MIDGUT BACTERIAL DIVERSITY ANALYSIS OF LABORATORY REARED AND WILD *Anopheles gambiae* AND *Culex quinquefasciatus* MOSQUITO VECTORS IN AHERO, KENYA

ERASTUS MWARINGA MWADONDO

MASTER OF SCIENCE

(Biotechnology)

JOMO KENYATTA UNIVERSITY OF AGRICULTURE AND TECHNOLOGY

2018

Midgut Bacterial Diversity Analysis of Laboratory Reared and Wild *Anopheles gambiae* and *Culex quinquefasciatus* Mosquito vectors in Ahero, Kenya

Erastus Mwaringa Mwadondo

A Thesis Submitted in Partial Fulfilment for the Degree of Master of Science in Biotechnology in the Jomo Kenyatta University of Agriculture and Technology

2018

DECLARATION

This thesis is my original work and has not been submitted for a degree in any other University.

Signature: _____ Date: _____

Erastus Mwaringa Mwadondo

This thesis has been submitted for examination with our approval as the University supervisors.

Signature: _____ Date _____

Dr. Remmy W. Kasili, PhD

JKUAT, Kenya

Signature: _____ Date _____

Dr. Amos E. Alakonya, PhD

JKUAT, Kenya

DEDICATION

I dedicate this work to my father Thomas Osman Mwaringa, my mother Mariam Kwekwe Mwadondo, my wife Phemina Deche and all my brothers and sisters. You always believed that I can do marvellous when I put my mind to.

ACKNOWLEDGEMENTS

First and foremost, I would like to express my heartfelt thanks to Moses Ogugo Head of Molecular Laboratory, Institute of Livestock Research Institute (ILRI), for his technical guidance and allowing me to use the available facilities in the laboratory. I am highly grateful to Amanuel Ghilamicael for his kind and prompt response to all enquiries during the data analysis. I express my sincere thanks to Dr. Shadrack Muya Zoology Department, JKUAT for granting me the opportunity to carry out my postgraduate studies.

My sincere thanks to my supervisors, I greatly appreciate their meticulous guidance, patience, encouragement, leadership. I am extremely thankful for their valuable support, encouragement during the course of the study.

I wish to convey my sincere thanks and acknowledgements to the JICA/ICMoB /Africa-*ai*-Japan for the financial support without which the study would not have been possible.

I wish to extend my gratitude to members of my family, my beloved mother, father, wife, brothers and sisters for their moral support, prayers and encouragement.

Above all, I thank the Almighty ALLAH, for giving me the inner strength and ability to accomplish this study.

MAY GOD BLESS YOU ALL!

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

LIST OF ABBREVIATIONS AND ACRONYMS

a.s.l	above sea level
ARCU	Animal Rearing and Containment Unit
CDC	Centre for Disease Control
DNA	Deoxyribonucleic acid
IBR	Institute for Biotechnology Research
ICIPE	International Centre for Insect Physiology and Ecology
ILRI	International Livestock Research Institute Nairobi
JKUAT	Jomo Kenyatta University of Agriculture and Technology
MPM	Meconial Peritrophic Matrix or Membrane
NCBI	National Council for Biotechnology Institute
OTU	Operational Taxonomic unit
PBS	Phosphate Buffered Solution
PCR	Polymerase chain reaction
QIIME	Quantitative Insight in Microbial Ecology
RDP	Ribosomal Database Project
rRNA	ribosomal Ribonucleic acid
UCLUST	Universal Clustering
UV	Ultra Violet rays
SSU	Small Sub Unit
TE	Tris Ethylene diamine tetra acetic acid
SRA	Sequence Reads Archive

ABSTRACT

Mosquitoes transmit a wide range of pathogens that cause diseases in human and other animals. Midgut symbiotic bacteria are known to play fundamental roles in the biology of mosquitoes, however knowledge of midgut bacterial communities associated with mosquitoes is scanty due to limitation of the isolation techniques based on culturing. The available culture techniques reduce the chances in determination of the microbial diversity, since they sometimes miss out on non-culturable microbes. High throughput methods that involve direct isolation and analyses of nucleic acids from samples have been found more feasible in microbial diversity studies. The main objective of this study, was the application of metagenomics to study the composition and diversity of midgut bacteria in field collected and laboratory reared adult female *Anopheles gambiae* and *Culex quinquefasciatus* mosquitoes. Female adult mosquitoes were dissected and their total microbial deoxyribonucleic acid (DNA) isolated from the pooled midgut extracts by using Purelink genomic DNA mini kit (Invitrogen). The 16S rRNA gene variable regions V4 of the extracted DNA were amplified. Library construction was performed following Illumina sequencing protocol. Sequences were analyzed using QIIME pipeline, taxonomy was assigned using BLASTn against SILVA 119. The R programming language and Vegan package were used to calculate Bray-Curtis dissimilarities between datasets, hierarchical clustering and diversity indices. Phylogenetic analysis was done using MEGA 6.0 software. Results showed that 145 operational taxonomic units (OTUs) were realized at 3% genetic distance based on 16S rRNA gene sequence. The 145 OTUs spanned 12 phyla; *Proteobacteria, Firmicutes*, *Bacteriodetes*, *Actinobacteria*, *Eukaryota*, *Gemmatimonadetes*, *Spirochaetae*, *Verrucomicrobia*, *Chloroflexi*, *Acidobacteria*, *Archeabacteria*, *Cyanobacteria* and the no blast hits. Microbial community composition based on OTUs showed significant difference between field collected and laboratory reared mosquitoes (χ^2=45.0799, p=3.2 x 10^{-5}). Similarly, there was a significant difference in community composition at OTU level between *Anopheles gambiae* and *Culex quinquefasciatus* (χ^2=31.2257,p=7.7 x 10^{-4}). This study demonstrates a high microbial composition and diversity among field collected *Anopheles gambiae* and *Culex quinquefasciatus* than the laboratory reared mosquitoes. The bacterial composition and diversity appeared to be influenced by the environment and the species of the mosquitoes.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Background of the study

Mosquitoes are primary transmission hosts for diseases like malaria, dengue, lymphatic filariasis and yellow fever in the tropics. These diseases cause millions of deaths every year (Chandel *et al*., 2013). Among the diseases, malaria is the most important vector born disease globally with an estimated 3.3 billion people at risk of being infected as reported by World Health Organization WHO, (2015). According to WHO, there were 214 million cases of malaria in 2015. The African region accounted for most of the global cases of malaria (88%) followed by South-East Asia region (10%) and Eastern Mediterranean region (2%) (WHO, 2015). Further, in 2015 there were an estimated 430,000 malaria death worldwide. Most of these deaths occurred in African region (90%) followed by South-East Asia region (7%) and Eastern Mediterranean region (2%). Majority of these deaths are caused by the parasite *Plasmodium falciparum* whose major vector in Africa is the mosquito species *Anopheles gambiae*. The *Anopheles gambiae* is widely distributed throughout the Afro-tropical belt (Boissière *et al*., 2012).

Culex quinquefasciatus is a mosquito vector for *Wuchereria bancrofti*, the filarial worm that causes filariasis and Japanese encephalitis virus (Pidiyar *et al*., 2004). Lymphatic filariasis is a major public health problem worldwide, it is estimated that 1.3 billion people from 83 countries are living with the disease or are at risk of infection (Chandel *et al*., 2013). On the East African coast including Kenya, the urban mosquito *Culex*

quinquefasciatus, is an important vector for *Wuchereria bancrofti* that causes lymphatic filariasis (Njenga *et al*., 2011).

Effective mosquito vector control strategies currently include insecticide treatment delivered through spraying houses or insecticide-impregnated mosquito nets. While these methods are effective at decreasing mosquito numbers, they as well contribute to an increase in resistance of the insect vector species (Bando *et al*., 2013).

Few studies have been conducted to identify bacterial species in field-collected *Anopheles* mosquitoes, using microbe culturing techniques. These studies have highlighted the breadth of bacterial flora associated with mosquitoes. Bacteria, like *Pseudomonas cepacia*, *Enterobacter agglomerans* and *Flavobacteria* sp. were found in higher abundance in laboratory-reared *A. stephensi*, *A. gambiae* and *A. albimanus* mosquitoes (Rani *et al*., 2009). Jadin *et al*. (1966) identified *Pseudomonas* sp. in the midgut of *Anopheles* mosquitoes from Democratic republic of Congo. Gonzalez-Ceron *et al*.(2003) isolated various *Enterobacter* and *Serratia* sp. from *Anopheles albimanus* mosquitoes captured in southern Mexico. Field-captured *A. gambiae* mosquitoes in Mwea in Kenya were reported to consistently associate with a *Thorsellia anophelis* lineage that was also detected in the surface microlayer of rice paddies (Briones *et al*., 2008). *Enterococcus faecalis*, *Acinetobacter soli* and *Enterobacter cloacae* were some of the bacterial species frequently isolated from midgut of *Culex quinquefasciatus* mosquitoes (Chandel *et al*., 2013). The mosquito midgut poses a critical challenge to the survival and development of the parasites and is therefore, the most attractive site to target malaria parasites (Whitten

et al., 2006). The symbiotic bacteria in the midgut of a mosquito can be genetically modified to express effector molecules then reintroduced into the mosquito for control of the disease carrying parasites (Chavshin *et al*., 2012).

The available conventional culture techniques limit the scope in determination of the diversity of microbes since it sometimes misses out on non-culturable microbes (Pidiyar *et al*., 2004). Amplicon sequencing, in particular that of the small subunit rRNA gene (16S rRNA gene in Bacteria and Archaea or 18S rRNA gene in Eukarya), is a widely applied approach to study the composition, organization and spatiotemporal patterns of microbial communities, due to its ubiquity across all domains of life (Head *et al*., 1998). In the last decades, 16S rRNA gene amplicons were analyzed using fingerprinting techniques such as (terminal restriction fragment length polymorphisms) TRFLP (Liu *et al*., 1997) and (automated method of ribosomal intergenic spacer analysis) ARISA (Fisher & Triplett, 1999) in combination with clone library construction and Sanger sequencing. However, this method often provided insufficient coverage to describe and compare microbial communities (Curtis *et al*., 2006).

High-throughput sequencing (HTS) technology and the application of barcode indexing are allowing the collection of thousands of sequences from a large number of samples simultaneously (Hamady *et al*., 2008). These approaches have revealed deeper insights into the diversity of microbial communities (Herlemann *et al*., 2011; Sogin *et al*., 2006) and by increasing sample numbers, have expanded the possibilities to study community and population dynamics over much finer temporal (Eiler *et al*., 2012) and spatial scales (Herlemann *et al*., 2011). New technology, such as pyrosequencing of hypervariable

regions of the 16S rRNA gene, is a cost-effective and a better alternative to examine the phylogenetic diversity of microbial populations in different ecosystems.

This study involved the use of Illumina Sequencing of PCR products of 16S rRNA gene to obtain a less biased estimation of the microbial community in the midgut of laboratory reared and field collected *Anopheles gambiae* and *Culex quinquefasciatus* mosquitoes.

1.2 Statement of the problem

Mosquito control still remains the primary strategy for controlling mosquito-borne diseases like malaria and filariasis/elephantiasis. However, insecticide resistance by mosquitoes, cost of new drug development, drug resistance of some parasites, environmental hazard of pesticide application and limitation of vaccines are factors that necessitate the need for the development of novel disease control strategies. Furthermore, global warming is anticipated to affect abundance and distribution of the *Anopheles gambiae* and *Culex quinquefasciatus* mosquito vectors. The mosquito midgut poses a critical challenge to the survival and development of the parasites and is therefore, the most attractive site to target malaria/filariasis parasites. The symbiotic bacteria in the midgut of a mosquito can be genetically modified to express effector molecules then reintroduced into the mosquito for control of the disease carrying parasites. Therefore, there is a need to explore the midgut microflora and develop novel control measures for mosquito borne diseases.

1.3 Justification

The knowledge on *Anopheles gambiae* and *Culex quinquefasciatus* midgut bacterial communities remains largely scanty, due to limitations of isolating techniques which are based on culturing. The available conventional culture techniques limit the scope in determination of the diversity of microbes since they sometimes miss out on non-culturable microbes. Further, culture dependent approach cannot be used for analysis of populations within natural communities because, less than 1% of the observed diversity can be cultured in the laboratory. It is therefore doubtful that such culture dependent approaches would help in the accurate description of microorganisms as they occur within natural environment.

This study involved application of metagenomic (DNA) analysis to determine the bacterial diversity in the midgut of laboratory reared and wild *Anopheles gambiae* and *Culex quinquefasciatus* mosquitoes to identify potential candidate bacteria that can be used in the control of mosquito borne diseases.

1.4 Null hypotheses

1.4.1 There is no difference in midgut bacterial diversity of field collected adult female *Anopheles gambiae* and *Culex quinquefasciatus* mosquito vectors from Ahero.

1.4.2 There is no difference in midgut bacterial diversity of the laboratory reared adult female *Anopheles gambiae* and *Culex quinquefasciatus* mosquito vectors.

1.5 General objective

To determine midgut bacterial diversity of adult female laboratory reared and wild *Anopheles gambiae* and *Culex quinquefasciatus* mosquito vectors.

1.6 Specific objectives

1.6.1 To determine midgut bacterial diversity of field collected adult female *Anopheles gambiae* and *Culex quinquefasciatus* mosquito vectors from Ahero.

1.6.2 To determine midgut bacterial diversity of laboratory reared adult female *Anopheles gambiae* and *Culex quinquefasciatus* mosquito vectors.

1.7 Research output

1. An analysis of midgut bacterial diversity of field collected adult female *Anopheles gambiae* and *Culex quinquefasciatus* mosquito vectors from Ahero.
2. An analysis of midgut bacterial diversity of laboratory reared adult female *Anopheles gambiae* and *Culex quinquefasciatus* mosquito vectors.
3. A comparison of midgut bacterial diversity of field collected verses laboratory reared mosquito vectors.

CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria prevalence in Kenya

For over the last 10 years, Kenya has made progress in controlling malaria. However, the country is still far from combating the disease. The prevalence of malaria in Kenya is shown on figure 2.1. Compromising the fight against malaria are factors such as poor knowledge of the disease and the lack of effective diagnostic equipment in many health facilities around the country. Furthermore, people do not take preventative measures seriously such as sleeping under insecticide treated nets.

