape, margin, elevation and other distinct characteristics observed per each colony

3.9 Biochemical assays

Gram stain tests were carried out on each of the bacterial isolate to help design the specific biochemical tests for the bacterial isolates in accordance to the outcomes (Zulfiqar *et al*., 2012). For Gram-positive bacilli the following biochemical tests were carried out: starch hydrolysis, catalase test, citrate test, growth in 6.5% sodium chloride, nitrate reduction, arabinose fermentation and TSI. For Gram-positive cocci the following biochemical tests were carried out: catalase test, mannitol salt fermentation, blood hemolysis, glucose fermentation and TSI. For the Gram-negative bacilli the following biochemical tests were carried out; oxidase test, glucose fermentation, motility, nitrate reduction and TSI.

3.9.1 Starch hydrolysis

A sterile wire loop was used to pick a few colonies from pure culture (24 hours old) for a bacterial isolate that was a Gram-positive bacillus and streaked onto a starch plate. This was repeated for each of the bacterial isolate that was for Gram-positive bacilli. The plates were incubated at 37 ℃ for 48 hours. After the incubation period 3 drops of 10 % iodine solution directly onto the edges of the colonies followed by observation after 10 minutes. Positive result was indicated by presence of the blue-black colour while a negative result was indicated by no blue-black coloration (Hemraj *et al.*, 2013).

3.9.2 Catalase test

A loop-full of colonies from a pure culture (24 hours old) was picked and spread on a clean glass slide. Two drops of 3 % hydrogen peroxide were added onto the spread colonies followed by observation after 10 seconds. This was repeated for each of the bacterial isolates that were Gram-positive bacilli. A positive result was indicated by presence of bubbles while no bubbles indicated a negative result (Hemraj *et al.*, 2013).

3.9.3 Citrate utilization test

24-hour old isolates were used. A sterile wire loop was used to streak the fresh inoculum over the slant of Simmon's citrate agar in a bijou bottle. This was repeated for the other Gram-positive bacilli followed by incubation at 37 ℃ for 24 hours. Positive result was indicated by an immerse blue colour of the medium while a negative result was indicated by the medium retaining the deep forest green colour (Hemraj *et al.*, 2013).

3.9.4 Sodium chloride (6.5 %) tolerance test

Growth on nutrient agar containing 6.5 % sodium chloride was done by adding 6 % more sodium chloride to the nutrient agar during media preparation. 2 colonies of the test bacterial isolates, 24-hour old, were inoculated onto the nutrient agar plates. The plates were incubated for 24 hours at 37 °C. positive result for this test was indicated by bacterial growth on the medium while a negative result was indicated by no microbial growth on the medium.

3.9.5 Nitrate reduction test

A sterile wire loop was used to pick a heavy inoculum from a 24-hour old pure culture and inoculated it onto a nitrate agar slant. The slants were incubated at 37 °C for 48 hours. 3 drops of Reagent A (0.8 % sulfanilic acid in 30 % acetic acid) were added followed by

addition of 3 drops of Reagent B (0.6 % N, N-dimethyl-α-naphthylamine in 30 % acetic acid). A positive result was indicated by a red colour after addition of Reagent A and B while a negative result was indicated by no colour after addition of Reagent A and B with a Red colour after addition of zinc powder (Mishra *et al.*, 2013).

3.9.6 Arabinose sugar fermentation

A sterile needle was used to pick an inoculum for 24-hour old bacterial culture and stab a nesla tube with arabinose medium within ¼ inch of the bottom. The tubes were incubated at 37 °C for 48 hours. A positive result was indicated by the culture changing to yellow while a negative result was indicated by a magenta or hot pink colour of the culture medium.

3.9. 7 Triple sugar iron tests

A cool, sterile inoculating needle was used to pick an isolated colony from a 24-hour old pure bacterial isolate followed by stabbing into the medium, first in the butt of the tube and then streaking back and forth along the surface of the slant. The tubes were incubated with the caps loosened, at 35 °C for 24 hours and each tube was examined for carbohydrate fermentation, hydrogen sulphide production and gas production. A positive result for large amount of acid production was indicated by a yellow colour of the butt and slant with possible generation of gas. For minimal lactose fermentation with small amount of glucose fermented, the butt will be yellow while the slant will be pink. Where neither lactose nor glucose are fermented, both the slant and butt will be pink (Mishra *et al.*, 2013).

3.9.8 Mannitol salt fermentation test

Using a sterile wire loop a light line of inoculum from the pure culture of the test organisms was streaked on a MSA plate. The plates were then incubated at 37 °C for 24 hours. Positive result was indicated by microbial growth followed by colour change from pink to yellow while a negative result was indicated by microbial growth but media doesn't change colour (Shields and Tsang, 2006).

3.9.9 SIM test

A sterile inoculating needle was used to pick fresh colonies of the test bacterial isolates and stabbed into SIM media within the bottom half of the nesla tubes. The tubes were incubated for 24 hours at 37 °C after which the growth patterns along the streak lines were checked for to determine motility. H_2s production was also checked for. Detection of indole production was done by adding3 drops of Kovac's reagent and the observed changes recorded. A positive result for H_2S was denoted by blackening of the medium while a negative H_2S was indicated by absence of the blackening. A positive result for motility was indicated by a diffuse zone of growth along the streak line while a negative result was indicated by confined growth along the streak line. A positive result for indole production was indicated by red colour after addition of Kovac's reagent while a negative result was indicated by a yellow colour after addition of Kovac's reagent (Hemraj *et al.*, 2013).

3.9.10 Methyl red/ Vogues proskauer test

MR-VP broth was prepared and dispensed into tubes and inoculated with a fresh inoculum, 24-hour old, of the test organisms. The test tubes were incubated at 37 °C for 48 hours. Two ml of the suspension was transferred into sterile bottles, one for the Mr test and one

for the VP test. This was done for all the test organisms. For the MR test, two drops of the Mr reagent were added followed by observation and recording of the results. For the VP test, two drops of 10% KOH were added followed by gentle shaking of the tubes. The tubes were held at room temperature and any changes observed were recorded. Positive results for Methyl red test were indicated by a red colour after addition of methyl red reagent while a negative result was indicated by a yellow colour of the medium. A positive result for Vogues Proskauer is indicated by a red colour within 15 minutes of addition of the reagents while a negative result is indicated by no production of a red colour after addition of the reagents for more than 15 minutes (Hemraj *et al.,* 2013).

3.9.11 Indole test

Tryptone broth was prepared and dispensed into tubes followed by sterilization. Light inoculums of the test organisms, 24-hour old, were inoculated into the tubes. The tubes were incubated for 48 hours at 37 °C. After the incubation period, 1 ml of Kovac's reagent was added to all the tubes followed by gentle shaking. The tubes were allowed to stand for 2 minutes. The tubes were then observed and the changes recorded. Positive test was indicated by formation of a pink colour after addition of Kovac's reagent while a negative result was indicated by no colour formation after addition of the reagent (Hemraj *et al.,* 2013).

3.9.12 Oxidase test

The test organisms were sub cultured and after 48 hours, a sterile swab was used to pick a generous amount of each of the test organism. A dry clean slide card was saturated with Kovac's oxidase reagent (1% N, N, N', N', tetra-methyl-p-phenylene diamine dihydrochloride). Each swab was rubbed on a separate slide card and the immediate

reaction and changes were recorded. A positive result was indicated by formation of a dark purple colour within ten seconds while a negative result was indicated by no colour formation (Hemraj *et al.,* 2013).

3.9.13 Glucose fermentation

A sterile inoculating wire was used to pick a single colony of the 24-hour old test organism and used to stab a nesla tube with glucose medium, with phenol red indicator within ¼ inch of the bottom of the tube. The tubes were then incubated for 48 hours at 37 °C. Positive result was indicated by production of acid which turns the medium to yellow while a negative result was indicated by the medium retaining the red colour (Hemraj *et al.,* 2013).

3.9.14 Blood hemolysis

Blood agar was prepared and supplemented with 5% sheep's blood. 24-hour old cultures were used. A sterile wire loop was used to pick an inoculum from the test organisms and inoculated onto the blood agar plates. The plates were incubated at 37 °C. for 48 hours after which the observations were recorded. B-hemolysis was indicated by clear zones around the bacterial growth, α-hemolysis was indicated by greenish colour around the bacterial growth while γ-hemolysis was indicated by lack of clearing around the bacterial growth.

3.9.15 Urease test

Slants of urease medium were prepared in bijou bottles. Using a sterile wire loop, an inoculum was picked from the test organisms and inoculated onto the slant surface. The slants were then incubated at 37 °C for 18 hours with overnight incubation. The slants were then observed and changes recorded. A positive result for the experiment was

indicated by changing of the colour from orange to pink while a negative result was indicated by the medium retaining the orange colour (Hemraj *et al.,* 2013).

3.10 Molecular characterization of human feet derived bacterial isolates

3.10.1 DNA extraction

Prior to DNA extraction, the nineteen grouped bacterial isolates were cultured on nutrient agar for 24 hours at 37 °C. Pure single colonies of the bacterial isolates were picked with a sterile wire loop and placed in Eppendorf tubes with 100 µl normal saline for washing the cells, followed by vortexing the tubes. The normal saline was drained off followed by addition of three hundred microliters of the lysis buffer (0.12 M Na_2HPO_4 [pH 8.0], 1% sodium dodecyl sulphate [SDS], 0.1 mg of proteinase K per ml) (Biolab) to the washed cells. The tubes were vortexed for 30 seconds followed by incubation at 65 °C for 20 minutes. Three hundred microliters of chloroform-isoamyl alcohol (Biolab) was added and the mixtures were inverted several times until a milky solution formed. The samples were then centrifuged at 13,000 xg for ten minutes and the supernatant was transferred into sterile 1.5 ml Eppendorf tube. DNA was precipitated by adding 300 µl of 100 % ethanol (HI Media, India) followed by cold incubation at -20 °C for 30 minutes. The samples were centrifuged at 13,000 xg for 10 minutes and the supernatant discarded to remain with the DNA pellet. The DNA pellet was air dried at room temperature and then re-dissolved in 30 µl deionized water. Detection of the extracted total genomic DNA from the bacterial isolates was done using gel electrophoresis. 3 µl of the DNA sample was loaded on 1 % agarose gel containing ethidium bromide in 1X Tris-acetate-EDTA (TAE) buffer (Biolab) and was run at 70V for 45 minutes (Fazhan *et al.*, 2016).

3.10.2 PCR amplification

The 16S rRNA gene amplification was carried out in accordance with the PCR protocol (Haas *et al*., 2011). Oligonucleotide primer pairs that target bacterial small sub unit (SSU) 16S rRNA genes were used. The primers used were 27F (5-'AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACG ACTT-3') (Li *et al*., 2011). The PCR was conducted out in a 25-µL reaction mixture containing; 1 µl template DNA, 12.5 µl dNTPs, 1X Taq DNA polymerase buffer (Mg^{2+} plus), 0.25 µl of 10 µM of each primer and 11 µl PCR water. The run conditions for the PCR amplification (35 cycles) were carried out under the following conditions; initial denaturation step at 94 °C for 2 minutes, followed by denaturation at 94 °C for 45 s, annealing step at 62°C for 45 s, and extension at 72 °C for 2 min, with a final extension at 72 °C for 5 min and a final hold at 4 °C. Final PCR products aliquots were checked by gel electrophoresis on a 1 % agarose gel and visualized after staining with ethidium bromide (Tewari *et al.*, 2011).

3.10.3 Sequencing

The amplicons were first taken through sequence PCR. The sequence PCR was carried out in a 25 µl reaction mixture that contained; 3 µl template DNA, 1.5 µl 5X buffer, 1.5 µl forward primer, 1.5 µl reverse primer, 2 µl Big Dye and 10.5 µl distilled water. The PCR amplification was run for 25 cycles under the following conditions; initial denaturation at 96 °C for 5 minutes, denaturation at 96 °C for 10 seconds, annealing at 62 °C for 45 seconds and extenstion at 60 °C for 4 minutes. The PCR products were then purified using Qiaquick reagents (Qiagen) following the manufacturer's instructions. The PCR products were then cycle sequenced using Big Dye V3.1 reagents following the manufacturers protocol (Platt *et al.*, 2007). The sequencing products were then purified using the

CleanSeq Sequence Purification System and automated sequencing done by capillary electrophoresis on an ABI3700. The sequences were finally aligned and then examined by use of visual inspection of the electropherogram by using the Sequencher software (Tsiatis *et al*., 2010).

3.10.4 Phylogenetic data analysis

Bacterial 16S rRNA gene-based sequence data, that is, the forward and reverse sequences were cleaned and edited using Finch-Tv software version 1.4.0 (Yang and Hong, 2013). The forward and reverse sequences were joined using CLC Genomics Workbench 10 software in which all the sequences for the 19 bacterial isolates had over 1,400 bases. Blasting of the bacterial isolate sequences was done using NCBI BLAST site (https://blast.ncbi.nlm.nih .gov/Blast.cgi). The reference strains having the highest max score, total score, query cover, and identity were considered as the closest matches and were selected alongside their accession numbers.

Fasta formats of the bacterial isolate sequences and the reference strain sequences were aligned using MEGA7 software using the ClustalW option. For the construction of the phylogenetic tree, *Methanoculleus thermophillus* Accession number AB065297 was used as the outgroup. The phylogenetic tree was constructed using MEGA7 software with the following algorithms: Bootstrap method for test of phylogeny with 1,000 bootstrap replications: Nucleotide substitution type; UPGMA; Homogeneous pattern among lineages; Complete deletion of gaps and a Neighbour-joining statistical method. The phylogenies were inferred by comparing the bacterial sequences to the main lines of descent within the bacterial phyla of more than 98 % threshold to assign sequences to the

same operational taxonomic units (OTUs). The bacterial isolates were categorized into either species or genus based on the 98 % threshold match *(Tsiatis et al*., 2010).

3.11 Antimicrobial susceptibility testing

3.11.1 Antibiotic susceptibility testing

Antibiotic susceptibility testing was conducted using the BAUER-KIRBY agar diffusion method (Salem *et al*., 2014), on Mueller-Hinton agar medium. The test was carried out by applying 1×10^8 CFU/ml of the nineteen bacterial isolates onto the surface of the Mueller-Hinton agar plates. Six commonly used antibiotics; Gentamycin 10 mcg, Penicillin 10 mcg, Ampicillin 10 mcg, Tetracycline 30 mcg, Streptomycin 10 mcg and Ciprofloxacin 30 mcg were used. Escherichia coli ATCC 8739 was used as a control for the experiment. The antibiotic discs were then aseptically placed on the inoculated Mueller Hinton plates followed by incubation at 37 °C for 24 hours. Inhibition of bacterial growth by the antibiotics was indictated by measuring the occurring zones of inhibition to the nearest millimeter using a ruler (Haimour *et al.*, 2013).

3.11.2 Antiseptic susceptibility testing

Three commercially available antiseptics were also tested for their efficacy against the nineteen bacterial isolates using the Kirby-Bauer disc diffusion method (Salem *et al*., 2014). The antiseptics were designated the codes A, B and C. Eight dilutions for each of the antiseptic were prepared by serially diluting the antiseptics using 1 ml sterile distilled water. Discs, 6 mm in width, were prepared from filter papers and were then sterilized. The discs were then adsorbed with 0.1 ml of each of the dilutions for the three antiseptics. 1×10^8 CFU/ml of the nineteen bacterial isolates were inoculated onto the surface of the Mueller-Hinton agar plates. The discs were air dried and aseptically placed on the surface

of the agar media. The plates were then incubated at 37 °C for 24 hours. Inhibition of microbial growth was dictated by measuring the occurring zones of inhibition to the nearest millimeter using a ruler (Haimour *et al.*, 2013).

3.12 Data analyses

Effects of differential attraction of female *Anopheles gambiae sensu stricto* to human feet odour was analysed using three-way ANOVA. Effects of the antibiotics were analysed using one-way ANOVA through the measurement of their average inhibition zones while the effects of the three antiseptics on the bacterial isolates were analyzed using two-way ANOVA via measurement of their average inhibition zones separately. The levels of significance were compared to the respective positive controls at P value of < 0.05. Means of the inhibition zones were separated using Tukey's HSD test (Wong *et al.*, 2010). Data analyses was carried out on SAS software Ver.9.4.

3.13 Ethical considerations

Procedures for the study were followed in accordance with the standard ethics of the Helsinki Declaration and were approved by the Ethical Review Committees of Kenyatta University. All the sample collection activities were carefully explained followed by obtaining oral consents from the relevant authorities. Informed consents for the participation of adult males were obtained by engaging the volunteer males. The consent forms were drafted in English. For participants who were not well conversant with the English language, the consent forms were elaborated in both Kiswahili language and the native language where there was need using a local interpreter. Adult males who opted not to undertake the study were allowed to do so. Participant confidentiality was assured by assigning each male volunteer a numerical code to be used in part of their real names.

These numerical codes were used in the entire research period and during the dissemination of results. Also, any information gathered was used only for the purpose of this study and not to any other study.

CHAPTER FOUR

RESULTS

4.1 Differential attraction of female *Anopheles gambiae s.s* to foot odour

The differential attraction of female *An. gambiae s.s* to ten adult male participants were evaluated using second generation mosquitoes from the established mosquito colony (Plate 4.1). The effect of differential attraction was checked by counting the number of mosquitoes attracted to the socks specimens from the participants. In the evaluation, there was a significant difference in the differential attraction of the female adult mosquitoes to the male participants ($p=0.0001$). There was no significant difference between the attraction of the female *Anopheles gambiae s.s* between the left foot and right foot for the ten participants ($p=0.274$). Participant two had the highest mean number of mosquitoes attracted at 3.083±0.262. Participant six had the lowest mean number of female adult *An. gambiae s.s* standing at 1.333±0.098 (Table 1). The control for the experiment had a mean number of mosquitoes attracted being 0.250±0.090. There was no significant difference in

the mean number of mosquitoes attracted to the right and left feet of the ten male participants (p=0.2741). There was no significant difference in the interaction between the participant and leg (p=0.176), leg and site (p=0.511) and participant, leg, and site (p=0.244). The mean number of mosquitoes attracted to the left feet was 2.106±0.113 while the mean number of mosquitoes attracted to the right feet was 2.030±0.111(Table 1).

Table 4.1: Attraction of female *An. gambiae s.s* mosquitoes to feet odor

Participant	**Mean no. of mosquitoes attracted to the feet**
1	2.25±0.24 b c
2	3.08 ±0.26 a
3	2.42 ±0.26 b c
4	2.58 ±0.28 a b
5	1.92 ±0.20 c d
6	1.33 ±0.10 e
7	2.75±0.22 a b
8	1.42 ±0.10d e
9	2.00 ±0.21 c
10	2.75±0.27a b
Control	0.25 ±0.09f
Leg	
Right	2.03±0.11a
Left	2.11±0.11a
P- values	

Participant	0.001
Leg	0.2741
Participant x leg	0.1755
Leg x site	0.5114
Participant x leg x site	0.2436

Values are mean no. of mosquitoes attracted to the feet plus standard error of the means. Values within the same column without common letters differ significantly according to Tukey's HSD $p<0.05$. The control for the experiment was clean unworn socks.

Plate 4.1: Differential attraction experiment showing: A, modified olfactometer used for the experiment; B, collecting water with mosquito larvae; C, transferred mosquito larvae in a zip bag. D, mosquito pupa. E, mosquito larvae in distilled water with sodium chloride. E, plastic plates for rearing mosquito larvae with plastic cups and cage for holding adult mosquitoes.

4.1.1 Effect of site

There was a significant difference in the attraction of female *An. gambiae s.s* mosquitoes to odour from the front part of the feet (2.87±0.11) as compared to the back part of the feet

(1.27 ± 0.058). For instance, Participant 1 had a high mean number of mosquitoes attracted to the front part of the feet at 3.33 ± 0.21(Fig 4.1) whereas the back part of the feet had a mean number of mosquitoes attracted at 1.67 ± 0.17. Participant 2, who had the highest mean number of mosquitoes attracted as an individual among the ten participants also had the highest mean number of mosquitoes attracted to the front part of the feet at 4.17 ± 0.31 while the back part of the feet had a mean number of mosquitoes attracted at 2.00 ± 0.26. Participant 6, who had the lowest mean number of mosquitoes attracted among the ten participants, had a mean number of mosquitoes attracted to the front part of the feet at 1.67 ± 0.21 and a mean number of mosquitoes attracted to the back part of the feet at 1.00 ± 0.00. Participant 8 had a mean number of mosquitoes attracted to the front part of the feet at 1.83 ± 0.17 while the back part of the feet had a mean number of mosquitoes attracted at 1.00 ± 0.00 which is similar to Participant 6 (Fig 4.1).

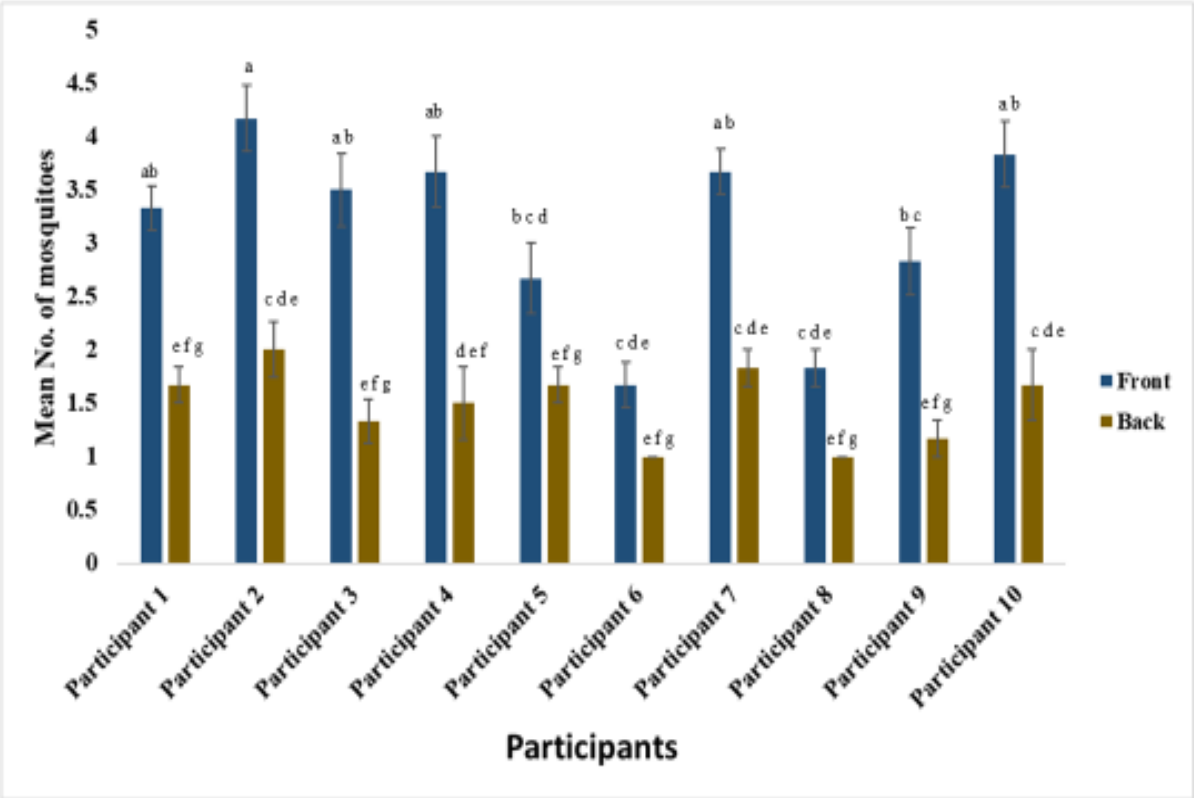

Figure 4.1: Mean number of mosquitoes attracted to the participants' feet sites, that is, the front (in between the toes) and the back (the sole region of the feet). Bars followed by the same letter are not significantly different using Tukey's HSD test at P≤0.05, while the error bars represent the standard error of the mean.

4.2 Isolation and morphological characterization of feet derived bacterial isolates

A total of 154 pure bacterial isolates were obtained from the human feet samples from the ten volunteer. Based on the morphological characteristics, a total of 19 morphological groups were obtained and were confirmed by biochemical characteristics. Purified isolates had varied cultural characteristics (Plate 4.3). Majority of the isolates (14 isolates) were majorly found in the front part of the feet (Table 4.2). Some of the isolates from the front part of the feet were white I colour with circular colonies that ranged in size from 1-6mm and were mainly opaque. Others had orange colonies with raised elevation and size ranging from 2-6mm in size. One isolate was pink in colour with a size of 3mm and was opaque

(Table 4.2). The back part of the feet had less number of bacterial isolates (5 isolates) as compared to the front part of the feet (Table 4.2). Majority of the isolates were white to watery white in colour ranging in size from 1-7mm with raised elevations. One isolate was pink in colour, had circular colonies and has a size of 2mm (Table 4.2).

Table 4.2: Morphological characteristics of bacterial isolates obtained from the feet of participants with differential attraction to *An. gambiae s.s*.

ISOLATE	PTI	PTII	PTV	PTVI	PTVI	PTVII	PTIX	PTX	PTXI	PTXI	PTXII	PTXIV	PTXV	PTXVI	PSI	PSII	PSII	PSIV	PSV
Color	W	CW	W	Y	W	W	W	W	W	P	O	LO	W	YO	P	WW	W	W	WW
Shape	C	C	IR	C	IR	C	C	IR	IR	IR	C	IR	C	IR	C	C	C	IR	IR
Size(mm)	1	2	6	2	6	3	2	5	5	3	2	4	2	6	2	1	2	4	7
Margin	S	S	NS	S	NS	S	S	S	NS	NS	S	NS	S	NS	S	S	S	NS	RH
Elevation	F	F	R	R	R	R	F	R	R	R	R	F	F	R	F	R	R	R	R
Transparency	O	O	O	O	O	T	O	O	O	O	O	T	O	O	O	O	T	O	T
Texture	FR	FR	FR	FR	M	FR	FR	FR	M	FR	FR	FR	FR	FR	FR	FR	FR	FR	M

Key: W, white; CW, cream white; Ww, watery white; Y, yellow; P, pink; O, orange; Lo, light orange; Wy, watery yellow; Yo, yellow-orange; C, circular; Ir, irregular; S, smooth; Ns, not smooth; F, flat; R, raised; RH, rhizoidal; O, opaque; T, translucent; Fr, firm; M, mucoid; SW, swarming. PTI-PTXVI bacterial isolates isolated majorly from in between the toes. PSI-PSV bacterial isolates isolated majorly from the sole.

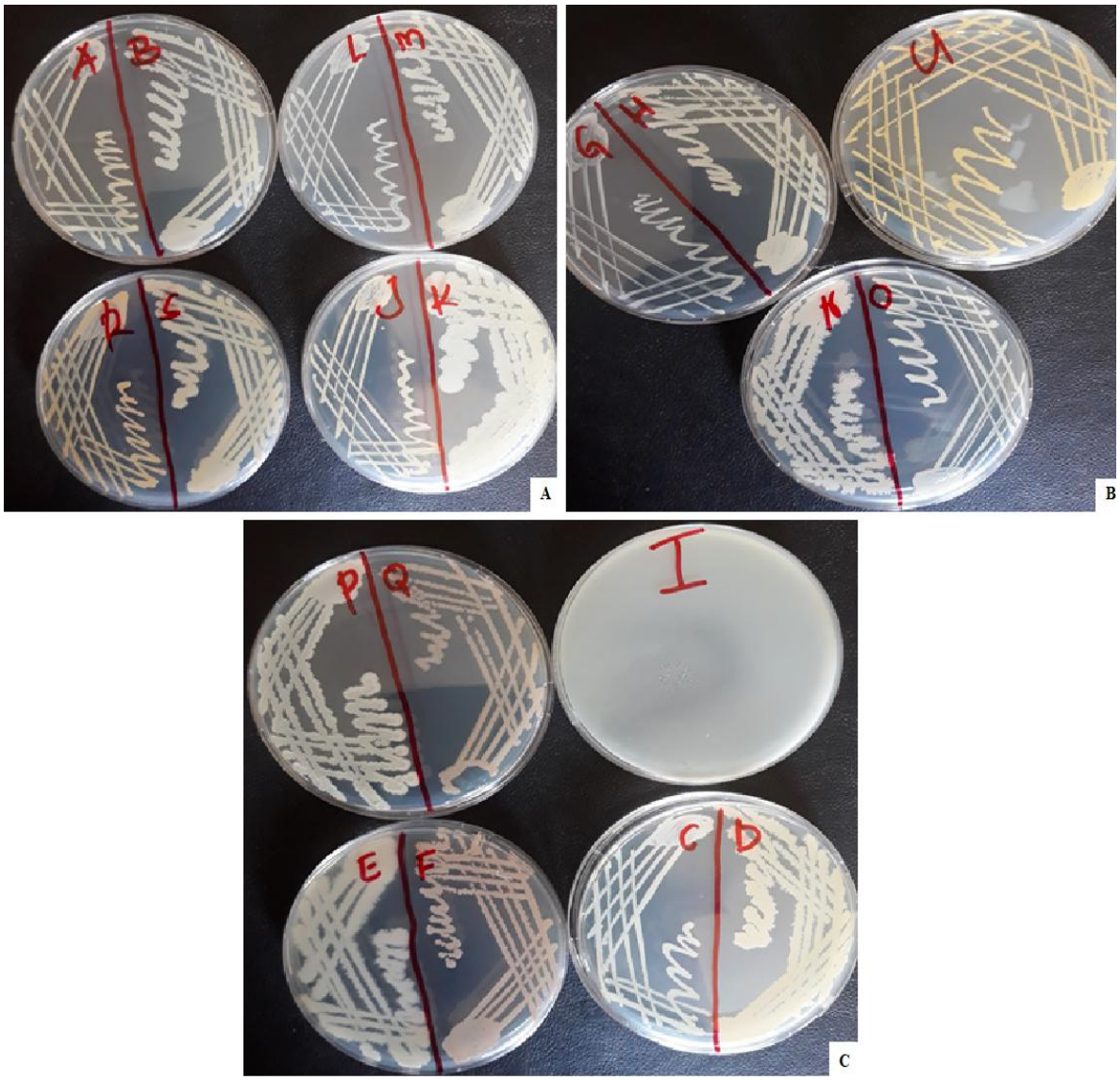

Plate 4.2: A – U showing pure bacterial isolates grown on nutrient agar: A-PTI, C-PTIII, E-PTV, F-PSI, G-PSII, H-PSIII, I-PSIV, J-PTVI, K-PTVII, L-PTVIII, M-PTIX, N-PSV, O-PTX, P-PTXI, Q-PTXII, R-PTXIII, S-PTXIX and U-PTXVI.