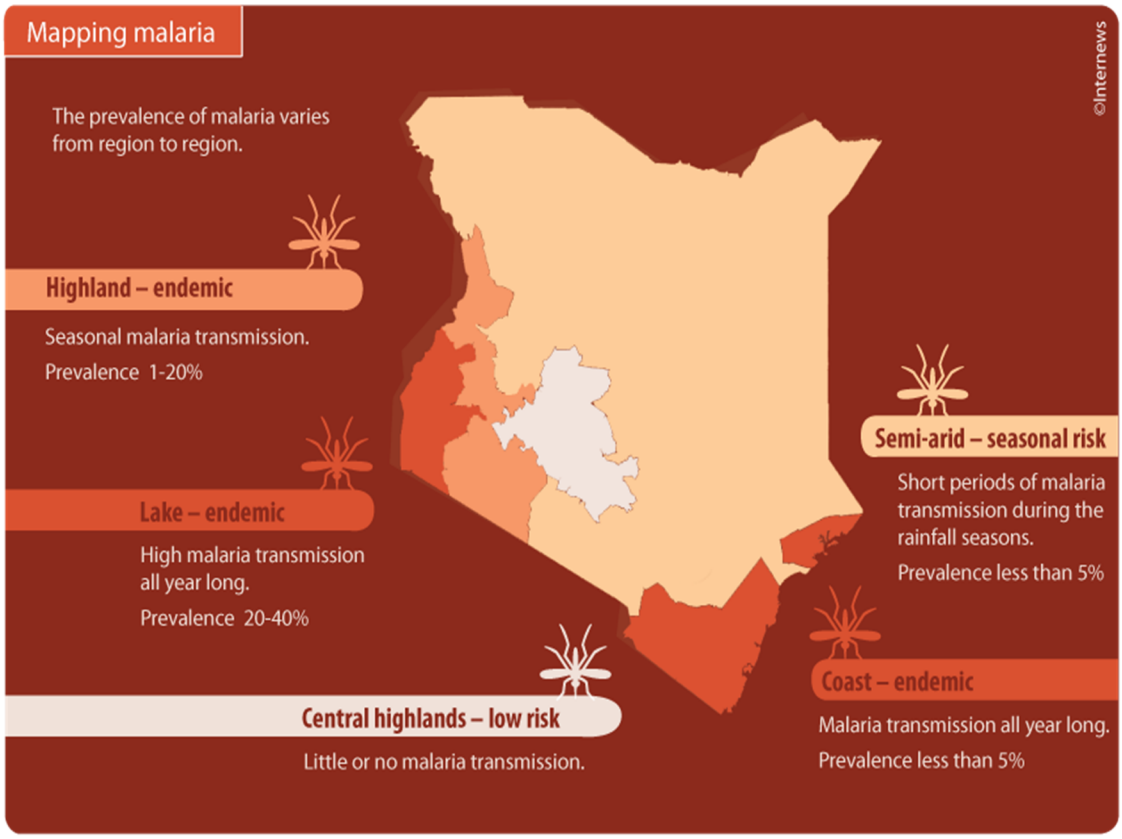

Figure 2.1: Map showing the malaria prevalence in Kenya. Source: Internews Data Dredger (2016)

A review of data reveals that there is currently less investment in malaria than in the past. Additionally, there is a plateau in the number of houses who own insecticide treated nets. In 2016 alone, Kenya lost more than 30,000 people due to malaria. To roll back malaria, the government must invest more in new initiatives and tools for fighting it even as it makes use of emerging epidemiological knowledge of the disease (Internews Data Dredger,2016).

2.2 *Anopheles gambiae* mosquito vectors

Anopheles gambiae Giles is the most efficient vector of human malaria in the Afrotropical region. Thus, it is commonly called the African malaria mosquito. The *Anopheles gambiae* complex sibling species (Fanello *et al*., 2002; Coetzee *et al*., 2013) comprises of eight reproductively isolated species that are almostly indistinguishable morphologically: *Anopheles amphariсus* Hunt *et al.*(2013), *Anopheles arabiensis* Patton 1905, *Anopheles bwambae* White 1985, *Anopheles gambiae* Giles 1902, *Anopheles coluzii* coetzee and wikerson 2013, *Anopheles melas* Theobald 1903, and *Anopheles mems* Donitz 1902. Collectively they are sometimes called *Anopheles sensu lato*, meaning 'in the wider sense'. Female *Anopheles gambiae* is shown on plate 2.1.

Adult female *Anopheles* can be differentiated from other mosquito genera because the palps (appendages found near the mouth) are as long as their proboscis (Coetzee *et al*., 2013). Adult *Anopheles* also have distinguishable resting position where their abdomen is raised into the air (Fanello *et al*., 2002). *Anopheles gambiae* have a variable body color, but it typically ranges from light brown to grey with pale spots of yellow, white or cream scales and dark areas on their wings (Gillies & De Meillon, 1968). In comparison to other

species, adults are considered small to medium sized mosquitoes with average wing length varying from 2.8 to 4.4 mm (Gillies & De Meillon, 1968).

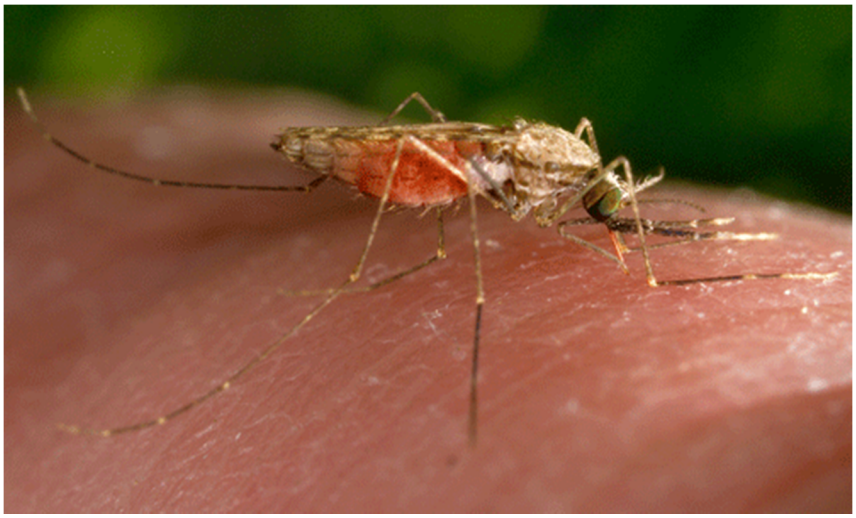

Plate 2.1: Female *Anopheles gambiae* Giles taking a blood meal (Source: CDC, 2010)

In Africa *Anopheles gambiae sensu lato*, is anthropophilic, endophagic and endophilic (prefer to feed on humans, and to feed and rest indoors) (Takken & Knols, 1999). These behavioural preferences together with its high susceptibility to *Plasmodium* infection provides a probable explanation why the African continent is more stricken by malaria than other continents (Besansky *et al*., 2004). *Anopheles funestus* is also anthropophilic, endophagic and endophilic. However this species is less susceptible to *Plasmodium* infection than *Anopheles gambiae* (Takken & Knols, 1999). *Anopheles arabiensis* varies from being anthropophilic to zoophilic in different studies from different areas (Takken & Knols, 1999). Both *Anopheles funestus* and *Anopheles arabiensis* are important vectors of malaria in some areas of the African continent (Fontenille & Simard, 2004).

2.3 Life Cycle of *Plasmodium falciparum*

The malaria parasites are host-specific, meaning that the four different species that can infect humans, *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*, do not live outside a human or an *Anopheles* mosquito host. Infections with *P. falciparum*, and to a much less extent *P. vivax*, cause severe disease and death from malaria in humans (Miller *et al*., 2002). The parasites have a complex lifecycle with several asexual stages in humans and sexual stages in mosquitoes (Miller *et al*., 2002).

The haploid sporozoites are the final stage in the mosquito and the infectious stage for humans. The sporozoites enter a human via the mosquito saliva during the bloodmeal and pass into the bloodstream. The sporozoites travel to the liver where they invade hepatocytes and start to divide mitotically.

Eventually the hepatocytes rupture and thousands of haploid merozoites are released into the bloodstream where they invade red blood cells and start the asexual erythrocytic lifecycle. Inside the erythrocytes, the parasites develop from merozoites to trophozoites and schizonts over a 48 h cycle. The schizonts divide into several merozoites and when the red blood cells rupture, the merozoites are released into the bloodstream and the cycle starts again. It is when the red blood cells rupture, that symptoms of malaria appear in the infected person, the most characteristic being fever (Miller *et al*., 2002). During the erythrocytic cycle, some of the parasites evolve into male and female gametocytes. When *Anopheles* mosquito takes up these gametocytes during a bloodmeal, they mate in the midgut of the mosquito to form a zygote. The zygote develops into an ookinete, which passes through the midgut epithelium and develops into an oocyst under the basal

membrane. Subsequently, the oocyst bursts and releases sporozoites into the hemolymph. The sporozoites travel to the salivary glands from where they can infect other humans during the next bloodmeal taken by the mosquito (Miller *et al*., 2002).The lifecycle of *Plasmodium* species lifecycle is illustrated on figure 2.2. The infection of the host erythrocytes by the parasites is responsible for the mortality and morbidity caused by this disease.

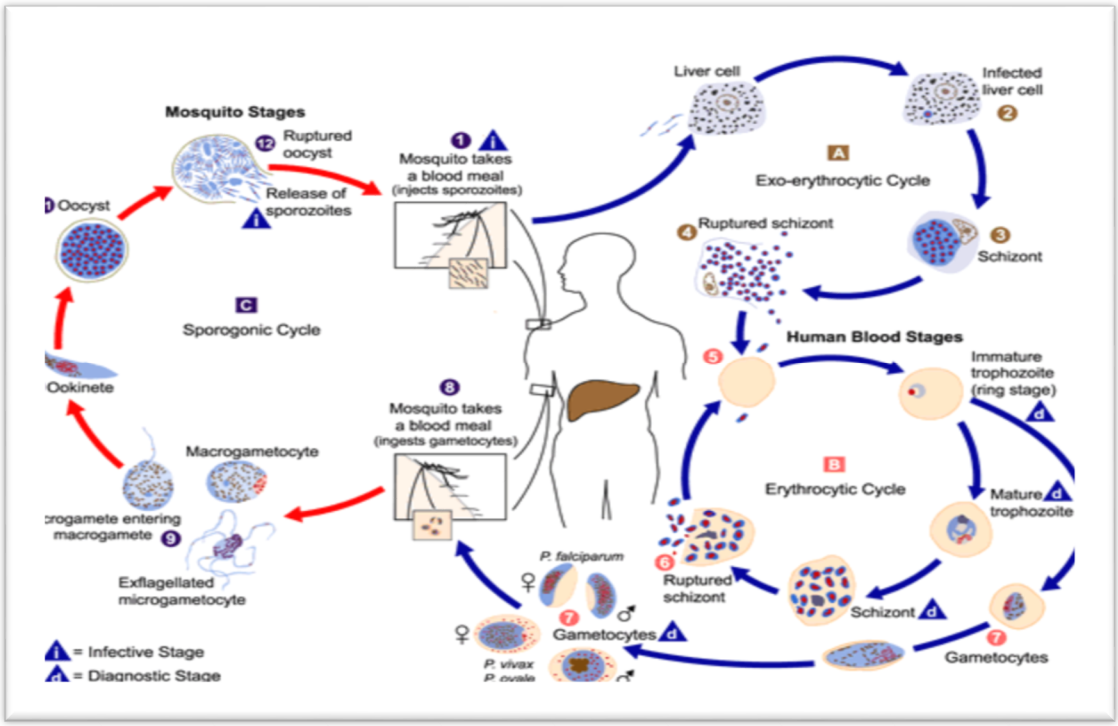

Figure 2.2: *Plasmodium* species lifecycle (Source: CDC, 2010)

2.4 *Culex quinquefasciatus* mosquito vectors

Adult *Culex quinquefasciatus* is a medium sized mosquito and is brown in colour. The body is about 3.96 to 4.25 mm long, while the main body is brown, the proboscis, thorax wings and tarsi are darker, than the rest of the body. The head is light brown with the

lightest portion in the centre. The antennae and the proboscis are about the same length but in the same cases, the antennae are slightly shorter than proboscis. The flagellum has 13 segments that may have few or no bands on the basal side of each targite. The female *Culex quinquefasciatus* is shown on plate 2.2. Males can be differentiated from female in having large palps and feathery antennae (www.ozanimals.com).

Plate 2.2: Female *Culex quinquefasciatus* mosquito (Source: CDC, 2011)

2.5 Life Cycle of *Wuchereria bancrofti*

During a blood meal, an infected mosquito introduces third-stage filarial larvae onto the skin of the human host, where they penetrate into the bite wound. They develop into adults that commonly reside in the lymphatics. The female worms measure 80 to 100 mm in length and 0.24 to 0.30 mm in diameter, while the males measure about 40 mm by 1 mm. Adults produce microfilariae measuring 244 to 296 µm by 7.5 to 10 µm, which are

sheathed and have nocturnal periodicity, except the South Pacific microfilariae which have no marked periodicity. The lifecycle of *Wuchereria bancrofti* is illustrated on figure 2.3.

The microfilariae migrate into lymph and blood channels moving actively through lymph and blood. A mosquito ingests the microfilariae during a blood meal. After ingestion, the microfilariae lose their sheaths and some of them work their way through the wall of the proventricus and cardiac portion of the mosquito's midgut and reach the thoracic muscles. There, the microfilariae develop into first-stage larvae and subsequently into third-stage infective larvae. The third-stage infective larvae migrate through the hemocoel to the mosquito's proboscis and can infect another human when the mosquito takes a blood meal.

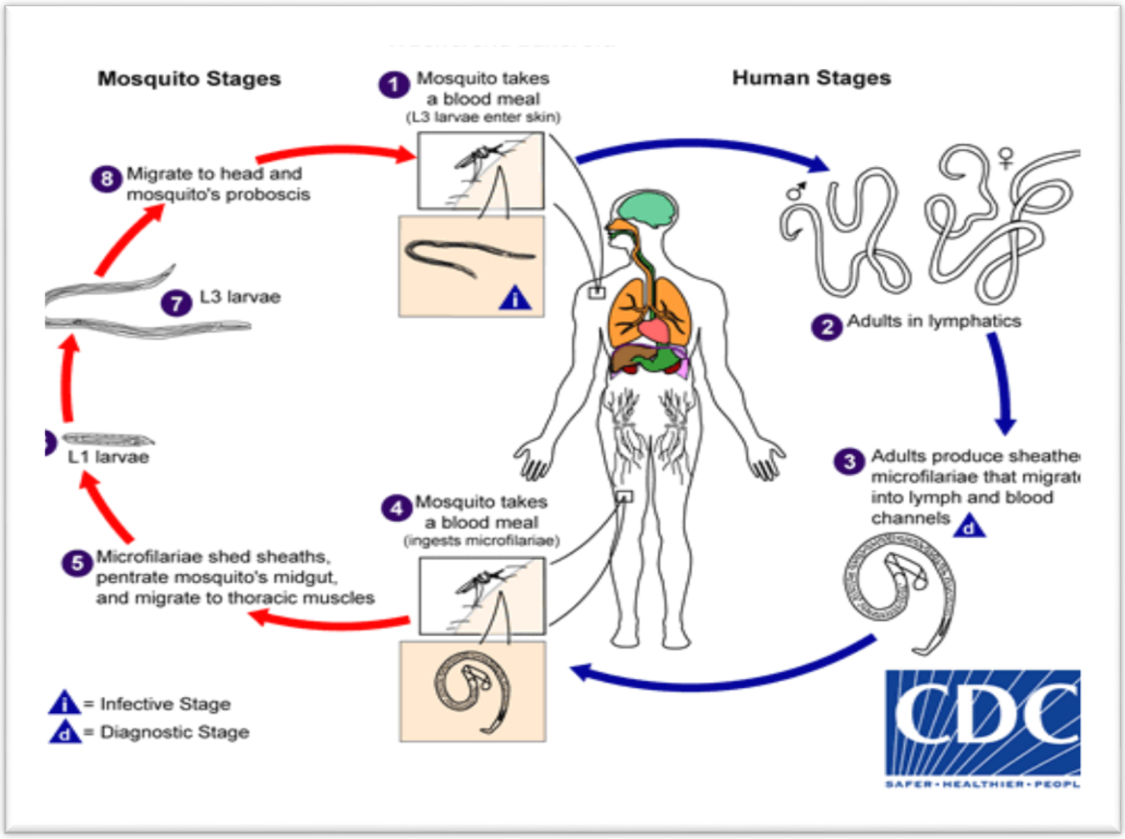

Figure 2.3: *Wuchereria bancrofti* lifecycle (Source: CDC, 2010)

2.6 Stages of mosquito development and microbial acquisition

Mosquitoes are holometabola that undergo four gradual stages of metamorphosis that is: egg, larvae, nymph, and adult that are intimately connected to their respective biotypes. Eggs, larvae and nymph stages are aquatic, whereas adult stage of mosquitoes live in terrestrial environments. The fraction of mosquito-associated microflora that is acquired from the surrounding environment is most likely to differ during the mosquito life cycle. At the larval stage, individuals consume bacteria and planktons as their sources of food. This allows a first stage bacterial colonization that adds to any inherited bacterial flora.