4.3 Biochemical characteristics of the bacterial isolates

Gram stain reactions for the nineteen bacterial isolates had varied results where eighteen isolates were Gram positive and one isolate was Gram negative. Eleven isolates were cocci in shape while eight were bacillus in shape (Table 4.3). This study revealed that of the nineteen isolates, only one isolate, PTXV, was able to produce hydrogen sulphide (H_2S) in both the TSI and SIM tests. Majority of the isolates were able to ferment both arabinose

and glucose as evidenced by the colour change from pink to yellow (Table 4.3). For the indole test only one isolate, PSIV, was positive while the rest were negative. In the motility test, seven bacterial isolates were positive exhibited by growth along the streak lines (Plate 4.4). Two isolates, PTIX and PSI were slightly positive to the motility test (Table 4.3)

For the urease test, there were varied outcomes where eight isolates were positive as exhibited by their ability to hydrolyse urea and the medium turning to pink colour from yellow (Plate 4.4). Eleven bacterial isolates were negative for urease test (Table 4.3). Eight bacterial isolates were positive for the citrate utilization test evidenced by the colour change from green to blue (Plate 4.4) while the remaining eleven isolates were negative to citrate utilization test. The bacterial isolates were then subjected the MR-VP test where eleven isolates were positive for the methyl red (MR) test with the colour changing from yellow to red (Plate 4.4). Seven isolates were positive for vogues-proskauer test (Table 4.3).

For the TSI test, isolate PTXV had a pink slant and a yellow butt with production of hydrogen sulphide (H_2S), black colour in the medium. Isolate PTVI had acid production in the slant and butt with colour change from pink to yellow (Plate 4.4). In the nitrate reduction test, seven isolates were positive, one had a variable result while eleven isolates were negative (Table 4.3).

Table 4.3: Cell and biochemical characteristics of the feet derived bacterial isolates

Isolates	I	H	M	CI	MR	VP	NR	A	G	O	S	B	H	G	CA	U	MS	A	G	SH	6.5	GS	Cell shape	Possible identity
PTI	-	-	-	+	-	-	-	+	-	-	P	Y	-	-	+	-	-	+	-	-	+	+	cocci	*Staphylococcus* spp
PTIII	-	-	+	+	+	-	+	+	-	-	P	Y	-	-	+	+	+	-	-	-	+	+	cocci	*Staphylococcus* spp
PTV	-	-	+	+	-	+	-	-	-	+	P	Y	-	-	+	+	-	+	-	+	-	+	rod- shaped	*Bacillus* spp
PTVI	-	-	-	-	-	-	+	-	-	-	P	Y	-	-	+	+	-	+	-	-	-	+	cocci	*Staphylococcus* spp
PTVII	-	-	-	+	+	-	+	+	-	+	P	Y	-	-	+	+	-	-	-	+	+	+	rod-shaped	*Bacillus* spp
PTVIII	-	-	+	-	-	+	-	-	-	-	P	Y	-	-	+	-	+	+	-	-	+	+	cocci	*Staphylococcus* spp
PTIX	-	-	S+	-	+	+	-	+	-	+	P	Y	-	-	+	+	-	+	-	-	+	+	rod- shaped	*Bacillus* spp
PTX	-	-	-	-	-	-	+	-	-	-	P	Y	-	-	+	+	+	+	-	-	-	+	cocci	*Staphylococcus* spp
PTXI	-	-	-	-	+	+	-	+	-	+	P	Y	-	-	+	-	-	+	-	-	+	+	rod- shaped	*Bacillus* spp
PTXII	-	-	-	-	+	-	-	+	-	-	P	Y	-	-	+	-	-	-	-	+	-	+	cocci	*Staphylococcus* spp
PTXIII	-	-	-	-	-	-	+	+	-	+	P	Y	-	-	+	-	+	+	-	-	-	+	cocci	*Staphylococcus* spp
PTXIV	-	-	+	+	-	-	-	+	-	-	P	Y	-	-	+	-	-	+	-	+	+	+	rod- shaped	*Bacillus* spp
PTXV	-	+	+	-	+	-	-	+	-	-	P	Y	+	-	+	-	-	+	-	-	-	-	rod- shaped	*Proteus* spp
PTXVI	-	-	-	-	-	-	V	+	-	-	P	Y	-	-	+	-	-	+	-	-	-	+	cocci	*Staphylococcus* spp
PSI	-	-	S+	+	+	-	-	+	-	+	P	P	-	-	+	-	-	+	-	+	-	+	cocci	*Staphylococcus* spp
PSII	-	-	-	+	+	+	-	+	-	V	P	Y	-	-	+	-	+	+	-	-	+	+	rod- shaped	*Bacillus* spp
PSIII	-	-	-	+	+	+	-	+	-	+	P	Y	-	-	+	-	-	+	-	-	+	+	rod- shaped	*Bacillus* spp
PSIV	+	-	+	-	+	-	+	-	-	+	P	Y	-	-	+	+	-	+	-	+	-	+	cocci	*Staphylococcus* spp
PSV	-	-	+	-	+	+	+	+	-	V	P	Y	-	-	+	+	-	-	-	-	+	+	cocci	*Staphylococcus* spp

Key: +, Positive; -, Negative; S+, Slightly positive; IS, Isolate; SIM, Sulfide indole motility; AR, Arabinose; TSI, Triple sugar iron; GL, Glucose; I, Indole; H, Hydrogen sulfide; M, Motility; CI, Citrate; MR, Methyl red; VP, Vogues Prausker; NR, Nitrate reduction; A, Acid; G, Gas; S, Slant; B, Butt; CA, Catalase; U, Urease; MS, Mannitol salt fermentation; SH, Starch hydrolysis; 6.5, 6.5 % Nacl; P, Pink; Y, YellowPTI-PTXVI, Isolates from the participants' toes; PSI-PSV, Isolates from the participants' sole; G.S-Gram Status.

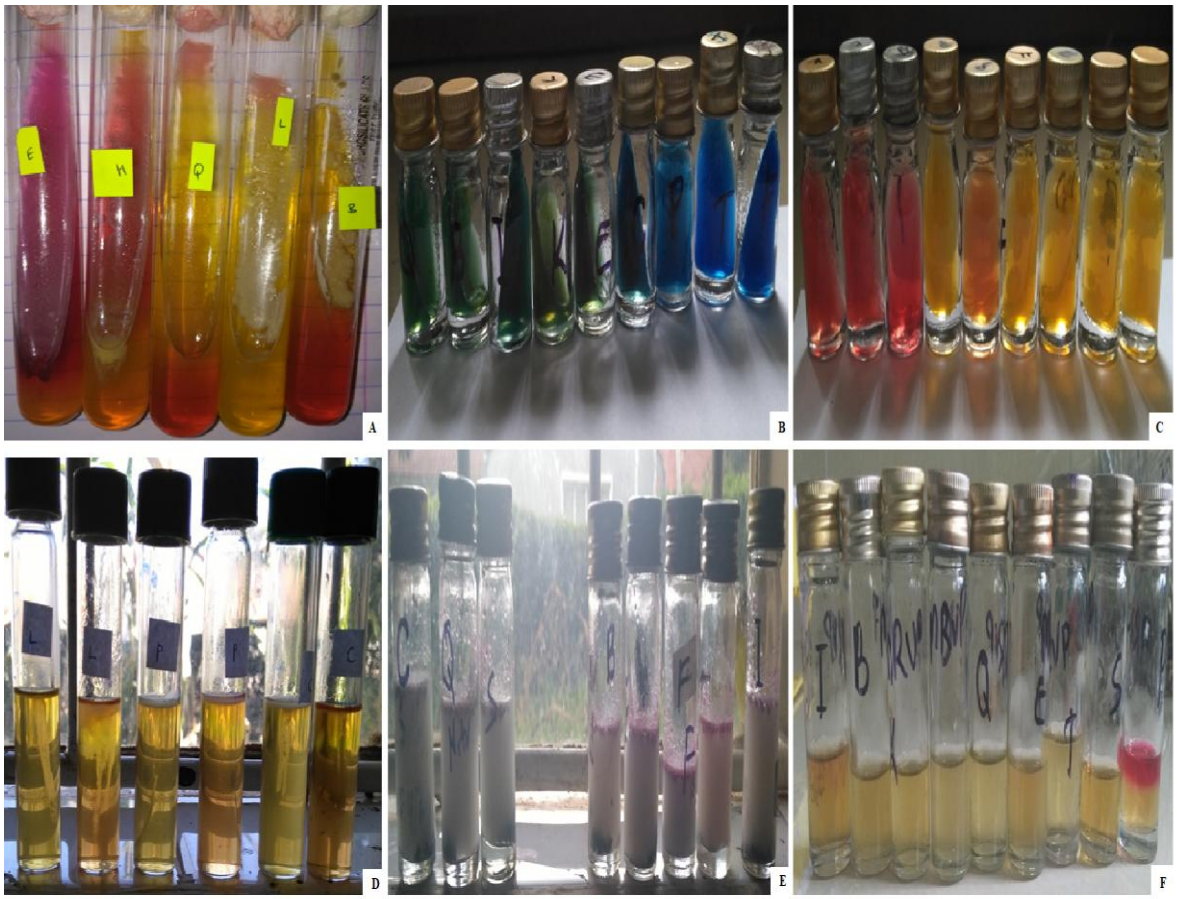

Plate 4.3: Biochemical test results showing A: Triple Sugar Iron test showing acid production (yellow slant and butt), hydrogen sulphide production (black colouration), B: Simmons's citrate test results with both the positive (blue slants) and negative (green slants), C: urease test with both the positive (pink slant) and negative result (yellow slant), D: SIM and MIU test showing different outcomes, E: nitrate reduction test showing different outcomes of the test and F: MR test showing positive (pink colour) and negative outcomes of the test.

4.4 Molecular characterization of bacterial isolates

4.4.1 DNA extraction and amplification

Genomic DNA was successfully extracted from all the grouped 19 bacterial isolates based on their morphological characteristics. The extracted DNA did have a size of 1,000 base pairs and visualized through gel electrophoresis on 1 % agarose gel using a 100 kb DNA ladder (Plate 4.4). PCR amplification of the 19 bacterial isolates produced single bands of 1,500 base pair in size

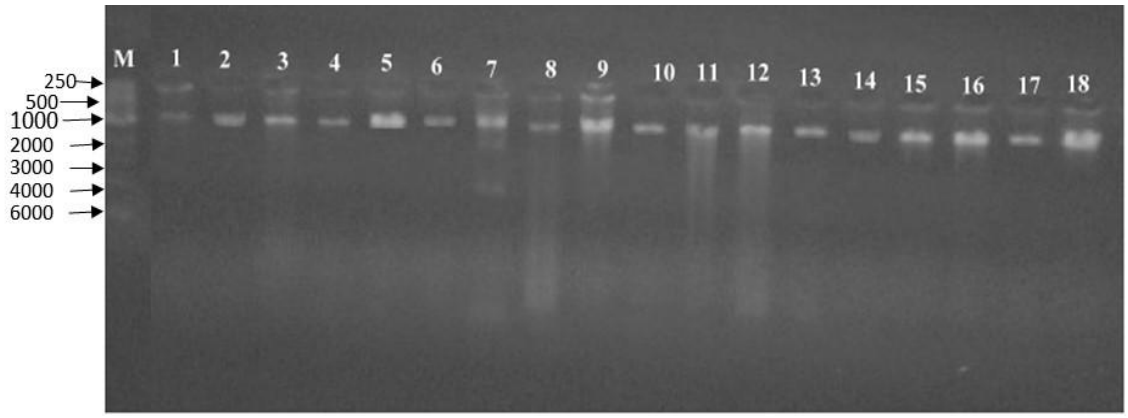

Plate 4.4: Feet derived bacterial isolates' DNA samples visualized on 1% agarose gel. Lane M, 1kb plus DNA ladder; lanes 1-18 bacterial isolates (1-PTI, 2-PTIII, 3-PTV, 4-PTVI, 5-PTVII, 6-PTVIII, 7-PTIX, 6-PTX, 8-PTXI, 9-PTXII, 10-PTXIII, 11-PTXIV, 12-PTXV, 13-PTXVI, 14-PSI, 15-PSII, 16-PSIII, 17-PSIV, 18- PSV).

4.4.2 Phylogenetic data analysis

Phylogenetic analyses of the 16S r RNA genes for the bacterial isolates revealed eight distinct clusters. Combined with the morphological and biochemical characteristics, the bacterial isolates were identified as follows; three bacterial isolates were *Staphylococcus capitis,* three isolates were *Staphylococcus xylosus,* four isolates were *Bacillus pumilus,* two isolates were *Bacillus safensis,* two isolates were *Staphylococcus simulans,* two isolates were *Staphylococcus sciuri,* one isolate was *Bacillus flexus,* while two isolates could not be identified via sanger based sequencing. All the nineteen isolates that were sequenced belonged to the Phylum Firmicutes. Eleven of the nineteen bacterial isolates were cocci and belonged to the Family *Staphylococcaceae* and Genus *Staphylococcus*. Eight of the nineteen bacterial isolates were bacilli and belonged to the Family *Bacillaceae* and Genus *Bacillus*. The first cluster consisted of isolates; PTV obtained from the front part of all participants' feet except for participant one, PTVII obtained from the front feet part of participants two and nine, PTXI obtained from the front feet part of participants two, ten, four, three and one, and PSII obtained from the sole part of the feet of participants

two, ten and seven. These four isolates clustered together (99% sequence similarity) with *Bacillus pumilus,* (Accession number CPO11007) (Fig 4.2) which was isolated from the lake water (Tambekar and Dhundale, 2012). The second cluster consisted of bacterial isolates; PTI obtained from the front feet part of all the participants, PTVIII obtained from the front feet part of participants two,ten, seven, four and three, and PTXII obtained from the front feet part of participants two and three. These isolates clustered together (99% sequence similarity) with *Staphylococcus capitis,* Accession number CP007601 (Fig 4.2) which was isolated in neonate blood. The third cluster consisted of bacterial isolates; PTVI obtained from the front feet part of participants ten and three and PTX obtained from the front feet part of participants two, ten and seven. These isolates clustered together (99% sequence similarity) with *Staphylococcus simulans,* Accession number CP014016, (Fig 4.2) which was isolated from urine catheters (Lewis *et al.*, 2018).

The fourth cluster comprised of bacterial isolates PTXIII obtained from the front feet part of participants ten, seven, four, three and four and PSV obtained from the sole part of the feet of participants two, ten, one, nine, five and eight. The two isolates clustered together (99% sequence similarity) with *Staphylococcus sciuri,* Accession number (CP022046) (Fig 4.2) which was isolated from homo sapiens (Couto *et al.*, 2000). In the fifth cluster, bacterial isolates PTXV obtained from the front feet part of participants one and eight and isolate PTXVI obtained from the front feet part of participants one and four clustered together but did not have any closest reference relative. In cluster six, bacterial isolates PTIII obtained from the front feet part of participants two, ten, seven, four, one, five, isolate PSI obtained from the sole part of participants two, seven and six while isolate PSIV was obtained from the sole part of participants ten, seven and four clustered together

(99% sequence similarity) with *Staphylococcus xylosus,* Accession number (CP013922) (Fig 4.2) isolated from mealy bugs (Indiragandhi *et al.*, 2011).

The seventh cluster only had bacterial isolate PTXIV obtained from the front part of participants two's and ten's feet and it clustered closely (99% sequence similarity) with *Bacillus flexus* Accession number (CP016790) which was isolated from industrial sludge (Wang and Zhao, 2013). The last cluster consisted of bacterial isolates PTIX obtained from the front part of participant two's feet and PSIII obtained from the sole part participants two's, ten's and seven's feet which clustered together (99% sequence similarity) with *Bacillus safensis* (Accession number CP015611) (Fig 4.2) which was isolated from corn rhizosphere (Reza *et al.*, 2014).

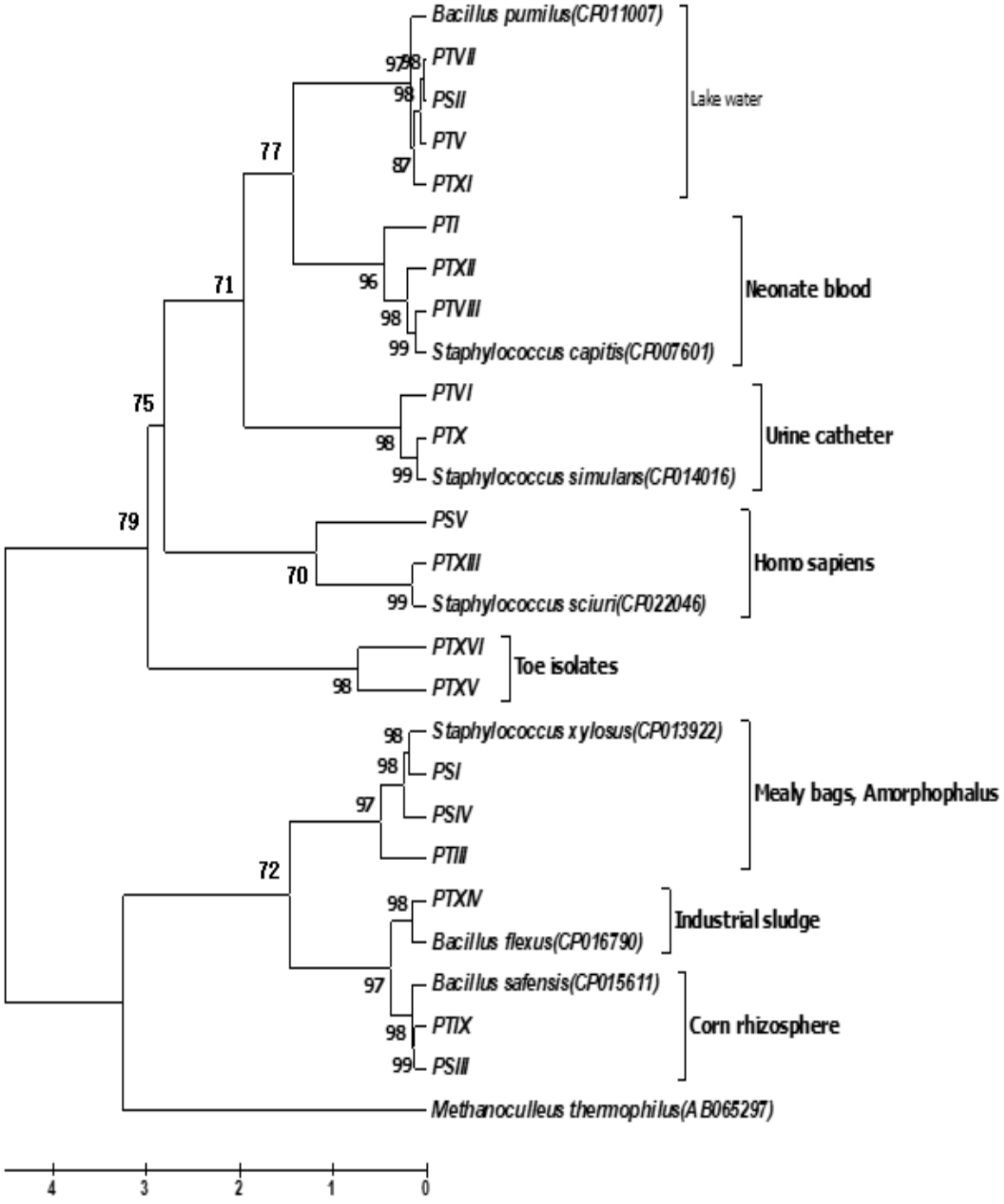

Figure 4.2: Phylogenetic tree showing the relationships among the bacterial isolates from feet samples of individuals with differential attraction to *An. gambiae s.s* and to representatives of reference isolates. Bootstrap values for the associated taxa that clustered together in the bootstrap test (1,000 replicates). The tree is based on Maximum Likelihood Composite. The scale bar indicates the rate of substitution per nucleotide position. The tree is rooted with 16S rRNA gene sequence of an archaeon, *Methanoculleus thermophillus.* Evolutionary analyses were conducted using MEGAX.

4.4.3 Relative diversity of bacterial isolates

Relative diversities of the bacterial isolates present in the feet of the participants were presented in form of pie charts. Participant two (2) who was the most attractive participant had the highest diversity with a total of fourteen bacterial isolates (Fig 4.3). Participant ten (10) had a total of thirteen bacterial isolates, participant seven (7) had a total of eleven bacterial isolates. Both participant nine (9) and five (5) had a similar diversity of six bacterial isolates each (Fig 4.4). Participant eight (8) had a relative diversity of five bacterial isolates while participant six (6) had a diversity of four bacterial isolates (Fig 4.4). Bacterial isolate PTI was shared among all the ten participants (Fig 4.3, Fig 4.4)

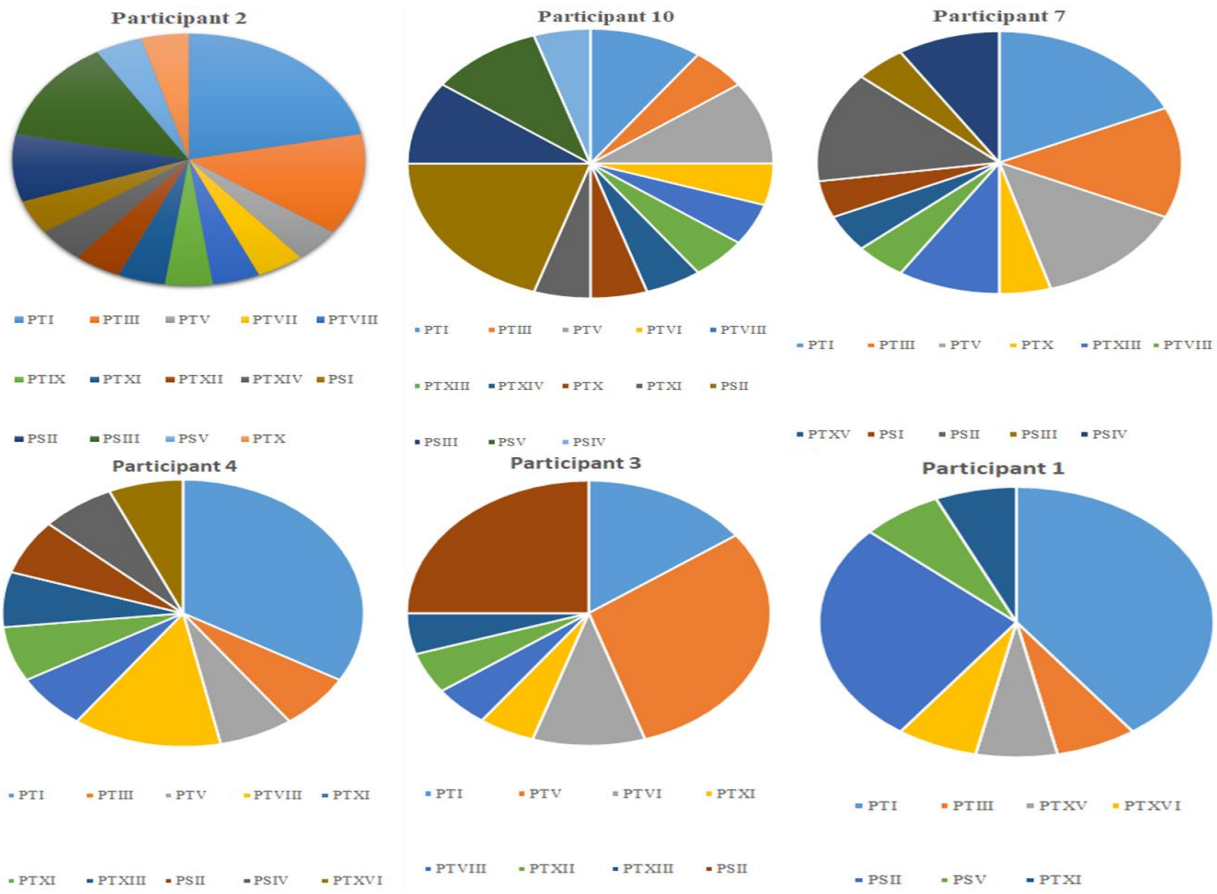

Figure 4.3: Relative abundance of bacterial isolates in the feet samples of Participants' 2, 10 ,7 ,4, 3 and 1. PT- bacterial isolates obtained from in between the toes. PS – bacterial isolates derived from the sole. Pie charts are arranged in the order of attraction from the most attractive (participant 2) to the least attractive (participant 1).

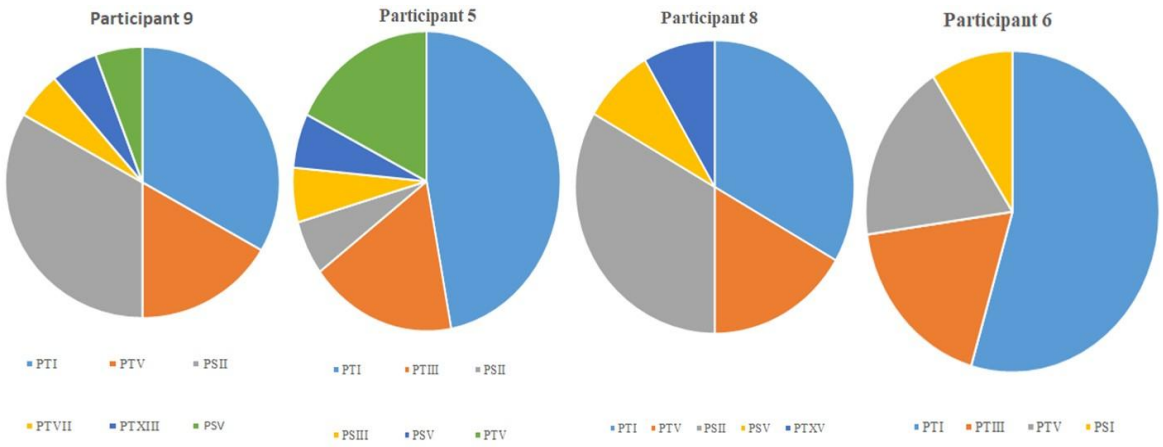

Figure 4.4: Relative abundance of bacterial isolates obtained from the feet samples of participants' 9, 5, 8 and 6. PT- bacterial isolates found mostly from in between the toes. PS – bacterial isolates obtained mainly from the sole. Pie charts are arranged in the order of attraction from the most attractive (participant 9) to the least attractive (participant 6).

4.5 Antimicrobial response patterns

4.5.1 Antibiotics

Six antibiotics; gentamycin 10 mcg, penicillin 10 mcg, ampicillin 10 mcg, tetracycline 30mcg streptomycin 10mcg and ciprofloxacin 30 mcg, were tested for their efficacy against the bacterial isolates obtained from the feet samples of individuals with differential attraction to *An. gambiae s.s*. There was a significant difference in the antimicrobial activity of gentamycin against the bacterial isolates (p=0.001), (Table 4.4). For instance, gentamycin was highly effective against isolate PTIX, obtained from the front part of the participant two's feet, with a mean zone of inhibition at 40.00 mm followed by isolate PTXII, isolated from the front feet part of participants' two and three, with a mean zone of inhibition at 35.67 mm, isolate PTI, isolated and present in the front part of feet from the ten participants, with a mean zone of inhibition at 31.33 mm and isolate PTXV, isolated from the front part of participants' seven and eight feet, with a mean zone of inhibition at 31.00 mm. On the other hand, gentamycin was least effective against isolate PTV, obtained from the front feet part of all the participants except for participant one, having a mean

zone of inhibition at 19.33 mm followed by isolate PTXIV, obtained from the front part of participants' two and ten feet, with a mean zone of inhibition at 21.33 mm, isolate PSII, obtained from the front part of all participants except for participant six, with a mean zone of inhibition at 22.00 mm and isolate PTXIII, obtained from the front part of participants' ten, seven, four three and nine feet, having a mean zone of inhibition at 22.33 mm. Gentamycin was also effective against the control, *E. coli* ATCC number 8739, which had a mean zone of inhibition at 24.00 mm.

There was a significant difference in the action of penicillin against the bacterial isolates (p= 0.001) (Table 4.4). Penicillin was highly effective against isolates PTI ,obtained from the front part of all the participants' feet and PTIX, isolated from the front part of participant's two feet, both having mean zones of inhibition at 40.00 mm, followed by isolate PTXII, obtained from the front part of participants' two and three feet, with a mean zone of inhibition at 38.00 mm and isolate PTXI, obtained from the front part of participants' one, two, three four and ten feet, with a mean zone of inhibition at 32.00 mm. On the other hand, penicillin was least effective against isolates PTV, obtained from the front part of the feet of all participants except for participant one, and PSIV, obtained from the back part of the feet of participants' ten, seven and four, with both having mean zones of inhibition at 6.00 mm followed by isolate PTIII, obtained from the front part of the feet of participants; two, ten, seven, four, one, five and six, having a mean zone of inhibition at 10.00 mm. Penicillin was also not effective against the control, *E. coli* ATCC 8739 with a mean zone of inhibition at 6.00 mm (Plate 4.5).

There was a significant difference in the antimicrobial activity of ampicillin against the bacterial isolates (p= 0.001) (Table 4.4). Ampicillin was highly effective against isolates

PTI, obtained from the front part of the feet of all participants, and PTIX, obtained from the front part of participant's two feet, both having mean zones of inhibition at 40.00 mm followed by isolate PTXII, obtained from the front part of the feet of participants' two and three, which had a mean zone of inhibition at 34.33 mm and isolate PTVIII, obtained from the front part of the feet of participants; two, ten, seven, four and three, with a mean zone of inhibition at 32.00 mm. Ampicillin on the other hand was least effective against isolate PTV, obtained from the front part of all the participants' feet except for participant one, with a mean zone of inhibition at 7.00 mm followed by isolate PTIII, obtained from the front part of the feet of participants; two, ten, seven, four, one, five and six, with a mean zone of inhibition at 10.33 mm, and isolate PSII, obtained from the back part of the feet of all the participants except for participant six, had a mean zone of inhibition at 16.67 mm. Ampicillin was also least effective against the control, *E.coli* ATCC 8739, with a mean zone of 6.00 mm.

Activity of tetracycline against the bacterial isolates also had a significant difference (p= 0.001) (Table 4.4). The highest antimicrobial activity for tetracycline was against isolates PTIX, obtained from the front part of the feet of participant two, and PTXII, obtained from the front part of the feet of participant's two and three, both having mean zones of inhibition at 40.00 mm followed by isolate PTI, obtained from the front part of all the participants' feet, which had a mean zone of inhibition at 38.67 mm. On the other hand, tetracycline was least effective against isolate PTVIII, obtained from the front part of the feet of participants; two, three, four, seven and ten, with a mean zone of 21.67 mm, isolate PTVII, obtained from the front part of participant's two and nine feet, with a mean zone of 23.33 mm, isolate PSII, obtained from the back part of the feet of all participants except for participant six, with a mean zone of inhibition at 24.67 mm and finally isolate PTV

with a mean zone of inhibition at 26.00 mm. Tetracycline was also least effective against the control, *Escherichia coli* ATCC number 8739, with a mean zone of inhibition at 6.00 mm.

In regards to streptomycin, there was a significant difference in the antimicrobial activity against the bacterial isolates (p= 0.001) (Table 4.4). The highest antimicrobial activity was against isolates PTIX, obtained from the front part of participant two's feet, and PTXII, obtained from the front part of the feet of participants two and three, with both having mean zones of inhibition at 40.00 mm followed by isolate PTVIII, obtained from the front part of the feet of participants; two, three, four, seven and ten, with a mean zone of inhibition at 30.00 mm, isolate PTX, isolated from the front part of the feet of participants two, seven and ten, with a mean zone of inhibition at 29.00 mm. The effectiveness of streptomycin was least against isolates PTV, isolated from the front part of the feet of all participants except participant one, and isolate PSIV, obtained from the back part of the feet of participants ten, seven and four, both having mean zones of inhibition at 19.33 mm. This was followed by isolate PTXIV, obtained from the front part of the feet of participants two and ten, having a mean zone of inhibition at 20.33 mm. Streptomycin was effective against the control, *Escherichia. coli* ATCC 8739 with a mean zone of inhibition at 21.00 mm.