Some of these bacteria are members of genus *Wolbachia* and are vertically acquired transovarially in *Culex Pipiens*, *Culex quinquefasciatus* or *Ae. Albopictus*. Venereal transmission of the bacterium *Asia* was reported in *Anopheles gambiae* and *Anopheles stephensi* (Damiani *et al*., 2010; Cook & McGraw, 2010).

The midgut of mosquito larvae also contains many photosynthetic *cyanobacteria* acquired from breeding site (Wang *et al*., 2011). The mosquito gut represents an ecosystem that accommodates a complex, intimately associated microbiome. It is increasingly clear that the gut microbiome influences a wide variety of host traits, such as fitness and immunity. Understanding the microbial community structure and its dynamics across mosquito life is a prerequisite for comprehending the symbiotic relationship between the mosquito and its gut microbial residents. Wang *et al*.(2011) showed that in the larval and pupal stages, *cyanobacteria* were very abundant accounting for 40% of an entire microbial community in *Anopheles gambiae*.

During metamorphosis, the mosquito anatomy is radically modified. In particular, a first meconial peritrophic matrix or membrane (MPM1) is found early in the pupal stadium and the second (MPM2) emerges sometimes around the time of adult emergence (Moncayo *et al*., 2005). A recent study suggests that the MPMs contribute to the sterilization of the adult midgut by sequestering microorganisms ingested during the larval stage, which, along with remaining meconial material, are egested after adult emergence (Moll *et al*., 2001; Moncayo *et al*., 2005).

This phenomenon could explain why the proportions of different bacterial classes or phyla alter drastically between immature and adult stages. For example, it was shown that the

number of bacterial operational taxonomic units (OTU) was 3 fold higher in larvae and pupae than in imagos of *Anopheles gambiae* (Wang *et al*., 2011). To date, comparative studies of bacterial composition between stages have only been done in *Anopheles* mosquitoes, in which transtidial maintenance of some bacterial genera such as *Acinetobacter*, *Bacillus*, *Enterobacter*, *Staphylococcus*, *Pseudomonas*, *Chryseobacterium* and *Serratia* spp. has been observed (Rani *et al*., 2009).

2.7 Sex of mosquito and microbial diversity

The sex of the mosquito is also an important factor that affects bacterial composition. Male and female mosquitoes exhibit different ecological behaviors in terms of nutritional and dispersal capabilities. Both sexes feed on nectar and plant saps and are able to hydrolyze sucrose, but females are also hematophagous. Indeed, female mosquitoes are anautogenous as they require blood from vertebrates for the completion of their reproductive cycles (Foster, 1995). Blood digestion in females is also favoured by the selection of bacteria for their hemolytic ability (Gusmão *et al*., 2010). Moreover, after a mosquito ingests a blood meal a temperature burst occurs and oxidative stress and immune responses are down regulated, which leads to an increase in the bacterial load (Oliveira *et al*., 2011).

A mosquito-associated bacteria rely on some of the nutrients brought in the meal for growth, the nutrient composition of food sources may directly impact the diversity of bacteria present (Rani *et al*., 2009). Zouache *et al*. (2011) showed that around half of the bacterial diversity in field populations of *Ae. albopictus* was explained by the sex of the

mosquito with greater diversity observed in females (Zouache *et al*., 2011). The effect of the sex of the mosquito on bacterial diversity was reported in field populations of the malaria vector *Anopheles stephensi*; bacteria from genera *Bacillus* and *Staphylococcus* were detected in males, whereas bacteria from genera *Chryseobacterium*, *Pseudomonas* and *Serratia* were present exclusively in females (Rani *et al*., 2009). Published data on mosquito–associated bacteria, shows that the midgut of females is mostly colonized by members of *Gammaproteobacteria*, as is found in other blood feeding insects. Interestingly, the genera *Pseudomonas*, *Serratia* and *Enterobacter* are frequently associated with females of several mosquito species (Gusmão *et al*., 2007). In contrast, the midgut of males is dominated by bacteria from the phylum *Firmicutes* including those from *Staphylococcus*, *Bacillus*, *Paenibacillus* and *Micrococcus* genera (Rani *et al*., 2009). It was also shown that diet, whether sugar or blood meals, significantly affects the bacterial population structure. Wang *et al*. (2011) demonstrated that blood meals drastically reduced the community diversity in favor of enteric bacteria in the *Anopheles gambiae*, while few changes were observed following sugar meals. However, irrespective of the type of meal after 4 days the bacterial microflora reestablishes itself as demonstrated by the genus *Elizabethkingia*. Male mosquitoes disperse less than females and tend to remain close to breeding sites which could be an additional factor constraining bacterial diversity in them (Foster, 1995).

2.8 Midgut microbiome of mosquitoes

Complex microbiotae have been described in mosquito midgut, these include Gram-

negative rods, including *Serratia marcescens*, *Klebsiella ozaenae*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Enterobacter* sp. (Azambuja *et al*., 2005).Three metagenomic studies provided a more comprehensive picture of the diversity of midgut microbiota in *Anopheles gambiae*, the main malaria vector in Africa (Boissière *et al*., 2012; Osei-Poku *et al*., 2012; Wang *et al*., 2011). In wild caught adults of *Anopheles* species, the microbiota revealed the presence of *Pseudomonas* and *Aeromonas* species. The five genera, *Asaia*, *Bacillus*, *Chryseobacterium*, *Klebsiella*, and *Pantoea* have been reported from four field collected *Anopheles* species, while *Serratia* and *Stenotrophomonas* were identified in three mosquito species. Three mosquito-specific bacterial species, isolated from the midgut of main malaria vectors of the *Gambiae* Complex, have been described, such as *Thorsellia anopheles* (Kampfer, 2006), *Janibacter anophelis* (Kampfer, 2006) and *Elizabethkingia anopheles* (Kämpfer *et al*., 2011).

Bacteria of the genus *Asaia* have also been associated with *Anopheles* species, in particular field-collected *Anopheles gambiae*, *Anopheles funestus*, *Anopheles coustani* and *Anopheles maculipennis*, as well as a colony of *Anopheles stephensi* in which *Asaia* bacteria was dominant and stably associated (Favia *et al*., 2007).

The presence of *Asaia* species in *Anopheles* could serve as candidate for malaria control based on the production of antiparasite molecules in mosquitoes for use in paratransgenic control of malaria (Damiani *et al*., 2010; Favia *et al*., 2007). Other bacterial species have been defined as antimalarial agents, especially those producing prodigiosin, a pigment produced by various bacteria, including *S. marcescens* (Azambuja *et al*., 2005). The

normal midgut microbiota of *Anopheles* mosquitoes need to be further identified as only few studies have reported the microbiota of wild caught malaria vectors (Chavshin *et al*., 2014). Further investigations of gut microbiota, especially of wild-caught insect vectors, might contribute to understanding the annual and regional variations recorded for vector transmitted diseases and yield novel vector-control strategies (Manguin *et al*., 2013).

2.9 Interactions between microbial communities

Bacterial interactions are important regulators of ecosystem characteristics and species density. The gut is naturally protected by a heterogeneous bacterial biofilm, a community of microorganisms living inside an adhesive matrix that forms a mutual structure. Pathogen colonization directly alters (dysbiosis) the biofilm structure (Reid *et al*., 2011). Some recent studies have focused on the positive and negative interactions between bacteria inside insect hosts. Terenius *et al*.(2012) tested bacterial interspecies competition with isolates from *Ae. Aegypti* and showed that *Serratia marcescens* could create an inhibition zone area on *Sphingomonas* and members of the family *Burholderriaceae*. It is suggested that a potential link exist between the presence of *S. marcescens* and the low bacterial diversity observed in the mosquito midgut. Competitive colonization was previously reported in the desert locust *Schistocerca gregaria* where bacterial diversity was shown to increase in the absence of *S. marcescens* (Dillon, 2002). Recent statistics show convincing association between the bacteria *Asia* and *Acinectobacter* in *Ae. Albopictus* (Minard *et al*., 2013). Even though additional analysis are still needed to better understand the degree of interactions between the two genera, bacterial interactions seem

to be synergistic because more *Asia-Acinectobacter* double-infections were observed than would be expected if bacteria acted independently.

Bacterial symbionts associated with mosquito vectors have recently been found to interact with pathogens they transmit, modifying the outcome of the multiple interactions. For instance, it was shown that removing bacterial communities from *Anopheles gambiae* increased its susceptibility to *Plasmodium falciparum* infection (Dong *et al*., 2009). On the contrary, Boissière *et al*.(2012) demonstrated that the presence of some bacteria could favor parasite infection, as they found a positive correlation between the abundance of members of the *Enterobacteriaceae* family in the mosquito midgut and the *Plasmodium* infection status. Conversely, Zouache *et al*. (2012) demonstrated that Chikungunya virus infection could modify the diversity of symbiotic bacteria in *Ae.albopictus*. Indeed, taxonomic microarray and quantitative PCR analyses showed that the abundance of *Enterobacteriaceae* increased with Chikungunya virus infection, whereas the abundance of some other bacterial genera such as *Wolbchia* and *Blattabacterium* decreased Chikungunya virus (Zouache *et al*., 2012). All these results suggest that complex microbial interactions (direct or indirect, co-operation or competition) occur between pathogens and microbiota that may affect mosquito traits such as vector competence.

2.10 Mosquito bacterial symbionts with potential in vector borne disease control

The mosquito-microbiota interaction has raised more interest in the possibility of genetically transforming mosquito symbionts to express anti-parasite effector molecules

to develop effective diseases-control strategies. One example of strategy involves the symbiont bacterium *Rhodococcus rhodnii*, which is naturally resident in the gut lumen of the triatomine vector *Rhodnius prolixus* contributing to vector nutrition. These symbionts have been transformed with some antiparasite effector genes. Increasingly, laboratory paratransgenic populations of triatomide unable to transmit the diseases have been generated for some years (Beard *et al*., 2001).

An approach aimed at introducing the genetically modified bacterial symbionts into natural populations of Chagas disease vectors has already been developed by the coprophagic behaviour (Beard *et al.*, 2001). A number of relevant interactions between symbionts and mosquito have already been described and a few symbionts have been identified as potentially effective for *Symbiotic Control Strategies* to combat mosquito-borne diseases. In this context *Asia* and *Pantoea* bacteria are potentially very useful (Beard *et al*., 2001).

Recent *Pantoea agglomerans*, another bacterial symbiont of *Anopheles* mosquitoes has been engineered to express and secret anti-plasmodium effector proteins, such as pectate lyase B (pelB) from *Erwinia carotovora* or hemolysin A (hlyA) secretion signals from the genes of related species and from *Escherichia coli* (Bisi & Lampe, 2011). These strains are now under evaluation for *plasmodium* activity in infected mosquitoes.

2.11 Paratransgenics in control of mosquito borne diseases

One approach in the fight against vector borne diseases is paratransgenics. In this approach bacteria are used to produce a molecule that kills, or stops the development of,

the causative agent of the disease (Beard *et al.*, 2002) . For a paratransgenic approach on mosquitoes, there are two main options for reaching mosquitoes in the field with transgenic bacteria in the gut. One of the options is mass rearing of mosquitoes that are fed on the transgenic bacterium and then releasing them in the field; the other option is mass cultivation of bacteria that are introduced to the mosquito in the field. Apart from the obvious controversy with releasing high numbers of female mosquitoes in the field, the insectary-reared mosquitoes may have a lower fitness than the mosquitoes present in the environment, leading to the extinction of the paratransgenic mosquitoes. In addition, it is much easier to cultivate bacteria than to rear mosquitoes. Therefore, the second option, introduction of transformed bacteria to mosquitoes in the field seems more likely to be successful (Minard *et al*., 2013).

Studies have shown that the paratransgenic technique is feasible in *Anopheles* mosquitoes (Riehle *et al.*, 2007). Yoshida*et al*.(2001) transformed *Escherichia coli* with a plasmid expressing a Cecropin A fusion protein. Cecropin A are antimicrobial peptides active against both Gram-positive and Gram-negative bacteria. The bacteria were fed to *Anopheles stephensi* and almost completely inhibited the development of *Plasmodium berghei* in the mosquito. Riehle *et al*.(2007) modified *E. coli* to display two different anti-*Plasmodium* molecules on the cell surface. These modified *E*. *coli* were fed to *Anopheles stephensi* mosquitoes and following an infected blood meal a clear reduction of *P*. *berghei* development was observed compared to mosquitoes fed on bacteria without the effector molecules. In the same study, an increase of the number of midgut bacteria post bloodmeal was observed. Similar bacterial growth after an ingested bloodmeal has

previously been observed in the same and other mosquito species. Pumpuni *et al*. (1996) showed an 11-fold to 40-fold increase 24 h after a bloodmeal for *Anopheles gambiae* and *Anopheles stephensi*, respectively. This is important since an increase in the number of bacteria leads to an increase of effector molecules at the same time when the parasite is present in the gut. Paratransgenic *Anopheles* mosquitoes can be developed in a relatively short time frame, although several significant hurdles still need to be overcome. Two of them are identifying a bacterium that is sustainable in the midgut of the mosquito (that can be genetically modified) and the delivery of the transformed bacteria into mosquito populations in the field.

2.12 Methods of investigating microbiota in mosquitoes

Complementary approaches are needed for in-depth analyses of microbial communities in complex ecosystems. Both culture-dependent and culture-independent techniques have been used to explore mosquito microbiota. Some microflora can be cultured by using various isolation procedures and media so that bacterial taxa can be identified (Apte-Deshpande *et al*., 2012). The main difficulty of the culture dependent approach is in the recreating the complex physiological environment of the insect body (Dillon & Dillon, 2004). To overcome this limitation and more thoroughly identify bacteria hosted by mosquito populations, culture-independent methods such as Denaturating Gradient Gel Electrophoeresis fingerprints, taxonomic microarrays, and meta-taxogenomics can be used.

The use of 16S rRNA gene sequence to study bacterial phylogeny and taxonomy has been

by far the most common housekeeping genetic marker used for a number of reasons: its presence in almost all bacteria, often existing as a multigene family or operon, the functions of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are more accurate measure of time evolution and the 16S rRNA gene is large enough for informatics purposes (Patel, 2001). The 16S rRNA gene is a component of the 30S subunit of prokaryotic ribosomes. It is 1,542 bp in length. Its acts as a scaffold defining the positions of the ribosomal protein. The 3' end contains the anti-Shine-Dalgarno sequence which binds upstream to the AUG start codon on the mRNA. The gene interacts with 23S, aiding in the binding of the two ribosomal subunits (50S+30S). It stabilizes the correct codon-anticodon pairing in the A site, via a hydrogen bond formation between the Nitrogen 1 atom of Adenine residues 1492 and 1493 and the 2'OH group of the mRNA backbone. The 16S rRNA gene is used for phylogenetic studies Weisburg*et al*.(1991),since it is highly conserved between different species of bacteria and archea (Coenye & Vandamme, 2003). In addition to highly conserved primer binding sites, 16S rRNA gene sequences contain hypervariable regions which can provide species-specific signature sequences useful for bacterial identification. As a result, 16S rRNA gene sequencing has become prevalent in medical microbiology as a rapid, accurate alternative to phenotypic methods of bacterial identification (Clarridge, 2004).

2.13 Next generation sequencing technology

Molecular based methods involving direct isolation and analysis of nucleic acids from samples have been revealed to help overcome some of the biased experiment in culture

dependent studies. These include metagenomics and Metatranscriptomic (Handelsman, 2004) and they assist in exploration of mixed microbial communities existing in various natural environments (Gifford *et al*., 2011). These approaches involve sequencing of random DNA profiles, determining taxonomic diversity and prospective genes related to environmental responses (Handelsman, 2004). Metagenomics enables discovery of interaction between microorganism and the environment and assignment of ecosystem functions to various communities (Handelsman, 2004; Frias-Lopez *et al*., 2008). Functional genes of uncultured organisms can be linked to phylogenetic groups by cloning and sequencing of large genomic DNA fragments (Simon & Daniel, 2009). This enables assessment of dominant biosynthetic pathways and primary energy sources (Frias-Lopez *et al*., 2008). A research on microbial assemblages from surface water at the Hawaiian Ocean Times-Series revealed community-wide metabolic activities and day-night patterns of differential gene expression (Poretsky *et al*., 2005). The transcript pools composition was found to be various models of prokaryotic gene expression (Poretsky *et al*., 2005).

These novel methods have been invigorated by the introduction of next generation sequencing technologies whereby more data can be practically generated in reasonably short time and in a cost effective way (Elahi & Ronaghi, 2004). They allow direct sequencing of DNA or cDNA, hence avoid possible cloning bias leading to large-scale studies (Adams *et al*., 2009). Advances in throughput and cost-reduction of sequencing technologies have also increased the number and size of metagenomic sequence projects. The data obtained helps in the exploration of diversity and performance of various organisms in diverse ecosystems (Simon & Daniel, 2009).

2.14 Recent advances in mosquito midgut bacteria diversity studies

Initially, molecular approaches for microbial diversity studies relied on cloning of target genes isolated from environmental samples (DeSantis *et al*., 2007). PCR-based 16S rRNA profile provides information about prokaryote diversity and allows identification of prokaryotes as well as the prediction of phylogenetic relationships (Pace, 1999). Therefore, 16S rRNA gene based PCR techniques such as denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), single-strand conformation polymorphisms (SSCPs), amplified ribosomal DNA restriction analysis (ARDRA), terminal restriction fragment length polymorphisms (T-RFLPs) and ribosomal intergenic spacer analysis (RISA), can provide detailed information about community structure of an ecosystem in terms of richness, evenness and composition and can be used to compare different species present in a sample such as compost (Rawat and Johri, 2014).

Nucleic acid sequencing provides larger discrimination than other methods and better characterization of a particular member of microbial community (McCaig *et al*., 2001). The 16S rRNA gene sequences have been used most extensively to classify the biodiversity. The difference in sequences can be used to construct a phylogenetic tree (Swofford *et al*., 1996). The phylogenetic approach for the systematic assessment of culturable microbial diversity up to the taxonomic level using nucleic acid hybridization and 16S rRNA gene sequences analysis has been of immense utility in the phylogenetic reformation of the classification of prokaryotic organisms (Woese, 1987). Currently, one of the easiest and more cost effective tools available for characterizing the microbial communities associated with mosquito midguts is sequencing the diversity of the 16S

rRNA gene using Next Generation Sequencing technology (NGS). NGS surveys have provided insights into the composition of mosquito midgut-associated bacterial communities, symbiont host-specificity and conditions conducive to the co-evolutionary dynamics of mosquitoes and their associated microbes (Martinson *et al*., 2012).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area

The study area was Ahero, Kisumu County, it is located at latitude0º 11'Sand longitude 34º 55'E), is approximately 1153 metres above sea level (a.s.l). The area has a tropical climate with a significant rainfall throughout the year and an average temperature of 23.0 ºC. Ahero is a malaria endemic zone (Figure 3.1).

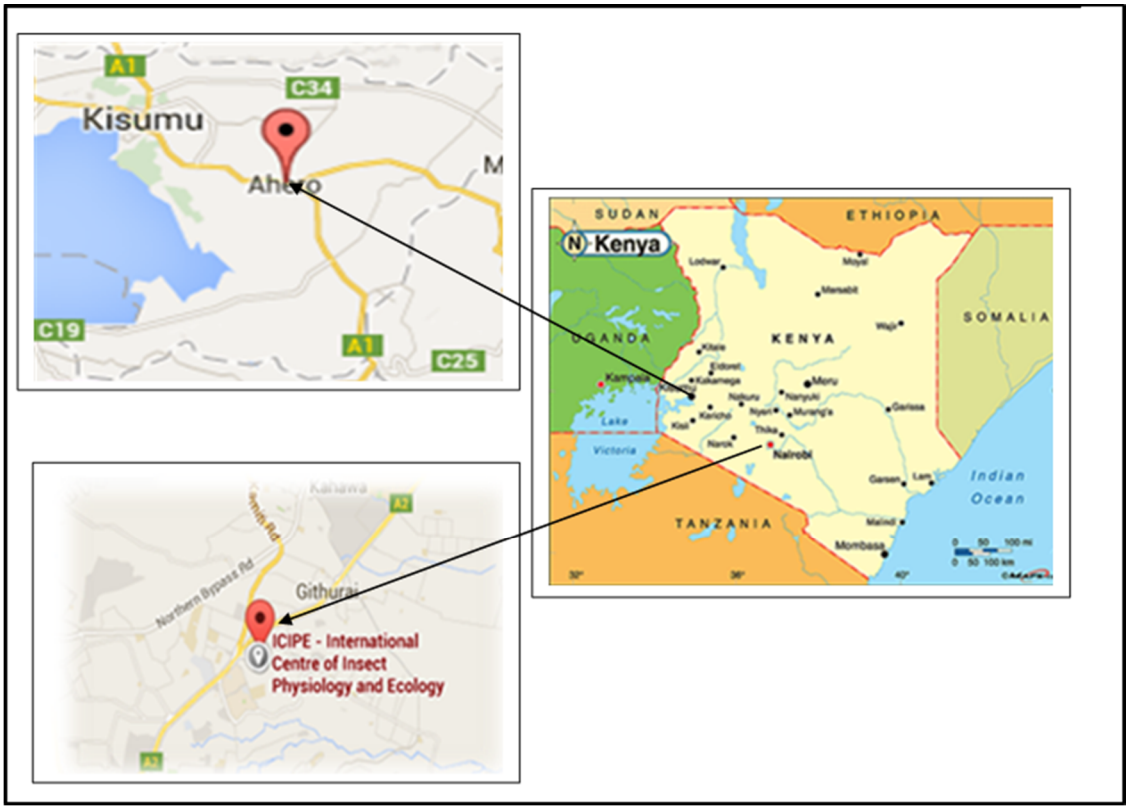

Figure 3.1: Map showing study area Ahero, Kisumu county.

3.2 Sample collection

3.2.1 Sample size determination

Fisher's exact formula was used to determine the minimum number of wild caught and laboratory reared mosquitoes. One hundred and thirty eight mosquitoes were included in this study as determined using fisher's exact formula.

Sample size; $n>=Z^2_{\alpha/2}\ (p\ (1-p))/d^2 = 138$

- Where $Z_{\alpha/2}$ is the corresponding value to the 95% confidence interval (1.96)
- p=10% estimated malaria prevalence among highly risk group Kenya. (Malaria survey indicator 2010. Children <5 prevalence =8% and >5=<14 =13%)
- confidence interval=95% and
- absolute precision d=0.05

  Therefore $n \geq 1.96^2\ (0.1(1-0.1)/0.05^2 = 138$

One hundred and thirty eight adult female *Anopheles gambiae* and *Culex quinquefasciatus* mosquitoes from Ahero, Kisumu county and were sampled. A Similar number of laboratory reared adult female *Anopheles gambiae* and *Culex quinquefasciatus* mosquitoes from ICIPE-Malaria Vector Control Insectary were sampled.

3.2.2 Acquisition of lab reared *Anopheles gambiae* and *Culex quinquefasciatus*

One hundred and thirty eight laboratory reared adult female *Anopheles gambiae* and *Culex quinquefasciatus* mosquitoes were purchased from International Centre for Insect Physiology and Ecology (ICIPE) Kasarani, Nairobi. They were transferred live to the

laboratory at the Institute for Biotechnology Research, Jomo Kenyatta University of Agriculture and Technology (JKUAT) and maintained in a hood at 28 ºC and 70-80% humidity until dissection. The mosquitoes were offered resins and 1% glucose solution only as a source of energy as and they were not blood fed.

3.2.3 Sampling of wild *Anopheles gambiae* and *Culex quinquefasciatus* mosquitoes

Adult *Anopheles gambiae* and *Culex quinquefasciatus* mosquitoes were captured from pit shelters by use of CDC light traps. The CDC light traps were hung at least one meter above the ground on a tree or pole between 6:00 pm and 7:00 pm in the evening and left overnight. The collection bags containing the mosquitoes were picked between 6.00 am and 6.30 am in the morning. The mosquitoes were then put into vial/jars from the collection bags using mouth aspirators and stored at 4 °C. One hundred and thirty eight adult female *Anopheles gambiae* and *Culex quinquefasciatus* mosquitoes were identified to species level using a standard morphological key according to Gillies and De Meillon. (1968). The specimens were transferred to the laboratory at the Institute for Biotechnology Research, JKUAT.

3.3 Laboratory procedures

3.3.1 Dissection of the mosquitoes

Dissection of mosquitoes was done according to Rani *et al*. (2009). Before dissecting the mosquitoes were chilled to death and surface sterilized with 70% ethanol, after which they were transferred into sterile distilled water in a sterile hood. The mosquitoes were dissected individually under sterile condition and the midguts were mashed and suspended

in100µl of sterile phosphate buffered solution (PBS). The mashed midguts were ground to homogeneity. Each midgut extracts consisted of approximately a pool of 20midguts of adult female mosquitoes. Each group of mosquitoes had seven of the pooled midgut extracts. The midgut extracts were stored at -80 ºC until further analysis.

3.3.2 DNA isolation

Total microbial DNA was extracted by using Purelink genomic DNA mini kit (Invitrogen), following the manufacturer's instruction manual (CAT number, K1820-02 Life technologies, California, USA). Genomic DNA concentration was quantified by using nano drop spectrophotometer by absorbance ratio at 260/280 nm, and the DNA suspension was stored at -20ºC until further analysis.

3.3.3 Polymerase chain reaction amplification

Polymerase chain reaction (PCR) amplification of the 16S rRNA gene V4 variable region was carried out on the extracted DNA using primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) that had a barcode (Caporaso *et al*., 2010). PCR amplification was carried out in 30 cycles using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 minutes of initial heating, followed by 30 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, followed by a final elongation step at 72°C for 5 minutes. PCR products were visualized on 2% agarose gel to determine the success of amplification and the relative intensity of bands. Multiple samples were pooled together

in equal proportions based on their DNA concentrations from the gel images. Pooled samples were purified using calibrated Ampure XP beads (Agencort Bioscience Corporation, MA, USA).

3.3.4 Amplicon Library Preparation

The pooled and purified PCR products were used to prepare DNA library by following Illumina TruSeq DNA library protocol (Yu and Zhang, 2012). Sequencing was performed at Molecular Research DNA (www.mrdnalab.com, Shallowater, TX, USA) on a MiSeq platform following the manufacturer's guidelines. The resulting raw sequences were submitted to NCBI (Sequence Read Archive) with the following study accession numbers; sequences for field collected *Anopheles gambiae* SAMN04386463; field collected *Culex quinquefasciatus* SAMN04386464; lab reared *Anopheles gambiae* SAMN04386465 and lab reared *Culex quinquefasciatus* SAMN04386466.

3.3.5 Sequence analysis and taxonomic classification

Sequences obtained from the Illumina sequencing platform were depleted of barcodes and primers using a proprietary pipeline (www.mrdnalab.com, MR DNA, Shallowater, TX) developed at the service provider's laboratory. Short sequences < 200bp, sequences with ambiguous base calls, and those with homopolymer runs exceeding 6bp were removed. The sequences were denoised, chimeras and singleton sequences removed (Caponeet *al*., 2011; Dowd *et al*., 2011; Eren *et al*., 2011). De novo OTU clustering was done with standard UCLUST method using the default settings as implemented in QIIME Version 1.8.0 at 97% similarity level (Caporaso *et al*., 2010). Taxonomy was assigned to each

OTU using BLASTn against SILVA SSU Reference 119 database at default e-value threshold of 0.001 in QIIME (Quast *et al*., 2013).

3.3.6 Diversity indices

Diversity indices (Shannon, Inverse Simpson, Evenness), Rarefaction, Venn diagram (to compare the shared OTUs between the samples of mosquitoes) and Hierarchical clustering were computed, using Vegan package version 1.16-32 in R software (Ihaka & Gentleman, 1996). Kruskal-Wallis rank sum test was used to compare the relative abundance of gut microflora among *Anopheles gambiae* and *Culex quinquefasciatus* from lab reared and field collected samples using R programming language (R development Core Team, 2012). Significance was tested at 95%confidence interval (p = 0.05). To support OTU-based analysis, taxonomic groups were derived from the number of reads assigned to each taxon at all ranks from domain to species using the taxa_summary.txt output from QIIME pipeline Version 1.8.0.

3.3.7 Phylogenetic tree construction

Sequences were compared with 16S rRNA gene sequence available in Gen Bank database by BLASTn search. Multiple sequence alignments of 16S rRNA gene sequences were aligned using MUSCLE (Edgar, 2004). The phylogenetic trees were constructed with Maximum Composite Likelihood (ML) and Neighbor Joining (NJ) methods using software MEGA 6.0 (Tamura *et al*., 2013). The robustness of the phylogeny was tested

by bootstrap analysis using 1000 iterations. Trees generated were analyzed with FIGTREE program version 1.4.2.

CHAPTER FOUR

RESULTS

4.1 Molecular characterization

4.1.1 Amplification of 16S rRNA gene from laboratory reared *Anopheles gambiae*

Amplification of 16S rRNA gene with bacterial universal primers bac 8F and 1492R (Brazelton *et al*., 2006) yielded amplification product of approximately 1500 bp (Plate 4.1). Amplicons were then stained with gel Reddy and visualized under UV on 1.5% agarose.

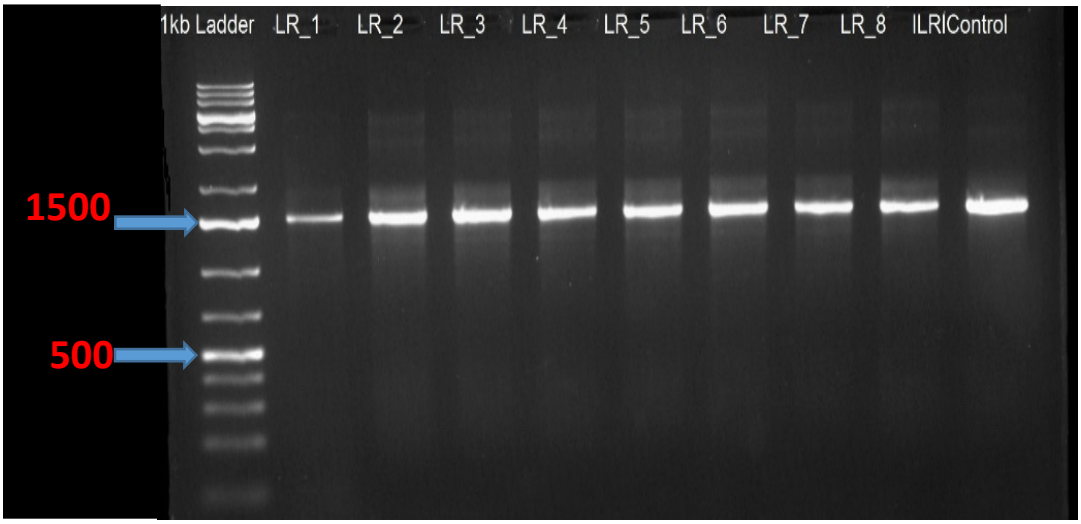

Plate 4.1: Gel picture showing PCR amplified 16S rRNA gene products from midguts of laboratory reared *Anopheles gambiae* using universal primers bac 8F and 1492R

.