There was a significant difference in the antimicrobial action of ciprofloxacin against the bacterial isolates (p= 0.001) (Table 4.4). Of all the antibiotics tested, ciprofloxacin recorded the highest mean zones of inhibition against the bacterial isolates. The highest mean zones of inhibition were recorded for isolates PTVII, obtained from the front part of the feet of participants two and nine, PTIX, isolated from the front part of participant two's

feet, PTX, isolated from the front part of the feet of participants, PTXII, obtained from the front part of the feet of participants two and three, and PSV, isolated from the back part of the feet of participants; eight, five, nine, one, ten and two, all having a mean zone of 40.00 mm, they were followed by isolate PTVIII, obtained from the front part of the feet of participants two, ten, seven, three and four, having a mean zone of inhibition at 38.00 mm. Ciprofloxacin was least effective against isolate PTV, obtained from the front part of the feet of all participants except for participant one, with a mean zone of 29.67 mm. Ciprofloxacin was also highly effective against the control with a mean zone of inhibition at 40.00 mm (Plate 4.5).

Table 4.4: Mean zones of inhibition (in mm) of the six antibiotics on the bacterial isolates obtained from the feet of participants with different levels of attraction to *Anopheles gambiae s.s.*

	Antibiotics					
Isolates	Gentamycin(mm)	Penicillin(mm)	Ampicillin(mm)	Tetracycline(mm)	Streptomycin(mm)	Ciprofloxacin(mm)
PTI	31.33 ±0.67 c	40.00 ±0.00 a	40.00 ±0.00 a	38.67 ±1.33 a	22.67 ±0.67 f g h	37.00 ±3.00 a b
PTII	24.67 ±0.33 g h i j	10.00 ±0.58 i	10.33 ±0.33 n	32.00 ±0.58 b c d e	24.67 ±0.33 e f	32.67 ±0.33 c d e
PTV	19.33 ±0.33 m	6.00 ±0.00 j	7.00 ±1.00 o	26.00 ±0.00 g h i	19.33 ±0.67 j	29.67 ±0.33 e
PTVI	27.33 ±0.33 e f	23.33 ±0.33 e	26.00 ±0.00 g h	33.00 ±0.58 b c	26.00 ±0.00 d e	36.33 ±0.33 a b c
PTVII	26.00 ±0.58 f g h	30.33 ±0.88 b c	30.00 ±0.58 c d e	23.33 ±0.33 i j	26.00 ±0.58 d e	40.00 ±0.00 a
PTVIII	30.00 ±0.00 c d	18.67 ±033 g	32.00 ±0.58 b c	21.67 ±0.33 j	30.00 ±0.00 b	38.00 ±0.58 a
PTIX	40.00 ±0.00 a	40.00 ±0.00 a	40.00 ±0.00 a	40.00 ±0.00 a	40.00 ±0.00 a	40.00 ±0.00 a
PTX	26.67 ±0.33 f g	14.67 ±0.33 h	27.00 ±0.58 f g	30.33 ±0.33 c d e f	29.00 ±0.58 b c	40.00 ±0.00 a
PTXI	27.00 ±0.58 e f	32.00 ±0.58 b	29.00 ±0.58 d e f	30.67 ±0.67 c d e f	27.00 ±0.58 c d	40.00 ±0.00 a
PTXII	35.67 ±0.33 b	38.00 ±0.58 a	34.33 ±0.33 b	40.00 ±0.00 a	40.00 ±0.00 a	40.00 ±0.00 a
PTXIII	22.33 ±0.33 k l	27.33 ±0.33 d	25.33 ±0.33 g h i	29.33 ±0.33 e f	20.67 ±0.33 h i j	31.67 ±0.33 d e
PTXIV	21.33 ±0.33 l m	24.67 ±0.33 e	23.33 ±0.33 i j k	28.33 ±0.33 f g	20.33 ±0.33 i j	30.33 ±0.33 d e
PTXV	31.00 ±0.58 c d	29.33 ±0.33 c d	25.33 ±0.33 g h i	29.67 ±0.33 d e f	27.67 ±0.33 c d	37.67 ±0.33 a
PTXVI	22 .67 ±0.33 k l	23.67 ±0.33 e	22.00 ±0.58 j k l	26.33 ±0.33 g h	20.67 ±0.33 h i j	30.00 ±0.00 d e
PSI	25.33 ±0.33 f g h i	19.00 ±0.58 f g	19.67 ±0.33 l	31.00 ±0.58 c d e f	24.33 ±0.33 e f	30.67 ±0.33 d e
PSII	22.00 ±0.58 k l	20.67 ±0.33 f g	16.67 ±0.33 m	24.67 ±0.33 h i	22.00 ±0.58 g h i	32.00 ±0.58 d e
PSIII	25.33 ±0.33 f g h i	30.00 ±0.58 b c	27.67 ±0.33 e f g	30.67 ±0.33 c d e f	25.67 ±0.33 d e	36.67 ±0.67 a b
PSIV	25.33 ±0.33 f g h i	6.00 ±0.00 j	30.33 ±0.33 c d	24.33 ±0.67 h i j	19.33 ±0.67 j	40.00 ±0.00 a
PSV	29.00 ±0.58 d e	21.00 ±0.58 f	23.67 ±0.33 h i j	26.33 ±0.33 g h	24.33 ±0.33 e f	40.00 ±0.00 a
E. coli ATCC 8739	24.00 ±0.00 h i j k	6.00 ±0.00 j	6.00 ±0.00 o	6.00 ±0.00 k	21.00 ±0.00 h i j	40.00 ±0.00 a
P -value	0.001	0.001	0.001	0.001	0.001	0.001

Key: Values present within the same column without common letters differ significantly based on Tukey's HSD $p<0.05$. Key: PTI-PTXVI, isolates from in between the participants' toes; PSI-PSV isolates from the sole of the participants' feet. Values within the same column followed by the same letter did not differ significantly. Values shown are mean zone of inhibition plus standard error of the means of three independent measurements.

The zones of inhibition were interpreted using the Clinical and Laboratory Institute Standards' table. All the isolates and control were susceptible to gentamycin 10 mcg (Table 4.5). As for penicillin, isolates PTIII, PTV, PTVIII, PTX, PTXVI, PSI, PSII, PSV and the control, *Escherichia coli* ATCC 8739, were all resistant whereas isolates PTI, PTXIII and PTXIV were intermediate. Isolates PTVII, PTIX, PTXII and PTXV were susceptible to penicillin (Table 4.5). Isolates PTIII, PTV and the control for the experiment, *Escherichia coli* ATCC 8739, were resistant to ampicillin 10 mcg while isolates PSI and PSII were intermediate for ampicillin. Isolates PTI, PTVII, PTVIII, PTIX, PTX, PTXIII, PTXIV, PTXV, PTXVI and PSV were all susceptible to ampicillin 10mcg. As for tetracycline 30 mcg, isolates PTI, PTIII, PTV, PTIX, PTX, PTXII, PTXIII, PTXIV, PTXV, PTXVI, PSI and PSV were susceptible. Isolates PTVII, PSII and PSIV were intermediate while isolates PTVIII and the control, Escherichia coli ATCC 8739, were resistant to tetracycline. All the bacterial isolates plus the control were susceptible to both streptomycin 10 mcg and ciprofloxacin 30 mcg (Table 4.5).

Table 4.5: Interpretation of the mean zones of inhibition (mm) of the six antibiotics against the 19 bacterial isolates.

ISOLATE	Gentamycin 10mcg	Penicillin 10mcg	Ampicillin 10 mcg	Tetracycline 30mcg	Streptomycin 10mcg	Ciprofloxacin 30mcg
PTI	+	+++	+	+	+	+
PTIII	+	+++	+++	+	+	+
PTV	+	+	+++	+	+	+
PTVI	+	+++	+	+	+	+
PTVII	+	+	+	++	+	+
PTVIII	+	+++	+	+++	+	+
PTIX	+	+	+	+	+	+
PTX	+	+++	+	+	+	+
PTXI	+	+	+	+	+	+
PTXII	+	+++	+	+	+	+
PTXIII	+	+++	+	+	+	+
PTXIV	+	++	+	+	+	+
PTXV	+	+	+	+	+	+
PTXVI	+	+++	+	+	+	+
PSI	+	+++	++	+	+	+
PSII	+	+	++	++	+	+
PSIII	+	+	+	+	+	+
PSIV	+	+++	+	++	+	+
PSV	+	+++	+	+	+	+
E. coli ATCC 8739	+	+++	+++	+++	+	+

Key: +++, resistant; ++. Intermediate; +, susceptible; PTI-PTXVI, bacterial isolates found in between the toes of the participants' feet; PSI-PSV, bacterial isolates found from the sole of the participants' feet; ATCC, American Type Culture Collection; mcg, microgram; *E. coli*, *Escherichia coli.*

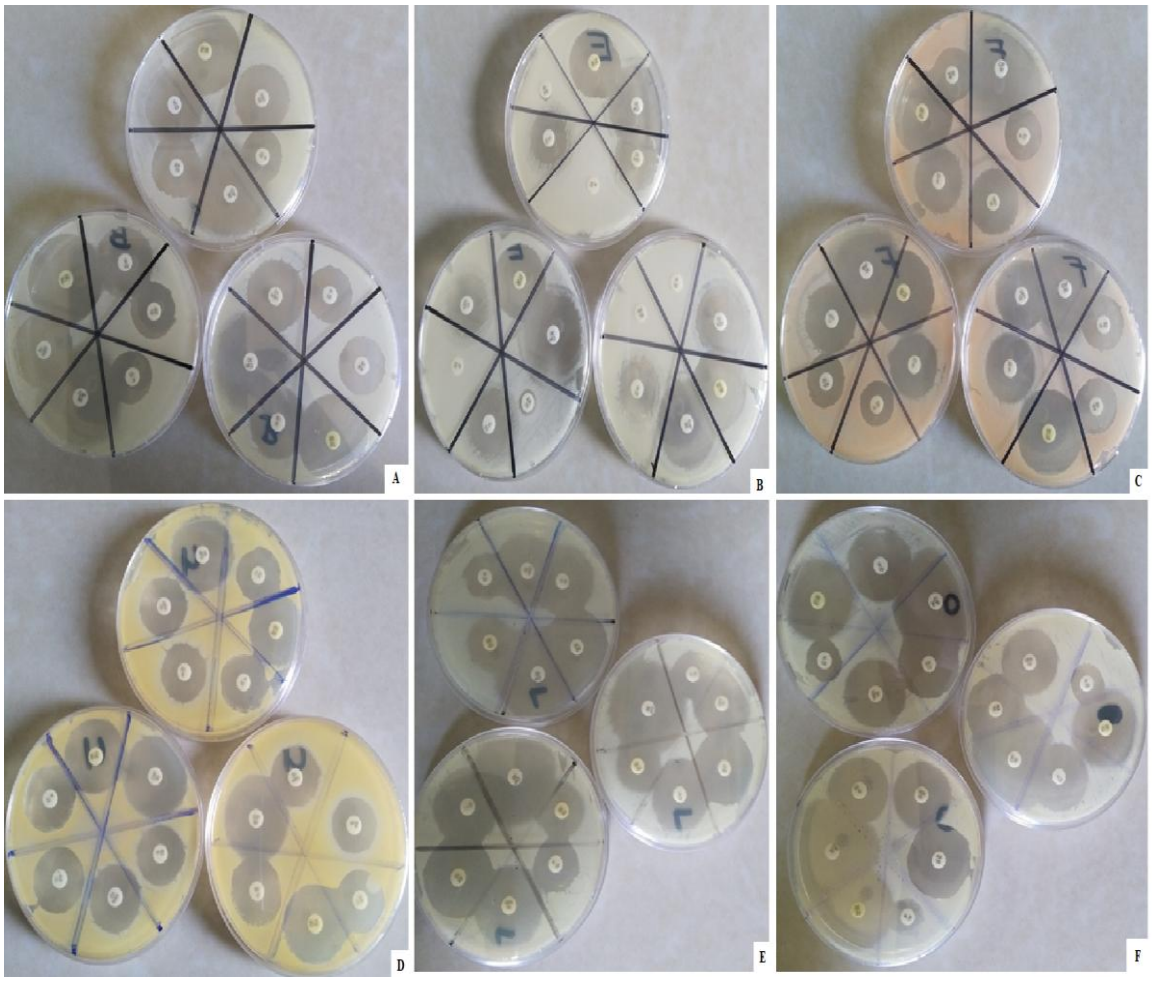

Plate 4.5: Triplicate Mueller Hinton Agar plates showing zones of inhibition (mm) for the six antibiotics against the bacterial isolates A: PTI, B: PTV, C: PSI, D: PTXVI: E: PTVIII and F: PTX.

4.5.2 Antiseptics

4.5.2.1 Effect of antiseptics on dilutions

Three antiseptics were tested for their antimicrobial activity against the bacterial isolates. Eight dilutions were prepared for each of the antiseptics. There was a significant difference in the effectiveness of the three antiseptics against the feet derived bacterial isolates, across the eight dilutions (P=0.001). Antiseptic A had the highest mean zone of inhibition in all the dilutions (Table 4.6). This was followed by antiseptic B with Antiseptic C being the

least effective antiseptic. In dilutions 10^{-5}, 10^{6} and 10^{-7}, the activity of antiseptic B was equal to that of antiseptic C (Table 4.6). Sterile distilled water, which was a control for the experiment in all of the eight dilutions, did not have any antimicrobial effect on the bacterial isolates with a mean zone of inhibition being 0.00 mm across board.

Table 4.6:Effect of antiseptics on dilutions

Antiseptic	10^{0} (mm)	10^{-1} (mm)	10^{-2} (mm)	10^{-3} (mm)	10^{-4} (mm)	10^{-5} (mm)	10^{-6} (mm)	10^{-7} (mm)
B	15.05 ±0.65 b	13.62 ±0.76 b	11.71 ±0.76 b	9.83 ±0.62 b	7.75 ±0.46 b	5.25 ±0.41 b	4.51 ±0.38 b	4.40 ±0.36 b
C	14.43 ±0.66 c	12.13 ±0.64 c	10.59 ±0.60 c	8.62 ±0.58 c	6.43 ±0.50 c	4.94 ±0.40 b	4.40 ±0.34 b	4.38 ±0.34 b
A	22.08 ±0.56 a	20.10 ±0.49 a	17.43 ±0.55 a	15.94 ±0.43 a	13.89 ±0.42 a	10.92 ±0.53 a	8.51 ±0.58 a	6.89 ±0.53 a
Water	0.00 ±0.00 d	0.00 ±0.00 d	0.00 ±0.00 d	0.00 ±0.00 d	0.00 ±0.00 d	0.00 ±0.00 c	0.00 ±0.00 c	0.00 ±0.00 c
P Value	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001

Key: Values shown are mean plus standard error of the means for three independent measurements. Values present within the same column without common letters differ significantly based on Tukey's HSD $p<0.05$. Values present in the same column followed by the same letter did not differ significantly. Sterile distilled water was the control for the experiment.

4.5.2.2 Effect of antiseptics on the bacterial isolates from the feet of participants with differential attraction to *Anopheles gambiae s.s.*

Out of the eight dilutions; 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, tested in the experiment, the first two dilutions were picked to demonstrate the effect of the interaction between the foot bacterial isolates and the three antiseptics tested in this study. The effect of bacterial isolates with antiseptic A was significant (P=0.001). The highest effect of antiseptic A with the foot bacterial isolates on the mean zones of inhibition was evident on isolate PTVI, obtained from the front part of the feet of participants three and ten, which had the highest zones of inhibition for both dilutions 10^{0} and 10^{-1} (Fig 4.5). This was followed by isolate PTVIII, obtained from the front part of the feet of participants; two, three, four, seven and ten. The lowest effect of the interaction between the feet microbial isolates with antiseptic

A on the mean zones of inhibition was seen in isolate PTXV obtained from the front part of participants' seven's and eight's feet which had the lowest mean zones of inhibition for both dilutions 10^0 and 10^{-1} (Fig 4.5). Antiseptic A was also least effective against isolate PSI, obtained from the back part of the feet of participants two, six and seven, in the two dilutions, 10^0 and 10^{-1}, (Fig 4.5).

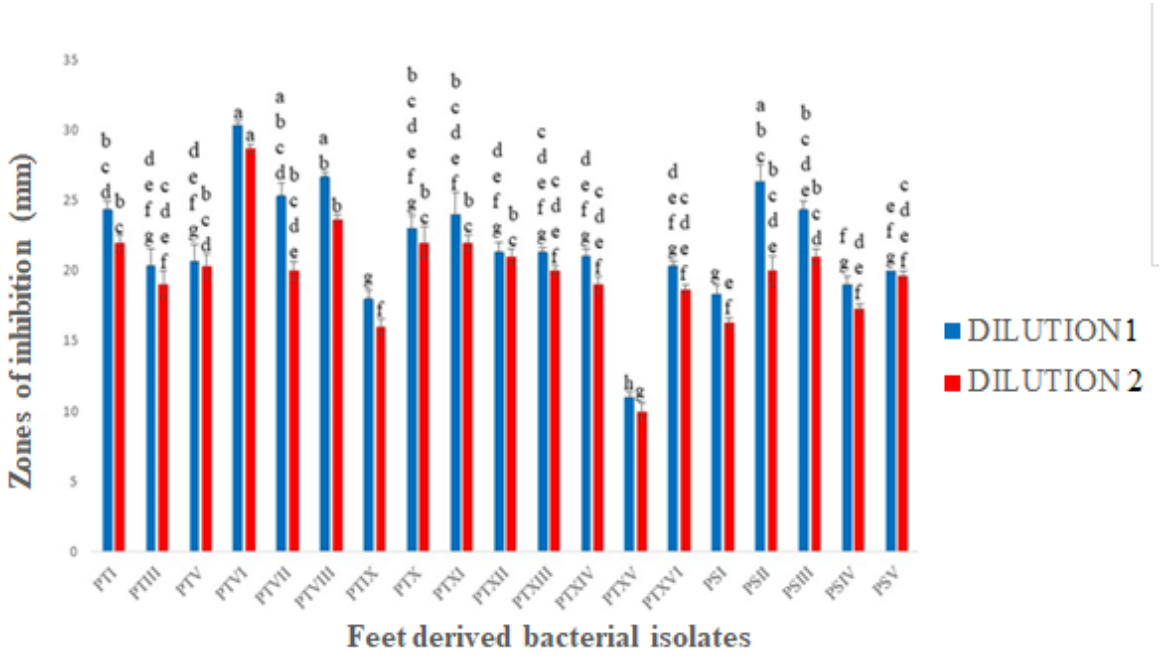

Figure 4.5: Interactive effects of feet bacterial isolates and antiseptic A on the mean zones of inhibition. Bars followed by the same letters are not significantly different using Tukey's HSD test at P≤0.05, while the error bars represent the standard error of the mean. PTI-PTXVI, bacterial isolates mainly found in between the toes of the participants. Dilution 1, 10^0. Dilution 2, 10^{-1} PSI-PSV, bacterial isolates mainly found on the sole of the participants.

The effect of the interaction of the isolated foot bacteria with the antiseptic C was significant (P=0.001). The highest effect of the interaction of the antiseptic C with the isolated feet bacteria on the mean zones of inhibition was seen on isolates PTIX obtained from the front part of participant two's feet, PTVIII, isolated from the front part of the feet of participants; two, ten, seven, four and three, and isolate PTXI, obtained from the front

part of the feet of participants; one, two, three, four and ten, which all showed high mean zones of inhibition, (Fig 4.6). The lowest effect of the interaction of the foot bacterial isolates with antiseptic C on the mean zones of inhibition was seen in isolates PTX, obtained from the front part of the feet of participants two, seven and ten, isolate PTXIII, obtained from the front feet part of participants; ten, seven, four, three and nine, and isolate PSII, obtained from the back part of the feet of all the participants except for participant six, as exhibited by the low mean zones of inhibition (Fig 4.6)

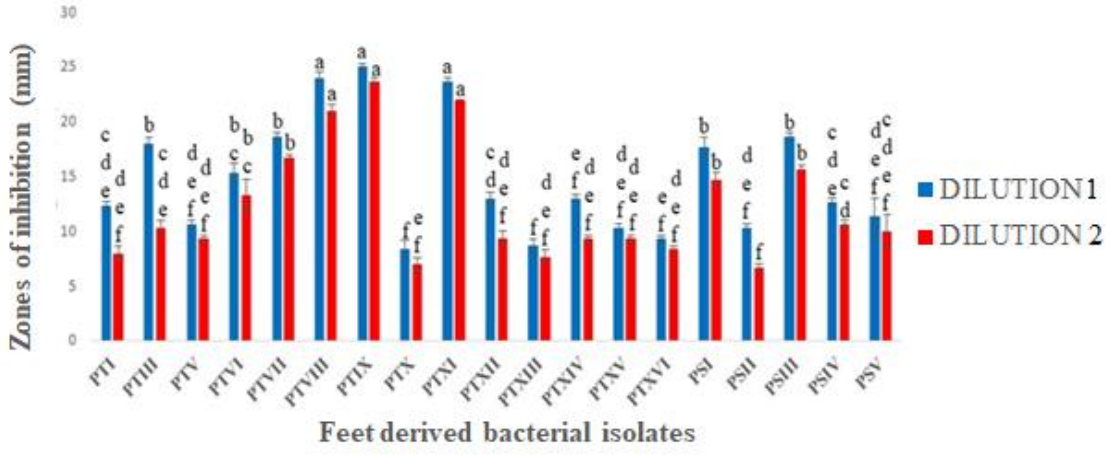

Figure 4.6: Interactive effects of feet bacterial isolates and two dilutions of antiseptic C on the mean zones of inhibition. Bars followed by the same letters are not significantly different using Tukey's HSD test at $P \leq 0.05$, while the error bars represent the standard error of the mean. PTI-PTXVI, bacterial isolates majorly found in between the toes of the participants. PSI-PSV, bacterial isolates majorly found on the sole of the participants. Dilution 1, 10^0. Dilution 2, 10^{-1}.

The effect of the interaction between the bacterial isolates and antiseptic B was significant (P=0.001). isolates PTVI, obtained from the front part of participants' three's and ten's feet, isolate PTIX, obtained from the front part of the feet of participant two, and isolate PTXII, obtained from the front part of participants' two's and three's feet, exhibited the highest effect in the interaction of antiseptic B with the foot microbial isolates on the mean

zones of inhibition. This was seen in the high zones of inhibition exhibited for the two dilutions, 10^0 and 10^{-1}, in all of the three isolates (Fig 4.7). The lowest effect in the interaction of the foot microbial isolates with antiseptic B on the mean zone of inhibition was exhibited in isolates PTV, obtained from the front part of all participants' feet except for participant one, PTXIII, isolated from the front part of the feet of participants; ten, seven, four, three and nine, and isolate PTXV, obtained from the front part of the feet of participants seven and eight. This was seen in the low mean zones of inhibition (Fig 4.7).

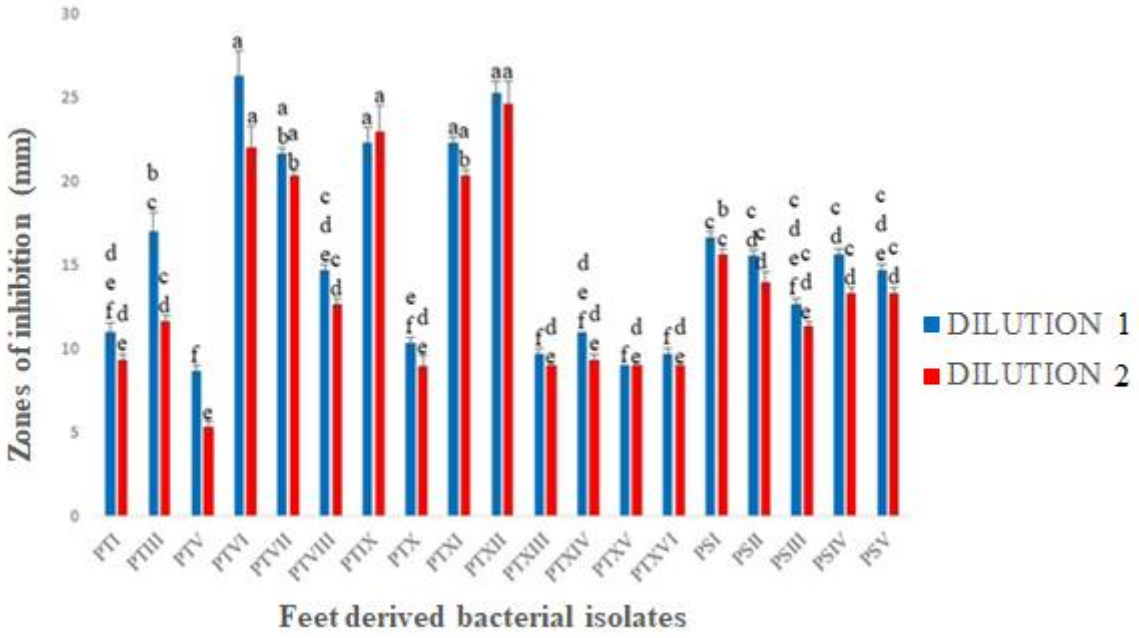

Figure 4.7: Interactive effects of feet bacterial isolates and two dilutions of antiseptic B on the mean zones of inhibition. Bars followed by the same letters are not significantly different using Tukey's HSD test at $P \leq 0.05$, while the error bars represent the standard error of the mean. PTI-PTXVI, bacterial isolates majorly found in between the toes of the participants. Dilution 1, 10^0. Dilution 2, 10^{-1}. PSI-PSV, bacterial isolates majorly found on the sole of the participants.

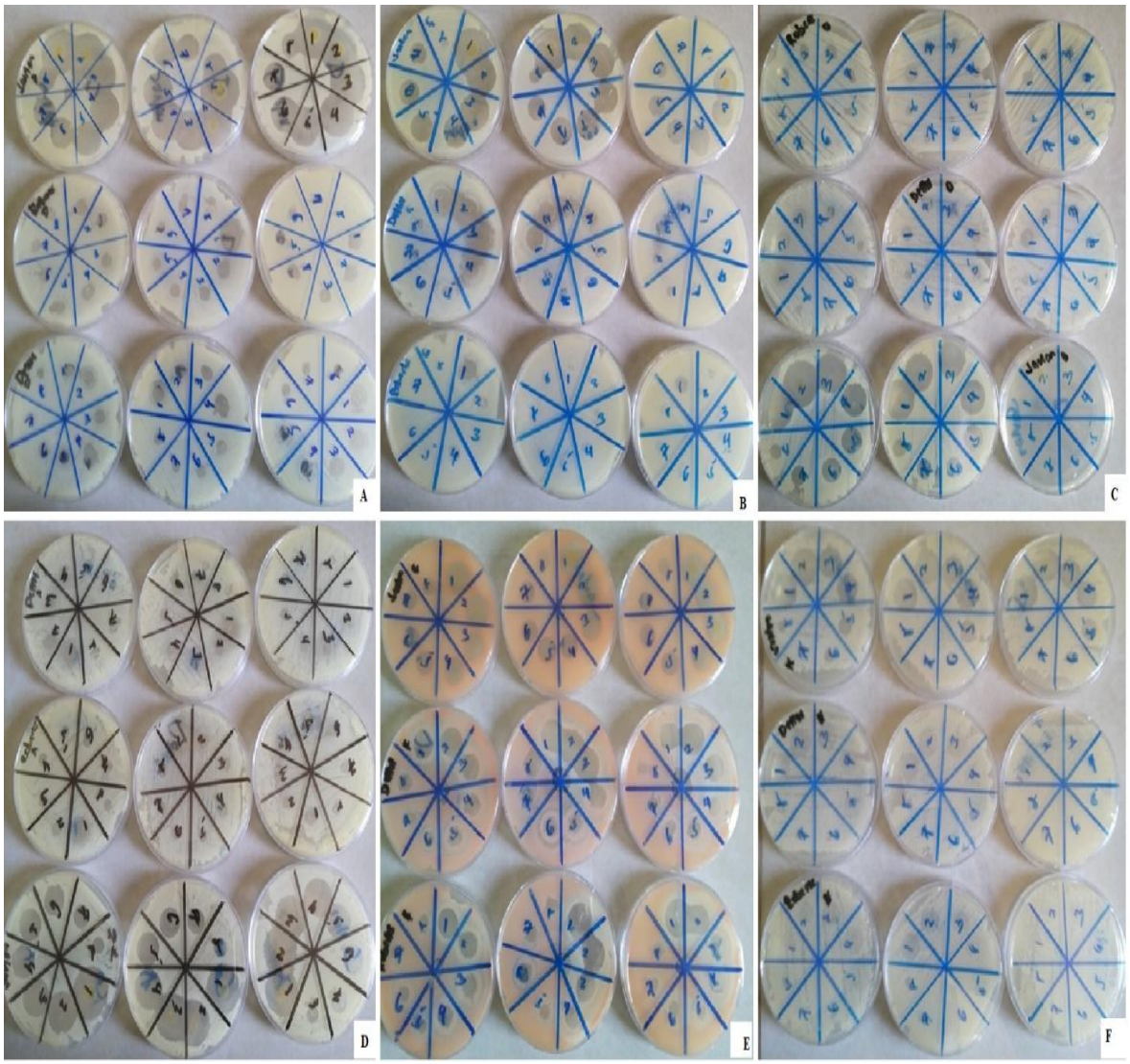

Plate 4.6: Triplicate plates for the three antiseptics A, B and C showing zones of inhibition against isolates A: PTIII, B: PSII, C: PTX, D: PTI, E: PSI and F: PSV.

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

5.1.1 Differential attraction of female *Anopheles gambiae s.s* to foot odour

The human skin is mainly inhospitable to majority of bacteria with few having the ability to survive (Omolo *et al*., 2013). The composition of skin microbiota is dependent on several factors such as pH, humidity, availability of nutrients and concentration of inhibitors (Ara *et al*., 2006). There is a correlation between an individual's body odour profile and the microbial composition in regards to the diversity, density and quantity (Kröber *et al.*, 2016). The variation among individuals was seen in this study by the rankings from the most attractive participant to the least. this is also in line with work done by (Verhulst *et al*., 2010; Verhulst *et al*., 2011; Omolo *et al*., 2013) where the collected foot odour did show varied differential attractiveness to the female *Anopheles gambiae s.s* mosquitoes. The different levels in the attractiveness of the human feet odours to *An. gambiae s.s* can be linked to the variations of the skin microbiota which does explain the differing qualitative and quantitative chemical profiles of the most to the least attractive individuals (Omolo *et al*., 2013).