4.1.2 Amplification of 16S rRNA gene for laboratory reared *Culex quinquefasciatus*

Amplification of 16S rRNA with bacterial universal primers bac 8F and 1492R (Brazelton *et al*., 2006) yielded amplification product of approximately 1500 bp (Plate 4.2). Amplicons were then stained with gel Reddy and visualized under UV on 1.5% agarose.

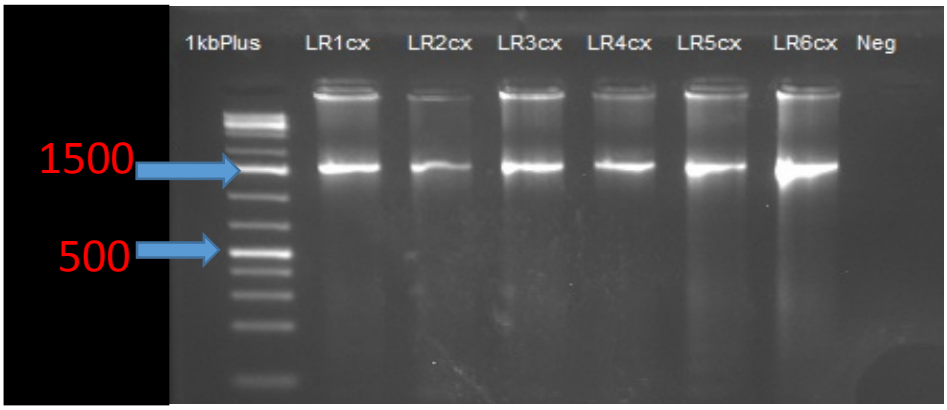

Plate 4.2: Gel picture showing PCR amplified 16S rRNA gene products from midguts of laboratory reared *Culex quinquefansciatus* using universal primers bac 8F and 1492R.

4.1.3 Amplification of 16S rRNA gene field collected *Anopheles gambiae* and *Culex quinquefansciatus*

Amplification of 16S rRNA with bacterial universal primers bac 8F and 1492R (Brazelton *et al*., 2006) yielded amplification product of approximately 1500 bp (Plate 4.3). Amplicons were then stained with gel Reddy and visualized under UV on 1.5% agarose.

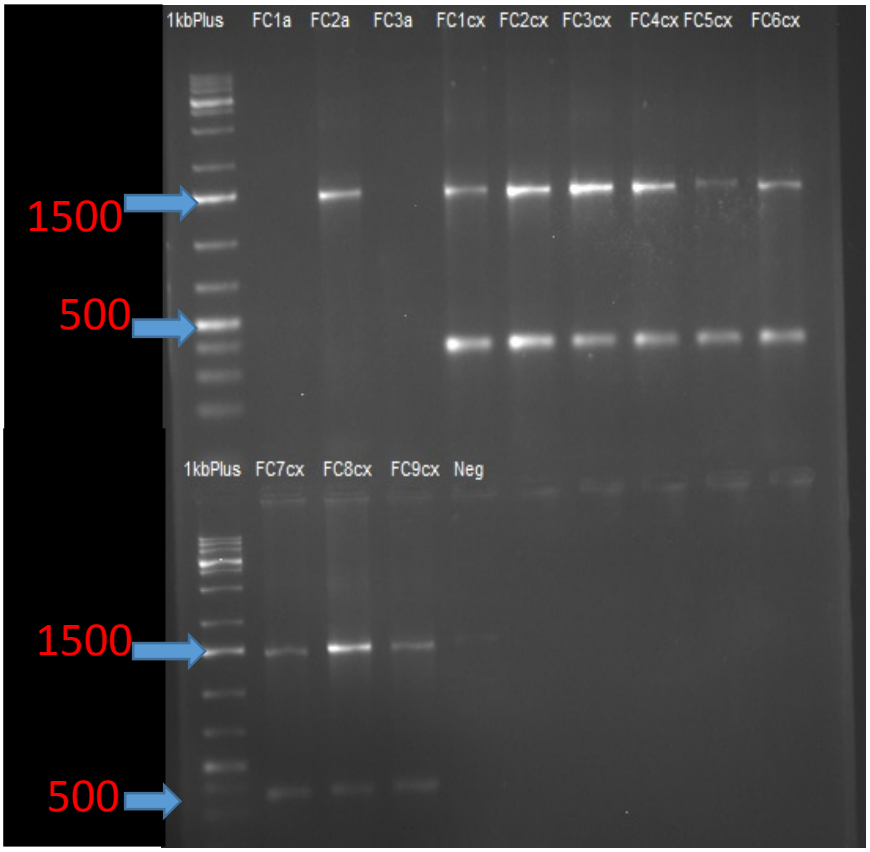

Plate 4.3: Gel picture showing PCR amplified 16S rRNA gene products from midguts of field collected *Anopheles gambiae* (FC a) and *Culex quinquefansciatus* (FC Cx.) using universal primers bac 8F and 1492R.

4.2 Assemblage and diversity of the microbial communities

4.2.1 Sequence reads and operational taxonomic units

After removing chimeras, denoising and demutiplexing, a total of 24,025 sequence reads greater than 200 bp were attained, from the 16S rRNA gene. Total OTU richness at 3% distance amounted to 145. The OTUs per data set ranged between 26 and 102. OTUs comprised 87% bacteria, 0.7% Archaea, 2% Fungi, 1.4% Eukarya and 8% no blast hit

(sequences reads that were not assigned). Rarefaction curve was plotted in order to evaluate if all the diversity within the samples have been exhaustively recovered (Figure 4.1).

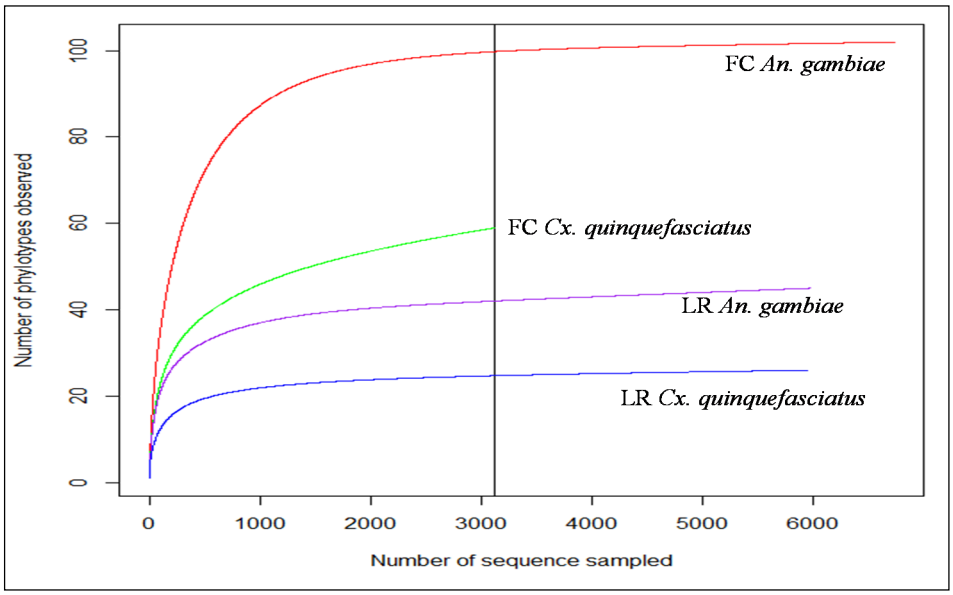

Figure 4.1: Rarefaction curve analysis in field collected (FC) and laboratory reared (LR) samples.

The slope of the lines flattens out in cases where full diversity has been detected. This indicates that even if more sequences were obtained, the number of OTUs detected in the samples would not increase. However, more sequences would be required to exhaust the full diversity within the samples if the slope does not flatten out (Chao *et al.*, 2014). The sequencing depth as shown by the rarefaction curve was exhaustive enough to ensure the inclusion of the entire diversity of the microbes in the midgut of the two species of mosquitoes collected from field and laboratory reared.

The distribution of shared OTUs across the two species of mosquitoes and the sample source (laboratory reared and field collected) is shown in figure 4.2. Seven OTUs were common in all the datasets, fifty four OTUs were only found in field collected *Anopheles gambiae* while 18 OTUs were detected only from the field collected *Culex quinquefasciatus* samples. Laboratory reared *Culex quinquefasciatus* and *Anopheles gambiae* samples had one and 10 unshared OTU's respectively.

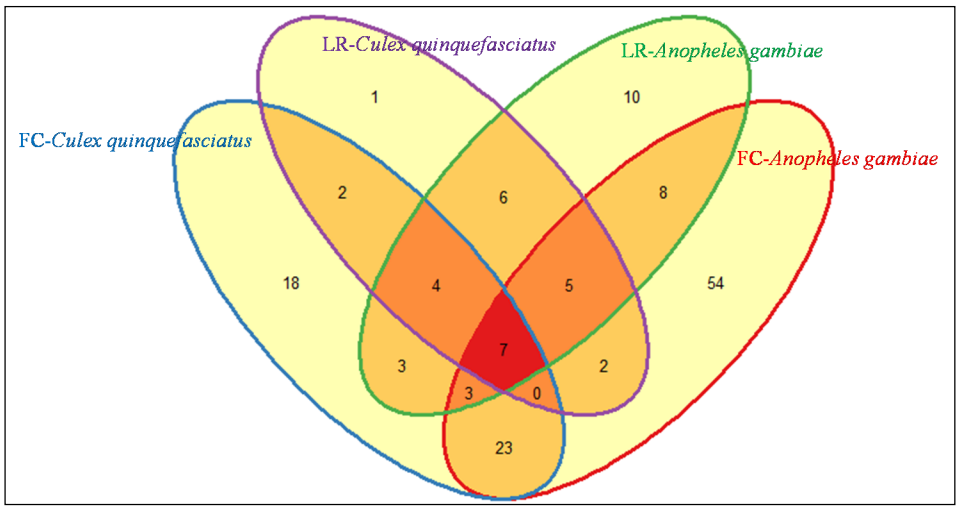

Figure 4.2: Venn diagram showing the distribution of shared OTUs across the 4 samples. Numbers indicate OTU's enumerated in field collected (FC) and laboratory reared (LR).

4.2.2 Bacterial diversity indices

A diversity index is a quantitative measure that reflects how many different types of species there are in a community and simultaneously takes into account how evenly the individuals are distributed among them.The estimated Shannon diversity index varied between (3.54) for field collected *Anopheles gambiae* and (1.93) for laboratory reared *Culex quinquefasciatus* (Table 4.1). The Shannon diversity index for field collected *Culex*

quinquefasciatus (2.73) was higher than laboratory reared *Anopheles gambiae* (2.52) and laboratory reared *Culex quinquefasciatus* (1.93) even though the number of sequence reads, after filtering of the laboratory reared samples (6669 and 6375, respectively) was approximately two folds higher than field collected *Culex quinquefasciatus* (3465). The Shannon index is a representation of both species abundance and evenness, when either of these two factors increase, the diversity index increase. Evenness was used to estimate how well the species are evenly distributed in a community. The highest evenness was recorded from field collected *Anopheles gambiae* (0.767) indicating that OTUs were evenly distributed as compared to other samples. The lowest evenness was recorded from lab reared *Culex quinquefasciatus* (0.593) indicating that bacterial species are less evenly distributed and some species are more dominant than the others. The value of InverseSimpson index ranged from 4.65 for lab reared *Culex quinquefasciatus* to 19.98 for field collected *Anopheles gambiae*. The value of Inverse Simpson index was observed to increase with increase in diversity**.**

Table 4.1 Diversity indices computed on all OTU-based microbial taxonomic units obtained from field collected (FC) and laboratory reared (LR) datasets.

Sample ID	No. of Sequences	Richness (OTUs)	Shannon (H)	Inverse Simpson	Evenness (J)	Effective No. of sp.
FC *Anopheles gambiae*	7516	102	3.54	19.98	0.767	34.47
FC *Culex quinquefasciatus*	3465	59	2.73	8.72	0.67	15.33
LR *Anopheles gambiae*	6669	45	2.52	5.98	0.661	12.43
LR *Culex quinquefasciatus*	6375	26	1.93	4.65	0.593	6.89
Total	**24,505**	**145**				

The microbial community composition, based on Kruskal-Wallis test, at OTU level showed significant difference between field collected and laboratory reared mosquitoes (χ^2=45.0799, p=3.2 x 10^{-5}). Similarly, there was significant difference in microbial community composition at OTU level between *Anopheles gambiae* and *Culex quinquefasciatus* (χ^2=31.2257,p=7.7 x 10^{-4}).

4.3 Microbial taxonomic community composition analysis

4.3.1 Relative abundance at phylum level

The SILVA SSU Reference 119 database (Quast *et al*., 2013) was used to assign reads to appropriate taxonomic ranks. The OTUs spanned 12 phyla (Figure 4.3). *Proteobacteria* (62.04–95.11 %)*, Firmicutes* (0.00–6.13 %), *Bacteriodetes* (0.42–4.89 %), *Actinobacteria* (0.00–4.97 %), *Eukaryota* (0.00–3.46 %), *Gemmatimonadetes* (0.00–0.86 %), *Spirochaetae* (0.00–0.21 %), *Verrucomicrobia* (0.00–0.76 %), *Chloroflexi* (0.00–0.80 %), *Acidobacteria* (0.00–0.68 %), *Archeabacteria* (0.00–0.39 %) and *Cyanobacteria* (0.00–0.10 %). The no blast hits had relative abundance ranging from 0.00 to 16.58%.

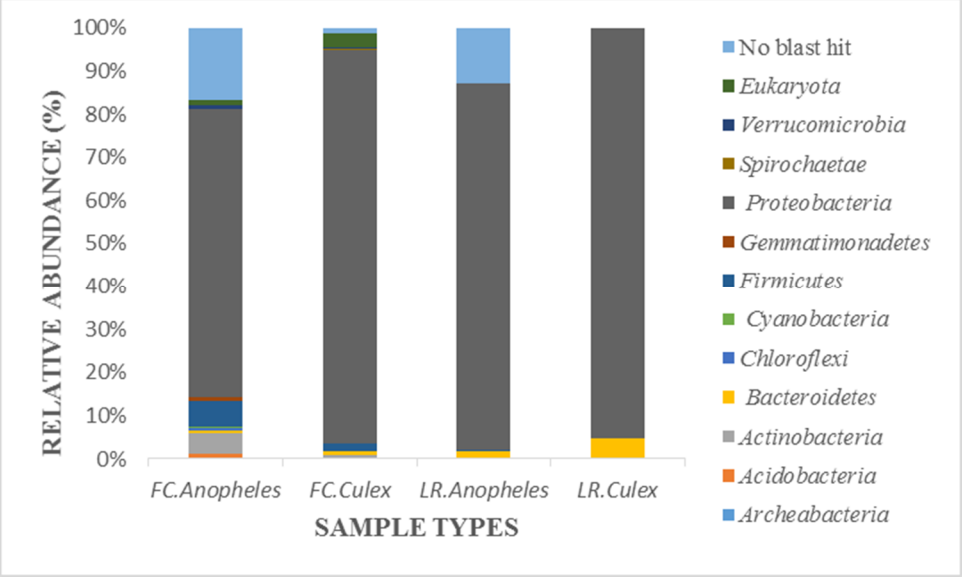

Figure 4.3: Relative abundance at phylum level from the field collected (FC) and laboratory reared (LR) samples.