This study revealed that among the ten participants, there was a higher level of differential attraction to foot odour from the front part of the feet as compared to the back part of the feet. The physical conditions on the different parts of the foot may influence the bacterial load and the rate of malodour production (James *et al*., 2013). As reported by James *et al*., 2013 there was a higher bacterial count within the toe cleft than on the plantar surface. This could explain why there was a higher differential attraction of the female mosquitoes to the front part (inter-toe spaces) as compared to the back part of the feet (plantar heel

surface) for all the ten participants. The front part of the foot is made up of five toes and four inter-toe spaces while the back part of the foot has the sole region. The space in between the toes does offer a good environment for accumulation of sweat which in turn encourages the growth of the foot bacteria. The bacteria then breakdown the sweat to obtain nutrients which in turn leads to the production of the volatiles which mediate the attraction of the female *Anopheles gambiae s.s* mosquitoes (Stevens *et al*., 2015).

In comparison, the sole area of the back part of the foot is less hospitable since there is no allowance for the build-up of sweat in this region (Stevens *et al*., 2015). This in turn does not facilitate a conducive environment for feet bacteria to breakdown sweat leading to the production the volatiles necessary for the attraction of the female *An. gambiae s.s* mosquitoes. Even though this study was able to show that there was a higher attraction of the female mosquitoes to the front part of the feet, it would be prudent in future to also investigate and determine which region among the four inter-toe spaces could have a higher differential attraction to the female mosquitoes.

5.1.2 Isolation and biochemical characterization of feet bacteria

During the research study, 154 pure bacterial isolates were isolated from feet samples of individuals with differential attraction to *An. gambiae s.s*. Human feet have been shown to harbour diverse bacterial isolates (Stevens *et al*., 2015). This could be attributed the presence of sweat which offers a conducive environment for the bacteria to thrive (Kröber *et al.*, 2016). Majority of the bacterial isolates (14 isolates) were obtained from the front part of the feet as compared to the back part of the feet (5 isolates). This is supported by studies done by (James *et al*., 2013). The feet derived bacteria had diverse morphological characteristics and were grouped into nineteen morphological groups. The grouped

bacterial isolates exhibited varied biochemical properties. Gram staining of the isolates showed that majority (eighteen isolates) were Gram positive while one was Gram negative. This is in agreement with studies done by (Cosseau et al., 2016) where they stated that the human skin is mainly dominated by Gram positive bacteria. Hydrogen sulphide production in the TSI test only elicited one positive reaction from isolate PTXV. This was indicated by the ability of the isolate to reduce compounds that contain inorganic Sulphur (Hemraj *et al.,* 2013). This was also in agreement with studies done by (Bolyard *et al.*, 2017) on production of hydrogen sulphide by bacteria.

Majority of the feet derived bacterial isolates (fifteen isolates) were able to hydrolyse both glucose and arabinose sugars. They are able to produce extracellular enzymes which enables them to ferment the carbohydrates. This is in agreement with studies done by (Okorie and Olasupo, 2013). Isolates; PTIII, PTV, PTVI and PTVIII were not able to hydrolyse the two carbohydrates and this could be attributed to them lacking extracellular enzyme required to ferment the sugars. Indole test from the SIM test showed that most of the feet derived bacterial isolates (18 isolates) were negative. This could be due to the fact that the isolates are not able to hydrolyse tryptophan so as to produce indole (Cheng *et al*., 2012). One isolate (PSIV) was positive for the indole test and as documented by Hemraj *et al.,* (2013), bacteria hydrolyse tryptophan to produce ammonia which leads to the change in colour.

In the citrate utilization test majority of the isolates (eleven isolates) were negative due to the lack of ability to utilize citrate in the growth medium. Eight bacterial isolates were positive for the test as evidenced by the color change from green to blue. This is due to the presence of citrate permease enzyme which facilitates the transportation of citrate in the

bacterial cells (Mehere *et al.*, 2018). For the 6.5 % sodium chloride tolerance test, ten bacterial isolates were positive. This shows that these isolates are not inhibited by the concentrations of sodium chloride. On the other hand, nine bacterial isolates were negative for the test which was an indicator that the concentrations of sodium chloride inhibit their growth as shown by (Murray *et al*., 2014)

5.1.3 Molecular characterization of the bacterial isolates

Diversity of skin microbiota was in the past mainly investigated via culture based techniques and recently has been subsequently confirmed using molecular approaches such as Sanger sequencing targeting the 16S rRNA gene (Foulongne *et al*., 2012). Isolates PTV, obtained from the front feet part of all participants except for participant one, isolate PTVII, obtained from the front part of participant two's and nine's feet, isolate PTXI, obtained from the front part of the feet of participants two, ten, four, three and one, and isolate PSII, obtained from the back part of the feet of all the participants except for participant six, feet formed the first cluster and they were closely related (99% sequence similarity) to *Bacillus pumilus* (Accession number CP011007) which was previously isolated from lake water (Tambekar and Dhundale, 2012). *Bacillus pumilus* has been investigated for the role it plays as a biocontrol agent. *Bacillus pumilus* has also been found in other environments including soil (Garbev *et al.*, 2003).

Isolates PTV (found in the least attractive participant), PTVII, PTXI and PSII were found in most of the participants and this could be attributed to contamination from the soil. With the possibility of the bacterial isolates having been picked up by the participants in their day to day activities which does expose them to soil leading to the contamination. This is as *Bacillus pumilus* does not play any role in the production of foot odour. Isolates' PTI,

obtained from the front feet part of all participants, PTVIII, obtained from the front feet part of participants two, ten, seven, four and three and PTXII, obtained from the front part of the second and third's feet, formed the second cluster and were closely related (99% sequence similarity) to *Staphylococcus capitis* (Accession number CP007601) which was previously isolated from neonate blood where a premature infant had developed a bloodstream infection while it was in a neonatal intensive care unit (NICU) (Zwet *et al*., 2002). A study done by (Hara *et al.*, 2014) showed that *Staphylococcus capitis* is part of the cutaneous bacteria on the human feet and that there was an association between foot odour and Staphylococci populations. *Staphylococcus capitis* has lipase enzymes that do break down the lipids in sweat from eccrine glands and could lead to strong feet odour (Marshall *et al*., 1987; Wood and Kelly, 2010). It has also been demonstrated that the isomers of the specific short-chain fatty acids do play an important role in the production of foot odour (Ara *et al*., 2006).

Isolate PTI in this cluster, obtained from the front feet part of all participants' feet was present in the feet of all the participants in this study and it is possible that it is involved in attraction of mosquitoes to the preferred feeding site. This opens up an avenue to do further investigations on whether odour production and attraction of the vector is done independently or synergistically. Isolates PTIII, PSI and PSIV in the third cluster were closely related (99% sequence similarity) to an opportunistic pathogen *Staphylococcus xylosus* (Accession number CP013922) which was previously isolated from the alimentary tract and integument of mealy bugs (Podgwaite *et al*., 2013). *Staphylococcus xylosus* is a commensal bacterium and is generally found inhabiting the skin and mucosal membranes of birds and mammals (Dordet-Frisoni *et al.*, 2007). *Staphylococcus xylosus* is also ubiquitous and can be found in various environments, it is present in soils and surfaces

(Gozalo *et al*., 2012). It is defined as a non-pathogenic Staphylococcus. However, a few strains of this species have been related to animal and human pathogenic infections (Kim *et al*., 2017). This bacterium is said to be transient on humans, having been acquired from domestic animals and their products (Vela *et al*., 2012). It has also been is naturally present in raw meat and milk and may be commonly utilized as a starter culture (Dordet-Frisoni *et al*., 2007). It is possible that through these sources; domestic animals, meat, milk and soil it would have been possible avenue for the presence of *Staphylococcus xylosus* on the human feet. The role of *Staphylococcus xylosus* in the production of foot odour remains clearly ununderstood.

Isolate PTXIV in the fourth cluster was closely related (99% sequence similarity) to *Bacillus flexus* (Accession number CP016790) which was previously isolated from industrial sludge close to petroleum smelter (Wang *et al*., 2013). *Bacillus flexus* has also been isolated from used toothbrushes as the highest occurring Gram-positive bacteria (Oluwole and Olumuyiwa, 2016) and it is possible that the isolate might have been passed to the feet of the participants through human related activities. This isolate was only found in two participants; Participant two and seven who were coincidentally the top two most attractive individuals with a differential attraction to the female *Anopheles gambiae s.s mosquitoes* and it is possible that it is one of the isolates potentially mediating attraction of *An. gambiae s.s* to the preferred feeding site in these individuals.

In the fifth cluster, isolates PTVI obtained from the front feet part of the third and tenth participants and PTX obtained from the front feet part of participants two, seven and ten were closely related (99% sequence similarity) to *Staphylococcus simulans* (Accession number CP014016) which was previously isolated from urine catheters and was linked to

urinary tract infections (Lewis *et al*., 2018). Some strains of *Staphylococcus simulans* have been shown to produce lipase enzyme which is known to breakdown lipids present in sweat (Long *et al*.,1992; Sayari *et al*., 2001), suggesting that it could potentially play a role in the production of volatiles that mediates the attraction of the female *Anopheles gambiae* mosquitoes to the human hosts for feeding purposes (Long *et al*.,1992).

Isolates PTXIII obtained from the front feet part of participants ten, seven, four, three and nine and PSV obtained from the sole part of participant's two, ten, one, nine, five and eight feet formed the sixth cluster and were closely related (99% sequence similarity) to *Staphylococcus sciuri* (Accession number CP022046), which was previously isolated from both hospitalized and healthy humans (*Homo sapiens*) (Couto *et al*., 2000). *Staphylococcus sciuri* was first described by Dakic *et al*., 2005 and it is considered as one of the most ancestral and dispersed staphylococcal species. It has a wide range of habitats which include skins of several animals, soil, water, furniture as well as marsh grass (Couto *et al*., 2000). It can also be found as a colonizing organism in the nasopharynx, skin and urogenital tract (Dakic *et al*., 2005) and its presence on the human feet can be attributed to cross transfer from many sources such as soil, water and skin of domestic animals. Since it was present in many participants, it is possible that it potentially plays a role in mediating differential attraction of *An. gambiae* to the preferred feeding site. However, from the previous investigations, there is no documentation on the role of *Staphylococcus sciuri* in the production of foot odour constituents, and hence it would be worth investigating. *Staphylococcus sciuri* does not show any lipolytic activity (Stepanović *et al*., 2001).

In the seventh cluster, isolates PTIX obtained from the front part of participant two's feet and PSIII obtained from the sole part of participants two, seven and ten's feet were closely

related (99% sequence similarity) to *Bacillus safensis* (Accession number CP01561) which was previously isolated from the corn rhizosphere (Cavaglieri *et al.*, 2009). *Bacillus safensis* has also been shown to be present in garden soil (Tarangini and Mishra, 2014) and it could have been picked from the soil to the human feet. Isolates PTXV obtained from the front part of participants one and eight's feet and PTXVI which was obtained from the front part of participants one and four's feet formed the eighth cluster and they did not have any close reference matches based on phylogenetic analysis of 16S rRNA gene sequences. They should be identified further to help elucidate their role in the production of foot malodour. As noted in the relative diversity of the feet bacteria, the number of the isolated bacteria decreased from the most attractive participant to the least attractive participant. This offers a new avenue to explore why this happened. The highest number and diversity of bacterial isolates was from the front part of the feet as evidenced by isolates PTI-PTXVI while the lower baterial diversity was from the sole part of the participants feet evidenced by isolates PSI-PSV.

5.1.4 Antimicrobial response patterns

5.1.4.1 Antibiotic response patterns

Ciprofloxacin was notably the most effective antibiotic of the six antibiotics tested in this study. This may be due to its high broad-spectrum activity against most bacteria and also the reduced instances of its toxic effects (Javeed *et al.*, 2011). Ampicillin and Tetracycline had mixed outcomes against the bacterial isolates, though twelve of the bacterial isolates were susceptible to the two antibiotics. Isolate PTI, which was closely related to *Staphylococcus capitis* (Accession number CP007601), was isolated from the front feet part of all participants except for participant six and was susceptible to five of the six antibiotics tested; gentamycin, ampicillin, tetracycline, streptomycin and ciprofloxacin.

This suggests that this bacterial isolate is susceptible to commonly commercially available antibiotics. However, isolate PTI was resistant to penicillin suggesting that penicillin cannot be used to eliminate this bacterial isolate from the human feet. Studies have shown that most CoNS to which this isolate was closely related are resistant to penicillin (Ghadiri *et al*., 2012). As evidenced by its appearance in all the participants in this study, its susceptibility to most of the antibiotics is a good approach to control measures since *Staphylococcus capitis* is known to play a role in the production of foot odour (Wood and Kelly, 2010).

Isolate PTVI, which was closely related to *Staphylococcus simulans* (Accession number CP014016) was obtained from the front feet part of participants three and ten and was susceptible to antibiotics; gentamycin, ampicillin, tetracycline, streptomycin and ciprofloxacin suggesting that this bacterial isolate can be eliminated using commercially available antibiotics. However, the isolate was resistant to penicillin indicating that penicillin cannot be used in any control measures against this bacterial isolate. This may also suggest abuse and misuse of penicillin are common in malaria endemic regions. Being closely related to a coagulase-negative staphylococci, which have been documented to be highly resistant to penicillin (Ghadiri *et al*., 2012), eliminates penicillin from any control measures that may be put in place against the bacterial isolate. Isolate PTVII, which was closely related to *Bacillus pumilus* Accession number CP011007, was obtained from the front part of the second and ninth participants' feet and was susceptible to four of the antibiotics while it was intermediate for Tetracycline. these results are in concurrent with studies done by (DebMandal, 2012) on the antimicrobial sensitivity of *Bacillus pumilus.*

Isolate PTVIII, which was closely related to *Staphylococcus capitis* Accession number CP007601 was obtained from the front feet part of participants two, ten, seven, four and three and was resistant to both penicillin and tetracycline and was susceptible to the remaining antibiotics. This implies that as control measures for this bacterial isolate, Penicillin and tetracycline cannot be used. As earlier stated *Staphylococcus capitis* is part of the CoNS which are highly resistant to penicillin, this is in agreement with studies done by (Qu *et al.*, 2010). Isolate PTX, obtained from the front feet part of participants two, ten and seven, which closely matched to *Staphylococcus simulans* Accession number CP014016, was resistant only to penicillin but susceptible to all of the other antibiotics. These results are in agreement with studies done by (Lilenbaum *et al.*, 2000). *Staphylococcus simulans* is a CoNS and has been documented to be resistant to penicillin (Ma *et al*., 2011). Isolate PTXII, obtained from the front feet part of participants two and three, which was closely matched to *Staphylococcus capitis* Accession number CP007601, was susceptible to ciprofloxacin, gentamycin, tetracycline, ampicillin and streptomycin. This is in agreement with studies carried out by (Akinkunmi and Lamikanra, 2010). Isolate PTXII, which is similar to isolates PTI and PTVIII, was resistant to penicillin which is a characteristic antibiotic profile for coagulase-negative staphylococci (Fowoyo and Ogunbanwo, 2017). Being that these isolates do play a role in the production of foot odour, having such effective antibiotics against them is a great remedy.

Isolate PTIII, obtained from the front feet part of participants two, ten, seven, four, one, five and six, which was closely related to *Staphylococcus xylosus* (Accession number CP013922), was susceptible to four of the antibiotics; gentamycin, tetracycline, streptomycin and ciprofloxacin suggesting that commonly used commercially available antibiotics can be used to control this bacterial isolate. Isolate PTIII was resistant to

penicillin and ampicillin. This results are in agreement with studies done by (Ma *et al.*, 2011) where the bacteria was resistant against penicillin but susceptible to both ciprofloxacin and gentamycin. As noted earlier most CoNS are resistant to penicillin (Duran, *et al.*, 2012) and so is isolate PTIII implying that as a control measure penicillin and ampicillin cannot be relied upon to effectively wipe out this isolate. Isolate PSI, which was closely matched to *Staphylococcus xylosus* Accession number CP013922, was resistant to penicillin. This is characteristic of most CoNS which was evident in isolate PTIII. Isolate PSI was intermediate to ampicillin and susceptible to gentamycin, Tetracycline, Streptomycin and ciprofloxacin. The control for the experiment was *Escherichia coli* ATCC number 8739 and was resistant to penicillin, ampicillin and tetracycline, this is same as reported by (Cambrea, 2014). *Escherichia coli* was susceptible to gentamycin, streptomycin and ciprofloxacin which was in tandem with studies done by (Kibret and Abera, 2011; Sukumaran *et al.*, 2012).

Ciprofloxacin antibiotic proved to be the most effective antibiotic in the study. This is exhibited by the high zones of inhibition for the majority of the bacterial isolates. This is also supported by the fact that all the bacterial isolates plus the control were all susceptible to the antibiotic. Majority of the bacterial isolates were resistant to penicillin antibiotic and this can be attributed to several resistance mechanisms that are employed by the bacterial isolates. These include expression of the low-affinity penicillin binding protein (PBP2a) that is encoded by the *mecA* gene, production of methicillinase enzyme and decreased binding capacity of PBP3.

5.1.4.2 Antiseptic response patterns

The results from this study indicate that the antibacterial effects of the antiseptics and disinfectants do not only depend on the types of the antiseptics and disinfectants, but also on their concentrations. This is similar to results that were found by (Saha *et al.,* 2011). Out of the three antiseptics tested in this study, antiseptic A was seen to be the most effective antiseptic. Antiseptic A has two active ingredients; Chlorhexidine and Cetrimide. Chlorhexidine, which is 0.5 % in 70 % alcohol, is categorized as a broad spectrum, bactericidal antiseptic. Chlorhexidine's mode of action is by the destruction of the bacterial cell membrane and also by precipitating the cell's cytoplasm. Chlorhexidine has been shown to have a broad range of activity combined with its longer residual activity (Horner *et al.*, 2012). The action of chlorhexidine is further improved when it is combined with Cetrimide, which is a quaternary ammonium compound. Cetrimide acts a cation surface disinfectant (GUNESER *et al.*, 2016). It usually dissociates in aqueous solutions into a small inactive anion and a relatively large complex cation. The large complex cation acts as an emulsifying bond between the lipid secretions and water on the skin surface. Coupled with this emulsifying detergent property, Cetrimide also has a bactericidal activity exhibited against both Gram-positive and Gram-negative bacteria (Haidar, 1977). The combined action of these two ingredients in antiseptic A could explain why it had high effects against the feet derived isolates. Of major importance are the bacterial isolates that were implicated in the production of foot odour. These isolates were PTI, PTVIII and PTXII, closely related to *Staphylococcus capitis* and isolates PTVI and PTX, closely related to *Staphylococcus simulans.* Antiseptic A was highly effective against all these five bacterial isolates as evidenced by the high mean zones of inhibition. The mean zones of inhibition did decrease from dilution one to dilution two attributed to the reduction in the

concentration of the two active ingredients due to dilution of the initial concentration with sterile distilled water.

Antiseptic B effect against the isolated feet bacteria came in second. It has one active ingredient, Chloroxylenol (4-chloro-3,5-dimethylphenol) (Shenoy *et al.*, 2012). Chloroxylenol is a phenolic compound whose mode of action is by acting on the cell membrane and also inactivates the intracytoplasmic enzymes. This is followed by formation of unstable complexes. The lipophilic molecules that are formed are then trapped in the phospholipids present in the membrane. Chloroxylenol action is more widespread and effective against Gram-positive bacteria than in Gram-negative bacteria (Ali *et al.*, 2015). Chloroxylenol as a phenolic-type antimicrobial agent also induces the progressive leakage of intracellular components which include the release of potassium ions leading to the death of the bacterial cells (Barah, 2013). The isolates that were implicated for their role in the production of volatiles were PTI, PTVIII and PTXII, closely related to *Staphylococcus capitis* and isolates PTVI and PTX closely related to *Staphylococcus simulans*. Antiseptic B was highly effective against isolates PTVI and PTXII as shown by the high mean zones of inhibition. Antiseptic performed dismally against isolates PTI and PTX as evidenced by the low zones of inhibition.

Of the three antiseptics tested, antiseptic C performed poorly in its efficacy against the isolated feet bacteria. The active ingredient in antiseptic C antiseptic is Parachloro-meta-xylenol (PCMX). Its mode of action is by disrupting the microbial cell wall (Brahma *et al.*, 2016). It also acts by inactivating enzymes. PCMX has good antimicrobial activity against Gram-positive bacteria but is not as effective when compared to other antiseptics (Brahma *et al*., 2016). Parachloro-meta-xylenol is usually considered intermediate to slow acting

and it has minimal persistent effects (Digison, 2007). The effect of antiseptic C on bacterial isolates implicated in the production of feet odour. Isolate PTVIII, closely related to *Staphylococcus capitis* did show high zones of inhibition for both dilutions one and two. This was seen similarly for the other two antiseptics effect on this isolate. However, the effect of antiseptic C on isolates PTI and PTXII, closely related to *Staphylococcus capitis* and isolates PTVI and PTX, closely related to *Staphylococcus simulans* was dismal as shown by the low zones of inhibition. Compared to the effects of antiseptics A and B on these isolates it is clear that antiseptic C antiseptic was not highly effective.

Antiseptic A aided by the combined action of its two active ingredients (Chlorhexidine and Centrimide) was shown to be the best and effective antiseptic in the control of the feet bacterial isolates as most of them were susceptible. This was evidenced by the high mean zones of inhibition. Antiseptic A should then be used by individuals in malaria-endemic regions to clean their feet as by eliminating these feet bacteria, it reduces the production of foot malodour. This in turn reduces the attraction of female *Anopheles gambiae s.s* to the feeding sites on the human hosts. This will then reduce the transmission of the *Plasmodium* parasites hence reducing malaria incidences in these regions.

5.2 Conclusions

i. Female *An. gambiae s.s* mosquitoes manifested different levels of attraction to the individual males' feet odour.

ii. This study showed that there was varied bacterial diversity in the feet of the individual males.

iii. Ciprofloxacin antibiotic was the most effective antibiotic of the six antibiotics tested against the nineteen bacterial isolates. Antiseptic A was the most effective antiseptic of the three antiseptics tested.

5.3 Recommendations

i. There should be further investigations on the different levels of attraction of female mosquitoes to site-specific volatiles (in between the toes and sole of the foot).

ii. There should be isolation and characterization of either attractive or repellent volatile blends produced by the feet derived bacterial isolates.

iii. Culture-independent techniques should be employed to clearly identify the community structures comprising of culturable and non-culturable microbiota present on the feet of individuals exhibiting different levels of attraction.

5.4 Further studies

i. The specific roles played by the feet derived bacterial isolates in the production of volatile blends, independently or in synergism should be investigated.

ii. There should be investigations on the effects of plant extracts on the feet derived bacterial isolates. This should be followed by formulation of a product that can be used in malaria endemic regions to eliminate the feet microbiota resulting in reduced production of volatile blends that mediate the attraction of female *An. gambiae s.s.*

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APPENDICES

Appendix I Participant Written Consent Forms

Informed Consents for Participation

Research Title: **Profiles and antimicrobial response patterns of human feet bacterial isolates of individuals with differential attraction to *Anopheles gambiae* in Kilifi, Kenya.**

Investigators: Gathiru James Muhunyu, Kenyatta University, Dr James Nonoh, Kenyatta University, Professor Ahmed Hassanali, Kenyatta University.

Purpose of The Study: the study is aimed at isolating and characterizing bacteria from feet of persons with differential attraction to mosquitoes. The study is also aimed at determining antimicrobial compounds viable against these bacteria. There will be no follow-ups of the participants. Feet swabs will be obtained from the participants in a one-time basis.

Procedure: the research study will entail swabbing of feet of the participants. Sterile cotton swabs will be used in swabbing to obtain the samples.

Risks and Discomforts.

There will be no risks or discomforts involved in swabbing of the feet.

Withdrawal from Participation.

If you feel are not in any way comfortable carrying on with the study, you are free to cancel your participation. You do have a choice to cancel your participation and your willingness to take part in the study will be highly appreciated. If consent for participation of the participant is withdrawn, samples will not be collected form the said participant.

Contact Information.

In case of any queries regarding this study, kindly contact Gathiru James Muhunyu, mobile number: 0719709702.

Queries regarding your participation rights should be addressed to the secretary:

Kenyatta University Ethics Review Committee,

P.O B.O.X 43844-00100.

Tel: 8710901/12

Fax: 8711242/8711575

Email: chairman.kuerc@ku.ac.ke , secretary.kuerc@ku.ac.ke , ercku2008@gmail.com

Participant's consent.

Having read and totally understood the intended purpose of the study, I willingly agree to take part in it.

Signature…….

Date……

Note: by appending your signature, you are agreeing that you have read this consent form and have had the opportunity to ask any questions.

Your partaking in this study is at your own free will.

Witness.

Signature……. Date……

The participant received a copy ☐

Appendix II Ethical approval letter

KENYATTA UNIVERSITY
ETHICS REVIEW COMMITTEE
Fax: 8711242/8711575
Email: kuerc.chairman@ku.ac.ke

Our Ref: **KU/R/COMM/51/616** Date: 24TH March 2016

Dear Muhunyu,

APPLICATION NUMBER- PKU/439/I348- ''PROFIES AND ANTIMICROBIAL RESPONSE PATTERNS OF HUMAN FEET BACTERIALISOLATES OF INDIVIDUALS WITH DIFFERENTIAL ATTRACTION TO *ANOPHELES GAMBLE* IN KILIFI COUNTY,KENYA''.

1. **IDENTIFICATION OF PROTOCOL**

The application before the committee is with a research topic **PKU/439/I348 "'PROFIES AND ANTIMICROBIAL RESPONSE PATTERNS OF HUMAN FEET BACTERIALISOLATES OF INDIVIDUALS WITH DIFFERENTIAL ATTRACTION TO *ANOPHELES GAMBLE* IN KILIFI COUNTY,KENYA''.** " received on February 2016th and deliberated on 12 March 2016.

2. **APPLICANT**

Gathiru James Muhunyu

3. **SITE**

Kilifi,Kenya

4. **DECISION**

The committee has considered the research protocol in accordance with the Kenyatta University Research Policy (section 7.2.1.3) and the Kenyatta University Ethics Review Committee Guidelines, and APPROVED that that the research proceed for aperiod of ONE yearfrom 22nd March,2016.

i. Progress reports are submitted to the KUERC every six months and afull report is submitted at the end of the study.
ii. Serious and unexpected study adverse events related to the conduct of study are reported to this board immediately they occur.
iii. Notify the Kenyatta University Ethics Committee of any amendments to the to protocal.
iv. Submit an electronic copy of the protocol to KUERC

When replying, kindly quote the application number above.
If you accept the decision reached and advice and conditions given please sign in the space provided below and return to KU-ERC a copy of the letter.

28 MAR 2019

PROF. JUDITH KIMIYWE
CHAIRMAN ETHICS REVIEW COMMITTEE

I ..... accept the advice given and will fulfill the conditions therein.

Signature..... Dated this day of... 30/3/2016.....2016.

cc.
DVC: Research Innovation and Outreach

Appendix III Approval Kenyatta university

KENYATTA UNIVERSITY
GRADUATE SCHOOL

E-mail: dean-graduate@ku.ac.ke
Website: www.ku.ac.ke

P.O. Box 43844, 00100
NAIROBI, KENYA
Tel. 810901 Ext. 57530

Internal Memo

FROM: Dean, Graduate School

DATE: 4th November, 2015

TO: Gathiru James Muhunyu
C/o Microbiology Department

REF: 156/28267/14

SUBJECT: APPROVAL OF RESEARCH PROPOSAL

This is to inform you that Graduate School Board, at its meeting of 4th November, 2015, approved your Research Proposal for the M.Sc Degree Entitled, "Profiles of Human Foot Microbiota of Individuals with Differential Attraction to *Anopheles gambiae* in a Malaria Endemic Region in Kenya".

You may now proceed with your Data Collection, subject to clearance with Director General, National Commission for Science, Technology and Innovation.

As you embark on your data collection, please note that you will be required to submit to Graduate School completed Supervision Tracking Forms per semester. The form has been developed to replace the Progress Report Forms. The Supervision Tracking Forms are available at the University's Website under Graduate School webpage downloads.

Thank you.

KENYATTA UNIVERSITY OFFICE OF DEAN 04 NOV 2015 P.O. Box 43844-00100 NAIROBI

JULIA GITU
FOR: DEAN, GRADUATE SCHOOL

c.c. Chairman, Department of Microbiology

Supervisors

1. Dr. James Nonoh
   C/o Microbiology Department
   Kenyatta University

2. Prof. Ahmed Hassanali
   C/o Chemistry Department
   Kenyatta University

JG/bkk

Appendix IV NACOSTI Research permit

NACOSTI

NATIONAL COMMISSION FOR SCIENCE, TECHNOLOGY AND INNOVATION

Telephone:+254-20-2213471,
2241349,3310571,2219420
Fax:+254-20-318245,318249
Email:dg@nacosti.go.ke
Website: www.nacosti.go.ke
when replying please quote

9th Floor, Utalii House
Uhuru Highway
P.O. Box 30623-00100
NAIROBI-KENYA

Ref: No **NACOSTI/P/16/41741/10423**

Date: **9th May, 2016**

James Muhunyu Gathiru
Kenyatta University
P.O. Box 43844-00100
NAIROBI.

RE: RESEARCH AUTHORIZATION

Following your application for authority to carry out research on ***"Profiles of human foot microbiota of individuals with differential attraction to anopheles gambiae in a malaria endemic region in Kenya,"*** I am pleased to inform you that you have been authorized to undertake research in **Kwale County** for the period ending **5th May, 2017.**

You are advised to report to **the County Commissioner, the County Director of Education and the County Coordinator of Health, Kwale County** before embarking on the research project.

On completion of the research, you are expected to submit **two hard copies and one soft copy in pdf** of the research report/thesis to our office.

BONIFACE WANYAMA
FOR: DIRECTOR-GENERAL/CEO

Copy to:

The County Commissioner
Kwale County.

The County Director of Education
Kwale County.

The County Coordinator of Health
Kwale County.

THIS IS TO CERTIFY THAT:
MR. JAMES MUHUNYU GATHIRU
of KENYATTA UNIVERSITY, 0-20100
Nakuru,has been permitted to conduct
research in *Kwale County*

on the topic: *PROFILES OF HUMAN FOOT MICROBIOTA OF INDIVIDUALS WITH DIFFERENTIAL ATTRACTION TO ANOPHELES GAMBIAE IN A MALARIA ENDEMIC REGION IN KENYA*

for the period ending:
5th May,2017

.....
Applicant's Signature

Permit No : NACOSTI/P/16/41741/10423
Date Of Issue : 9th May,2016
Fee Recieved :Ksh 1000

.....
Director General
National Commission for Science, Technology & Innovation

CONDITIONS

1. You must report to the County Commissioner and the County Education Officer of the area before embarking on your research. Failure to do that may lead to the cancellation of your permit
2. Government Officers will not be interviewed without prior appointment.
3. No questionnaire will be used unless it has been approved.
4. Excavation, filming and collection of biological specimens are subject to further permission from the relevant Government Ministries.
5. You are required to submit at least two(2) hard copies and one(1) soft copy of your final report.
6. The Government of Kenya reserves the right to modify the conditions of this permit including its cancellation without notice

REPUBLIC OF KENYA

National Commission for Science, Technology and Innovation

RESEARCH CLEARANCE PERMIT

Serial No. A 9053

CONDITIONS: see back page