4.3.2 Relative abundance at species level

OTUs belonging to the Phylum *Proteobacteria* were the most abundant and were represented by most genera as shown in figure 4.4. In lab reared *Culex quinquefasciatus* sample the OTUs comprised of 15 bacteria species which were affiliated to the following genera; *Aeromonas*, *Asaia*, *Elizabethkingia*, *Enterobacter*, *Pseudomonas*, *Rahnella*, *Serratia* and *Wolbachia*. *Serratia marcescens* was the most abundant species in this sample with a relative abundance of 64.29 %. Other species present in higher abundance were *Rahnella* uncultured bacterium 18.13%, *Serratia* uncultured bacterium 5.08%, *Wolbachia Embioptera* sp. 4.37% and *Elizabethkingia meningoseptica* 4.88% (Figure 4.4). However, in field collected *Culex quinquefasciatus* sample it consisted of 39 bacterial species, belonging to the following genera; *Wolbachia*, *Sphingomonas*,

Streptococcus, *Serratia*, *Rhizobium*, *Rahnella*, *Pseudomonas*, *Methylobacterium*, *Ixodes*, *Helicobacter*, *Gamma proteobacterium*, *Enterobacter*, *Corynebacterium*, *Bartonella*, *Bacteroidetes*, *Bacillus*, *Asaia*, *Arcobacter*, *Akkermansia*, *Agrobacterium*, and *Aeromonas*. The most abundant species in field collected *Culex quinquefasciatus* sample were *Arcobacter* uncultured bacterium with relative abundance of 34.83%, while *Bartonella grahamii* as4aup had 24.45 % (Figure 4.4). *Arcobacter* uncultured bacterium, *Bacteroidetes* uncultured bacterium, *Bartonella grahamii* as4up, *Gamma Proteobacteria* uncultured bacterium, *Helicobacter* sp. B52Seymour and *Ixodes scapularis* are unique species to the field collected *Culex quinquefasciatus* sample.

Lab reared *Anopheles gambiae* sample comprised of 21 bacterial species. *Asaia* uncultured bacterium was the most abundant species with 39.30% relative abundance. Other taxa represented in the sample include *Aeromonas* sp. DMA1, *Rahnella* uncultured bacterium and *Serratia marcescens* each scoring a relative abundance of 10%. The genera found in lab reared *Anopheles gambiae* sample include; *Aeromonas, Serratia, Bacillus*, *Asaia, Chryseobacterium, Gluconacetobacter, Delftia, Pseudomonas, Rahnella, Thorsellia, Enterobacter* and *Stenotrophomonas*. *Thorsellia anophelis* was unique to lab reared *Anopheles gambiae* sample (Figure 4.4). The field collected *Anopheles gambiae* sample was found to harbor a higher diversity of consisting 64 bacterial species. The most abundant species were *Agrobacterium* sp. 12.63% and *Methylobacterium* uncultured bacterium at 11.14% relative abundance. The most predominant genera found in field collected include; *Serratia, Bacillus*, *Agrobacterium Stenotrophomonas, Gluconacetobacter, Methylobacterium*. *Rahnella* (Figure 4.4). The unique species in field

collected *Anopheles gambiae* sample include *Agrobacterium tumefaciens*, *Gemmatimonadetes* uncultured bacterium, *Micrococcus* uncultured bacterium and *Rhizobium* sp. M51 (Figure 4.4).

Bacterial species which were recovered from all the four samples include, *Serratia marcescens*, *Asaia* uncultured bacterium, *Enterobacter* uncultured bacterium, *Pseudomonas* uncultured bacterium and *Rahnella* uncultured bacterium. *Parathelohania divulgata* and *Takaokaspora nipponicus* are fungal species recovered from the field collected *Anopheles gambiae* and *Culex quinquefasciatus* respectively (Figure 4.4). A detailed information on all the bacterial species recovered in this study is on appendix 1.

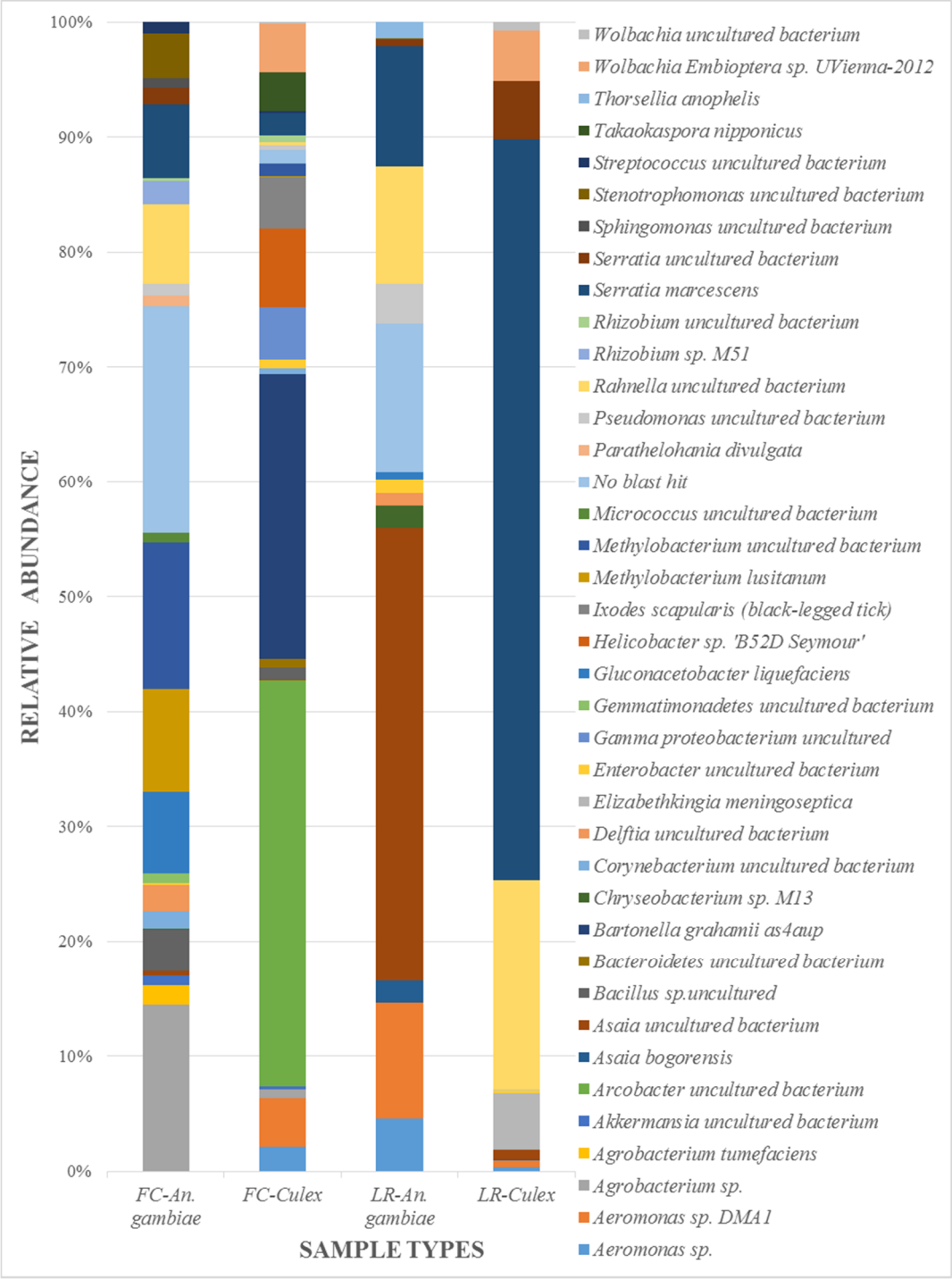

Figure 4.4: Relative abundance of the most predominant bacterial species from the four samples.

4.4 Hierarchical clustering

Hierarchical clustering, based on Bray-Curtis dissimilarity, showed two clusters (Figure 4.5). The dendogram shown on the top signify the relationship between the four samples. The bacteria composition of lab reared *Anopheles gambiae*, field collected *Anopheles gambiae* and field collected *Culex quinquefasciatus* samples were clustered together. Within this cluster the field collected *Anopheles gambiae* and field collected *Culex quinquefasciatus* samples were more closely related to each other. The bacterial community recovered from the lab reared *Culex quinquefasciatus* samples was observed to form a distinct cluster.

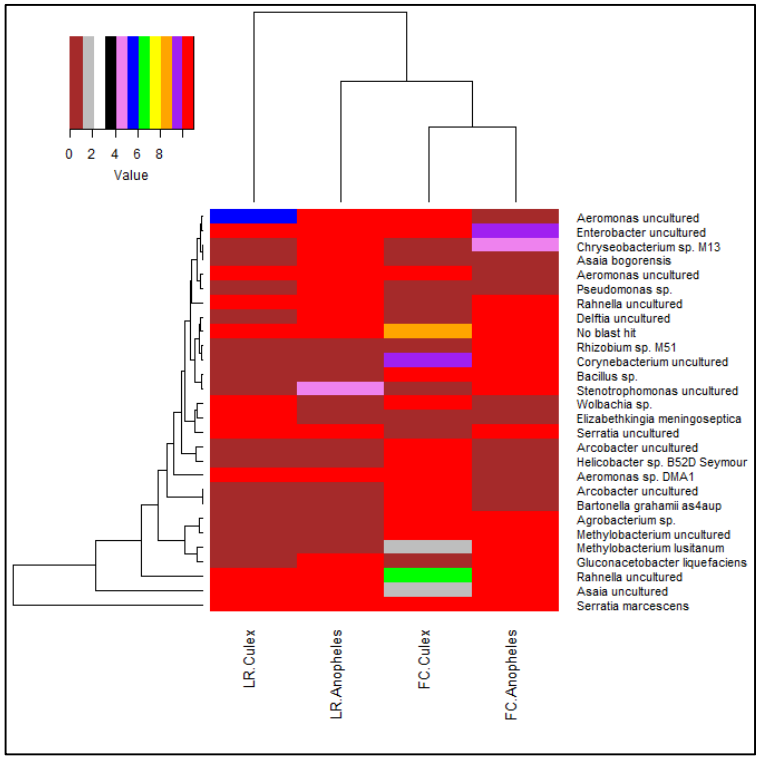

Figure 4.5: Hierarchical clustering of most abundant midgut bacterial species of the field collected and laboratory reared mosquitoes. Species level was chosen to be used in hierarchical clustering to assess the relationship between sample and taxa.

4.5 Phylogenetic analysis of 16S rRNA gene sequence of bacteria communities

The phylogenetic analysis was done using partial 16S rRNA gene sequence aligned homologus nucleotide sequences. On the basis of sequence similarities to the existing GenBank database entries, the bacteria species recovered from field collected *Anopheles gambiae* were more diverse, the major classes were, *Gammaprotobacteria*, *Alphaproteobacteria* and *Actinobacteria*, others include *Acidobacteria*, *Bacteroidetes*, *Bacilli*, *Betaproteobacteria*, *Verrucomicrobia*, *Flavobacteria*, *Methanobacteria* and uncultured bacteria (Figure 4.6).

In laboratory reared *Anopheles gambiae* sample the phylogenetic analysis revealed that the bacterial species were clustered in three major classes; *Gammaprotobacteria, Betaproteobacteria, Alphaproteobacteria*, with *Anoxybacillus* uncultured species that belong to class *Bacillus, Incertae sedis* uncultured bacterium species belong to Gram positive *Firmicutes* and *Chryseobacterium* belong to *Flavobacteria* (Figure 4.7). The *Gammaprotobacteria* had the dominant bacterial species, the representative species were; *Serratia marcescens*, *Rahnella* uncultured bacteria. *Aeromonas* sp, *Thorsellia anophelis*, *Enterobacter uncultured* bacterium*, Pseudomonas* uncultured bacterium.

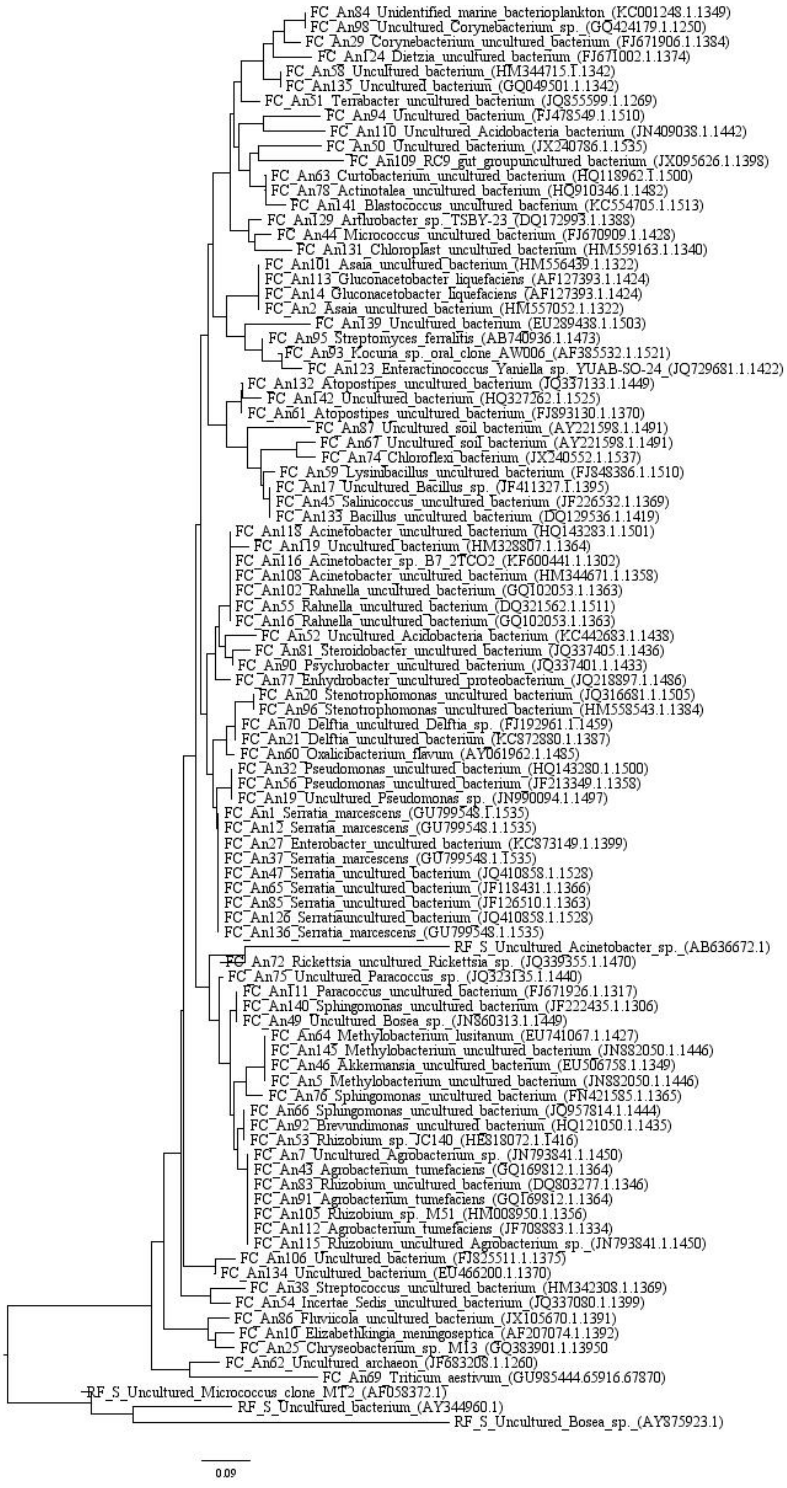

Figure 4.6: Phylogenetic tree constructed from 16S rRNA gene isolate from field

collected *Anopheles gambiae*. Entries with RF_S are from public database. Entries from this work are represented as FC *Anopheles gambiae*, OTU number, generic name, and accession number in (parenthesis).

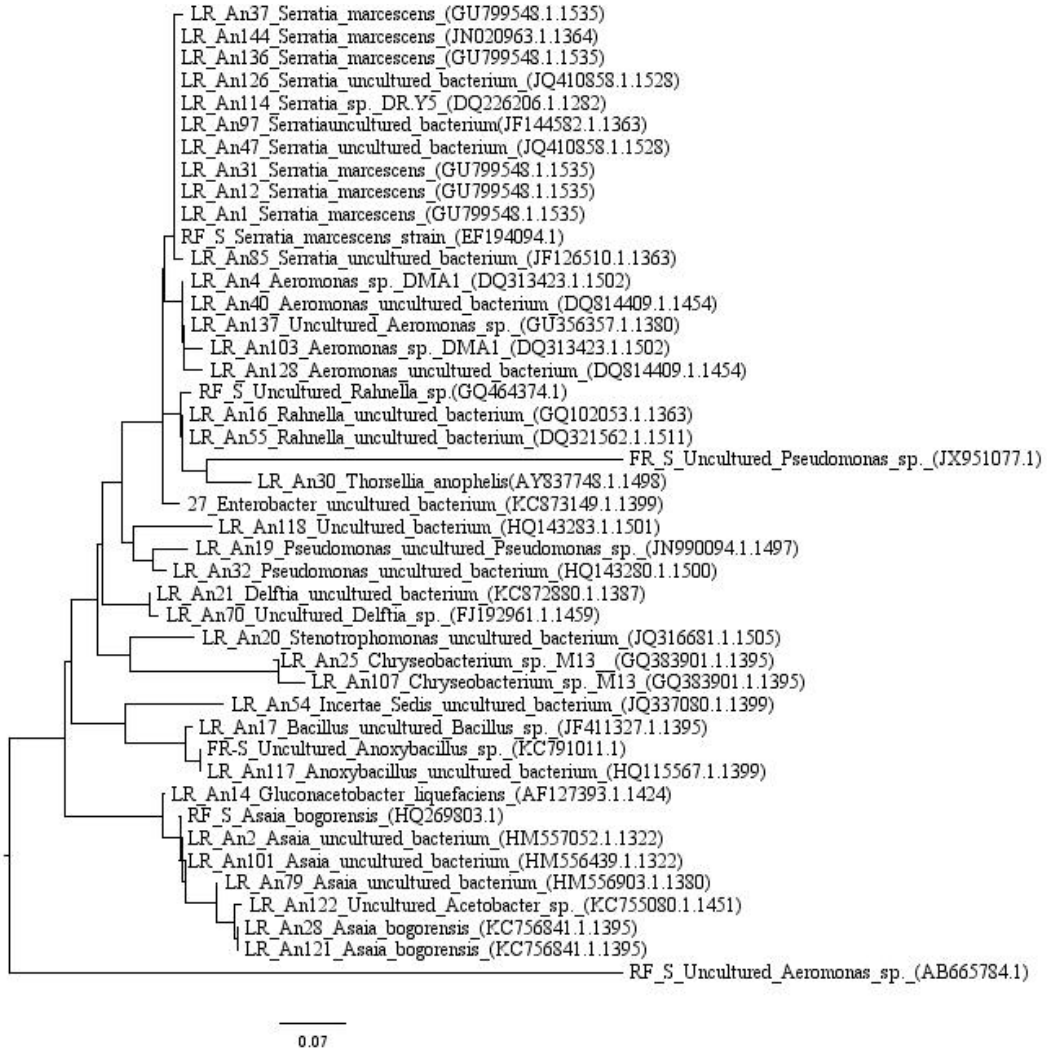

Figure 4.7: Phylogenetic tree constructed from 16S rRNA gene isolate from laboratory reared *Anopheles gambiae*. Entries with RF_S are from public database. Entries from this work are represented as LR, *Anopheles gambiae*, OTU number, generic name, and accession number in (parenthesis).

The phylogenetic analysis of the field collected *Culex quinquefansciatus*, the bacterial species were clustered in three major classes; *Gammaprotobacteria*, *Alphaproteobacteria* and *Actinobacteria*, with few other bacterial species affiliated to *Firmicutes, Epsilonprotobacteria, Bacilli, spirochetes* and *Verrucomicrobia* (Figure 4.8).

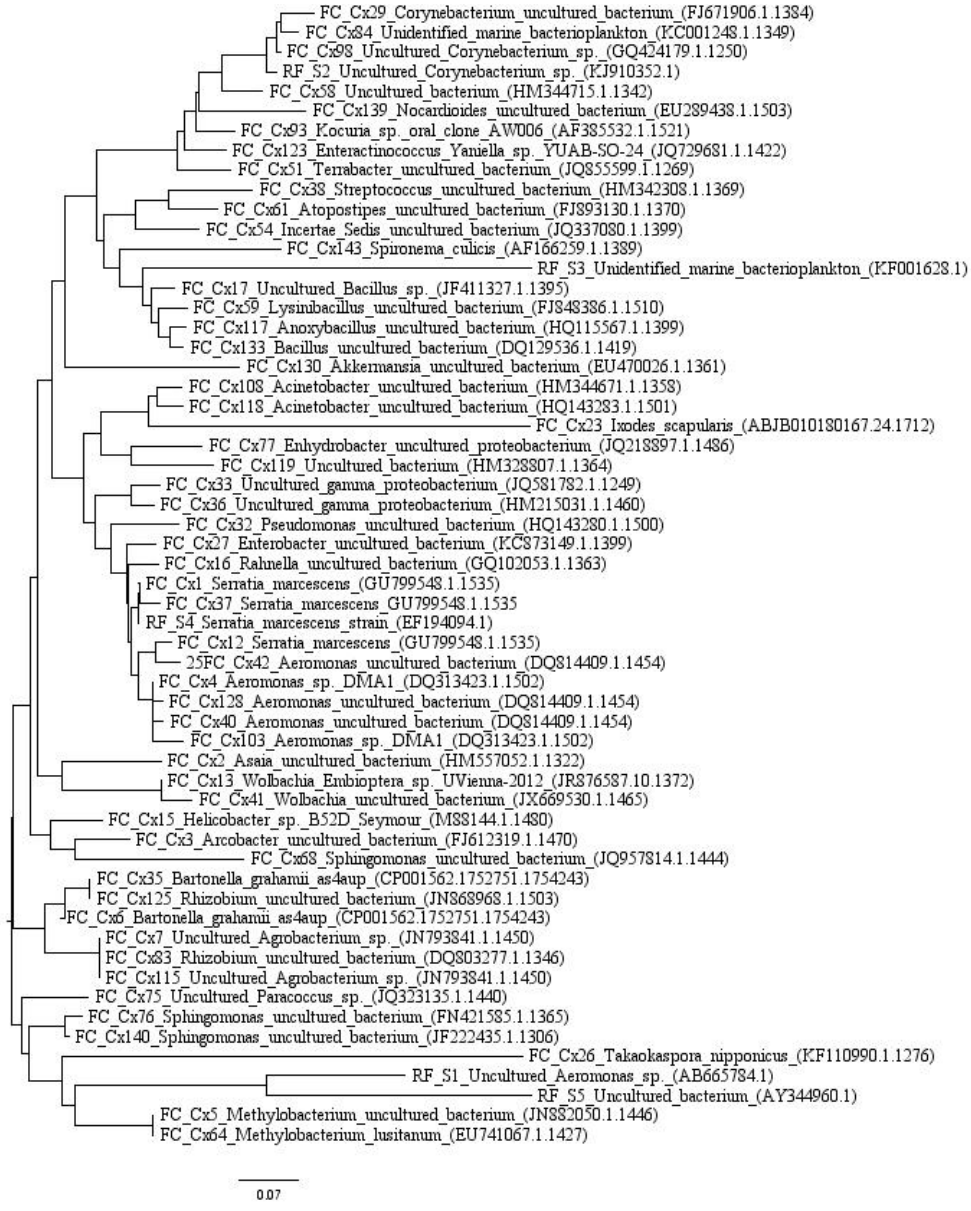

Figure 4.8: Phylogenetic tree constructed from 16S rRNA gene isolate from field collected *Culex quinquefansciatus*. Entries with RF_S are from public database.

Entries from this work are represented as FC *Culex quinquefansciatus,* OTU number, generic name, and accession number in (parenthesis).

Laboratory reared *Culex quinquefansciatus* clustered in two major classes; *Gammaprotobacteria* and *Alphaproteobacteria* while *Elizabethkingia meningoseptica* which belong to gram positive *Flavobacteria* (Figure 4.9). The dominant species in *Gammaproteobacteria* include *Serratia*, *Rahnella*, *Aeromonas*, *Pseudomonas* and *Enterobacter*, while *Wolbachia* and *Asaia* belong to *Alphaproteobacteria. Serratia marscences* was the dominant species in all the samples.

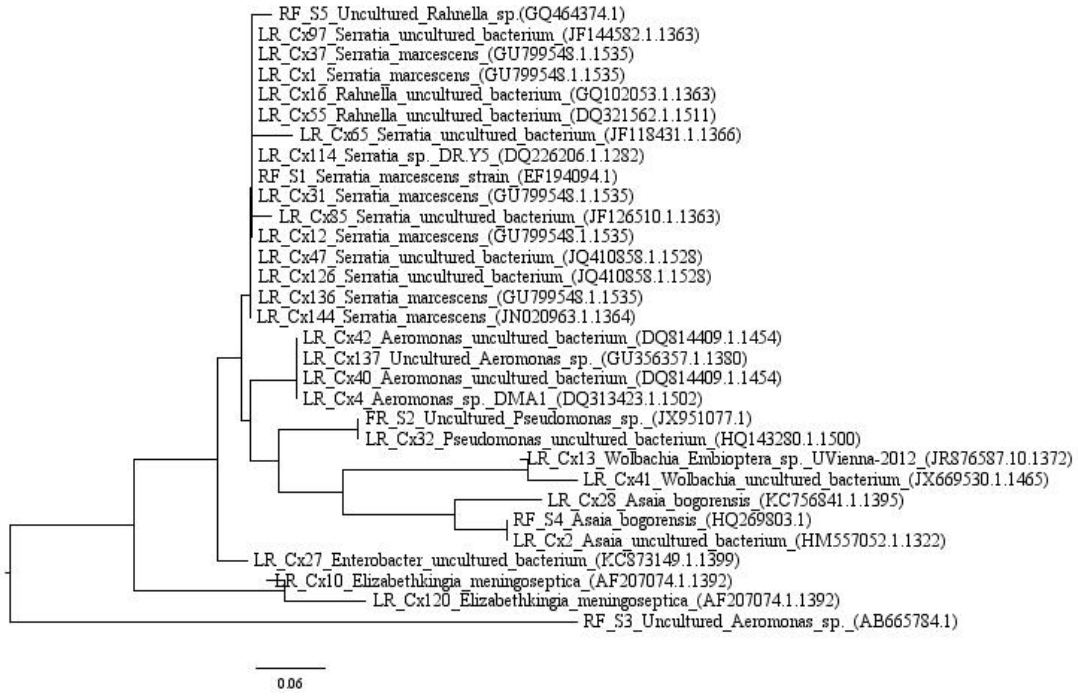

Figure 4.9: Phylogenetic tree constructed from 16S rRNA gene isolate from laboratory reared *Culex quinquefansciatus*. Entries with RF_S are from public database. Entries from this work are represented as LR *Culex quinquefansciatus*. OTU number, generic name, and accession number in (parenthesis).

CHAPTER FIVE

DISCUSSION

4.1 Discussion

The aim of this study was to identify microbes from midgut of laboratory reared and field collected *Anopheles gambiae* and *Culex quinquefasciatus* mosquitoes. The study contributes to the understanding of bacterial diversity in midgut of laboratory reared and field collected *Anopheles gambiae* and *Culex quinquefasciatus* mosquitoes. Identification was based on DNA isolation and 16S rRNA gene sequencing on Illumina platform. The high sensitivity of Illumina sequencing enables the detection of rare species, thus providing more detailed information on bacterial diversity in these mosquitoes.

Diversity indices analysis at OTU level from field collected and laboratory reared mosquitoes revealed a significant difference in microbial community composition. Field collected *Anopheles gambiae* had the highest value of Inverse Simpson index while laboratory reared *Culex quinquefasciatus* sample had the lowest. The value of Inverse Simpson increases with diversity, therefore bacteria in the field collected *Anopheles gambiae* were more diverse than in the other samples. The Shannon index is another widely used index for comparing diversity between various habitats (Chandel *et al*., 2013). The Shannon index is a representation of both species abundance and evenness, when either of these two factors increase, the diversity index increases. Evenness was used for the estimating how well the species were evenly distributed among the samples. The lowest evenness was recorded from laboratory reared *Culex quinquefasciatus* samples

indicating that the bacterial species in this sample were less evenly distributed i.e. some species are more dominant than others. Based on Kruskal-Wallis test *Anopheles gambiae* species revealed higher diversity indices than the *Culex quinquefansciatus* mosquito species. Interms of sample source field collected samples showed higher diversity indices than the laboratory reared samples. Therefore the bacterial diversity in all the four samples were influenced by the environment they inhabit and the type of species of the mosquito vector.

The field collected mosquitoes showed a higher midgut bacterial diversity than the laboratory reared mosquitoes. A similar observation was reported by Rani *et al*. (2009) when he studied bacterial diversity analysis of larvae and adult midgut microflora in laboratory reared and field collected *Anopheles stephensi* mosquito vectors. The higher bacterial diversity in field collected mosquitoes could be probably due to the fact that, the wild mosquitoes are exposed to the natural environment and fed on various natural foods whereas the laboratory reared mosquitoes are feed on artificial diet of glucose and resins. Furthermore, adult female mosquitoes require a bloodmeal for their egg development and the blood acquired in the field could also be a source of various bacterial flora, while on the other hand, the blood given to the adult female laboratory reared mosquitoes is from infection-free rabbits or rats.

The midgut bacterial community complexity was demonstrated in rarefaction curves. Field collected *Anopheles gambiae* had the highest numbers of species with the highest number of phylotypes, followed by field collected *Culex quinquefasciatus*, lab reared

Anopheles gambiae and *Culex quinquefasciatus*. The rarefaction curve leveled off in all the samples, indicating that adequate sampling had been achieved and most of the phylotypes had been retrieved. Phylotypes are environmental DNA sequence or group of sequences sharing more than an arbitrarily chosen level of similarity of particular gene marker. Rarefaction curves compare species richness among imperical samples that differ in number of individuals (Colwell *et al*., 2012) .

Venn diagram analysis indicates the OTUs shared among the four sample datasets. The field collected *Anopheles gambiae* samples had more unshared OTUs while lab reared *Culex quinquefasciatus* had only one unshared OTU. Field collected *Anopheles gambiae* and field collected *Culex quinquefasciatus* samples had the highest number of shared OTUs than the rest of samples, this could be due to their exposure in the same natural environment.

Comparative diversity was visualized using heatmap based on Bray-Curtis dissimilarities at species level. The microbial composition of the field collected samples at species level were more similar compared to the laboratory reared samples. However for the laboratory reared samples, the bacterial composition seemed to differ between *Anopheles gambiae* and *Culex quinquefasciatus*.

The relative abundance at phylum level comprised of twelve phyla; *Proteobacteria*, *Firmicutes*, *Cyanobacteria*, *Euryarchaeota*, *Gemmatimonadetes*, *Spirochaetae*, *Verrucomicrobia*, *Chloroflexi*, *Bacteriodetes*, *Acidobacteria* and *Actinobacteria*. These

phyla have also been identified in *Anopheles gambiae* mosquitoes in Kenya (Wang *et al*., 2011; Favia *et al*., 2007; Boissière *et al.*, 2012). It appears that members of *Proteobacteria*, were predominantly recovered from the field collected and laboratory reared than those of *Firmicutes*, *Actinobacteria* and *Bacteriodetes*. *Proteobacteria* were also shown to be dominant in a previous study conducted in Kenya in *Anopheles gambiae* mosquitoes (Wang *et al*., 2011). The relative abundance of *Proteobacteria* based on Kruskal-Wallis test, was significantly higher in *Anopheles gambiae* than *Culex quinquefasciatus*. *Proteobacteria* was the largest phylum represented by 43 species belonging to 26 genera. Some of the groups of bacteria recovered in the present study are similar to those recovered from previous culture dependent and culture-independent studies (Rani *et al*., 2009). *Firmicutes* consisted of ten species which were affiliated to nine genera. *Actinobacteria* represented fifteen species belonging to thirteen genera whereas *Bacteriodetes* consisted of five species belonging to five genera. Members of *Cyanobacteria*, *Gemmatimonadetes*, *Spirochaetae*, *Verrucomicrobia*, *Chloroflexi*, *Archeabacteria* and *Acidobacteria* represented only a small portion of the mosquito midgut communities.

Among the dominant genera recovered belong to *Serratia, Asaia*, *Arcobacter*, *Rahnella*, *Bartonella*, *Aeromonas*, *Agrobacterium*, *Methylobacterium* and *Wolbachia*. These genera have also been frequently reported from mosquito gut in previous studies and the results are consistent with those of earlier reports (Pidiyar *et al*., 2004; Demaio *et al*., 1996 ; Favia e*t al*., 2007; Dong *et al*., 2009). This suggests that at least a fraction of the mosquito midgut inhabitants could be common for different mosquito species inhabiting similar

environments and may represent evolutionary conservation of association between bacteria and mosquito gut. Members of the genera *Acinetobacter*, *Aeromonas*, *Bacillus*, *Enterobacter*, *Pseudomonas*, *Serratia*, *Asaia*, *Rahnella*, and *Stenotrophomonas* have been frequently reported in mosquito gut in previous studies (Pidiyar *et al*., 2004; Boissière *et al.*, 2012; Chandel *et al*., 2013). Sequences belonging to genera *Asaia*, *Enterobacter*, *Pseudomonas*, *Rahnella* and *Serratia* were recovered from all the samples and comprise of a major part of microbiota of *Anopheles gambiae* and *Culex quinquefasciatus* mosquitoes in the present study.

Serratia marcescens appeared to be the core species (23.6%) in the present study, as it was observed in both laboratory reared and field collected mosquitoes, suggesting that it possesses a competitive advantage over other bacterial species. *Serratia marcescens* is abundant in nature, more so in the artificial foods given to the laboratory reared mosquitoes. Similar results were reported in five generations of *Anopheles gambiae* (Dong *et al*., 2009).

The genus *Asaia*, recovered at (11.6%), was more abundant in laboratory reared *Anopheles gambiae*. *Asaia* has been associated with *Anopheles* species, in particular field collected *Anopheles funestus*, *Anopheles Maculipennis, Anopheles gambiae* and *Anopheles coustani* and *Anopheles stephensi* (Favia *et al*., 2007). The presence of *Asaia* species in *Anopheles* mosquito could be useful in malaria control given that this bacteria genus produces antiparasite molecules in mosquitoes that could be exploited in paratransgenic control of malaria (Damiani *et al*., 2010; Favia *et al*., 2007).

Elizabethkingia meningoseptica and *Wolbachia* sp. have been detected repeatedly in both laboratory reared and wild caught mosquitoes (Pumpuni*et al*., 1996) indicating their prevalent symbiotic association with mosquitoes. *Wolbacha* was detected in field collected and laboratory reared *Culex quinquefasciatus* in the present study, but previously it has been reported in several other mosquito vectors including, *Aedes*, *Coquillettida*, and *Masonia* (Ricci *et al*., 2012).

Bacillus sp., *Stenotrophomonas*, *Micrococcus Acinetobacter* and *Rhizobium* were recovered at significantly greater numbers from the field collected mosquitoes. These species have been frequently isolated from soil and environmental samples, suggesting that the local soil and water environment plays an important role in colonization of the mosquito midgut at breeding sites or during nectar/blood feeding (Chandel *et al*., 2013).

Parathelohania divulgata, *Parathelohania obesa* and *Takaokaspora nipponicus* are fungal species recovered in the field collected *Anopheles gambiae* and *Culex quinquefasciatus* but not in laboratory reared mosquitoes. *Aspergillus* spp. has been isolated from *Anopheles stephensi* larval gut, but not in adult mosquitoes (Tajedin *et al*., 2009). The presence of fungi in adult mosquito gut is in contradiction to the previous studies conducted using isolation methods (Chandel *et al*., 2013).

Phylogenetic analysis of bacteria from field collected *Anopheles gambiae* were the most diverse and the bacterial species were clustered in three major classes; *Gammaprotobacteria*, *Alphaproteobacteria* and *Actinobacteria*, others species were

clustered in *Acidobacteria*, *Bacteroidetes*, *Bacilli*, *Betaproteobacteria*, *Verrucomicrobia*, *Flavobacteria*, *Methanobacteria* and uncultured bacteria. While in the lab reared *Anopheles gambiae* samples, the bacterial species were clustered in *Gammaprotobacteria, Betaproteobacteria, Alphaproteobacteria*, *Bacillus, Firmicutes* and *Flavobacteria*.

In field collected *Culex quinquefansciatus*, phylogenetic analysis revealed that bacterial species were clustered in classes; *Gammaprotobacteria*, *Alphaproteobacteria* and *Actinobacteria*, with few bacterial species clustered in *Firmicutes, Epsilonprotobacteria, Bacilli, spirochetes* and *Verrucomicrobia*. The laboratory reared *Culex quinquefansciatus* clustered in two major classes; *Gammaprotobacteria* and *Alphaproteobacteria* with *Elizabethkingia meningoseptica* affiliated to class *Flavobacteria*.

Parasite-mosquito relationship is believed to have existed for many decades. It is likely that acquired microflora permits the maintance of parasites in mosquitoes. The microbes could be benefiting the mosquito by protecting it against harmful bacteria or benefiting the parasites by lowering the immunity of the mosquito against parasite. It has been reported that reduction in bacterial flora in the mosquito midgut increases *plasmodium falciparum* infection rates in experimentally infected *Anopheles* mosquitoes (Beier *et al*., 1994). Interactions between midgut bacteria and malaria parasites could explain how the vector potential for malaria parasite transmission is modulated by environmental factors such as acquisition of different types of bacteria.

Midgut bacterial infection in wild mosquito populations may influence parasitic infection and transmission and this could contribute to understanding the vectorial capacity of the various species because naturally existing microorganisms in mosquito midgut have important roles to determine parasite development and its survival in the natural environment (Chandel *et al*., 2013). Mosquitoes are known to respond to infection by disease causing pathogens, they elicit a specific immune response against them (Dimopoulos *et al*., 1997). Some immune response genes that are expressed in response to specific bacteria in the mosquito midgut may alter the vectorial capacity at which a pathogen is transmitted by the mosquito (Pumpuni *et al*., 1996). This shows that the midgut bacterial composition has a considerable effect on the survival of pathogens in the midgut environment.

Previous studies indicate that the susceptibility of *Culex quinquefansciatus* to Japanese Encephalitis virus increases when *Pseudomonas* sp. and *Acinetobacter* sp. were incorporated in the mosquito blood meal (Mourya *et al*., 2002). Similarly, a study on occurrence of *Klebsiella* sp. and *Pseudomonas* sp. in mosquito midguts reported that mosquitoes with *Pseudomonas* in their midguts showed a higher prevalence of malaria sporozoites, whereas females infected with *Klebsiella* sp. could not support parasite development (Chandel *et al*., 2013). The large number of bacteria present in the vector midguts are capable of producing factors that kill parasites. Haemolysin produced by *Enterobacter cloacae*, *Serratia marcescens*, *Escherichia coli*, *Enterococcus faecalis* exert activity against both prokaryotic and eukaryotic cells (Azambuja *et al*., 2005). The present study revealed that a relatively high number of *Serratia marcescens* were identified from

laboratory reared and field-populations of *Anopheles gambiae* and *Culex quinquefansciatus* which is known to produce haemolysin. The results of this study confirms earlier reports that indicate that the midgut of mosquito vectors harbors numerous microbial communities some of which could have originated from the terrestrial environments as well as the feeds given to the laboratory reared mosquitoes.

4.2 Conclusion

This study demonstrates a high bacterial composition and diversity in the field collected *Anopheles gambiae* samples than the *Culex quinquefasciatus*.

Among the laboratory reared samples the *Anopheles gambiae* revealed a high bacterial composition and diversity than the *Culex quinquefasciatus*.

The field collected *Anopheles gambiae* samples revealed a high bacterial composition and diversity than the laboratory reared. The field collected *Culex quinquefasciatus* samples demonstrated a high bacterial composition and diversity than the laboratory reared *Culex quinquefasciatus.*

Moreover, the field collected *Anopheles gambiae* and *Culex quinquefasciatus* samples had a high microbial composition and diversity than their laboratory reared counterparts.

The bacterial flora of both *Anopheles gambiae* and *Culex quinquefasciatus* midgut is diverse and dominated by *Serratia marcescens*. The results provide basic information about bacterial diversity in midgut of adult female laboratory reared and field collected *Anopheles gambiae* and *Culex quinquefasciatus*.

4.3 Recommendations

1. Since the most abundant bacterial species was *Serratia marcescens*, it should be further evaluated for its suitability in parasite control strategies.
2. Future study should include identification of midgut microbial flora from different areas that are filariasis and malaria endemic.

REFERENCES

Adams, I. P., Glover, R. H., Monger, W. A., Mumford, R., Jackeviciene, E., Navalinskiene, M., & Boonham, N. (2009). Next-generation sequencing and metagenomic analysis: a universal diagnostic tool in plant virology. *Molecular Plant Pathology*, ***10*(4), 537–545.**

Apte-Deshpande, A., Paingankar, M., Gokhale, M. D., & Deobagkar, D. N. (2012).*Serratiaodorifera* a Midgut Inhabitant of *Aedes aegypti* Mosquito Enhances Its Susceptibility to Dengue-2 Virus. *PLoS ONE*, ***7*(7).**

Azambuja, P., Garcia, E. S., & Ratcliffe, N. A. (2005). Gut microbiota and parasite transmission by insect vectors. *Trends in Parasitology*, ***21*(12), 568–572.**

Bando, H., Okado, K., Guelbeogo, W. M., Badolo, A., Aonuma, H., Nelson, B., & Kanuka, H. (2013). Intra-specific diversity of *Serratia marcescens* in *Anopheles* mosquito midgut defines *Plasmodium* transmission capacity. *Scientific Reports*, ***3*, 1641.**

Beard, C. B., Cordon-Rosales, C., & Durvasula, R. V. (2002). Bacterial Symbionts of the Triatominae and Their Potential Use in Control of Chagas Disease Transmission1. *Annual Review of Entomology*, ***47*(1), 123–141.**

Beard, C. B., Dotson, E. M., Pennington, P. M., Eichler, S., Cordon-Rosales, C., & Durvasula, R. V. (2001). Bacterial symbiosis and paratransgenic control of vector-borne Chagas disease. *International Journal for Parasitology*, ***31*(5–6), 621–627.**

Beier, M. S., Pumpuni, C. B., Beier, J. C., & Davis, J. R. (1994). Effects of Para-Aminobenzoic Acid, Insulin, and Gentamicin on Plasmodium falciparum

Development in Anopheline Mosquitoes (Diptera: Culicidae). *Journal of Medical Entomology*, ***31*(4), 561–565.**

Besansky, N. J., Hill, C. A., & Costantini, C. (2004). No accounting for taste: host preference in malaria vectors. *Trends in Parasitology*, ***20*(6), 249–251.**

Bisi, D. C., & Lampe, D. J. (2011). Secretion of Anti-Plasmodium Effector Proteins from a Natural *Pantoea agglomerans* Isolate by Using PelB and HlyA Secretion Signals. *Applied and Environmental Microbiology*, ***77*(13), 4669–4675.**

Boissière, A., Tchioffo, M. T., Bachar, D., Abate, L., Marie, A., Nsango, S. E., & Morlais, I. (2012). Midgut Microbiota of the Malaria Mosquito Vector *Anopheles gambiae* and Interactions with *Plasmodium falciparum Infection.PLoS Pathogens*, *8*(5).

Brazelton, W. J., Schrenk, M. O., Kelley, D. S., & Baross, J. A. (2006). Methane- and sulfur-metabolizing microbial communities dominate the Lost City hydrothermal field ecosystem. *Applied and Environmental Microbiology*, ***72*(9), 6257–6270.**

Briones, A. M., Shililu, J., Githure, J., Novak, R., & Raskin, L. (2008). *Thorsellia anophelis* is the dominant bacterium in a Kenyan population of adult *Anopheles gambiae* mosquitoes. *The ISME Journal*, ***2*(1), 74–82.**

Capone, K. A., Dowd, S. E., Stamatas, G. N., & Nikolovski, J. (2011). Diversity of the Human Skin Microbiome Early in Life. *The Journal of Investigative Dermatology*, ***131*(10), 2026–2032.**

Capone, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., & Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, ***7*(5), 335–336.**

Chandel, K., Mendki, M. J., Parikh, R. Y., Kulkarni, G., Tikar, S. N., Sukumaran, D., & Veer, V. (2013). Midgut microbial community of *Culex quinquefasciatus* mosquito populations from India. *PloS One*, ***8*(11), e80453.**

Chao, A., Gotelli, N. J., Hsieh, T. C., Sander, E. L., Ma, K. H., Colwell, R. K., & Ellison, A. M. (2014). Rarefaction and extrapolation with Hill numbers: A framework for sampling and estimation in species diversity studies. *Ecological Monographs*, ***84*(1), 45–67.**

Chavshin, A. R., Oshaghi, M. A., Vatandoost, H., Pourmand, M. R., Raeisi, A., & Terenius, O. (2014). Isolation and identification of culturable bacteria from wild *Anopheles culicifacies*, a first step in a paratransgenesis approach. *Parasites & Vectors*, ***7*, 419.**

Clarridge, J. E. (2004). Impact of 16S rRNA Gene Sequence Analysis for Identification of Bacteria on Clinical Microbiology and Infectious Diseases. *Clinical Microbiology Reviews*, ***17*(4), 840–862.**

Coenye, T., & Vandamme, P. (2003). Intragenomic heterogeneity between multiple 16S ribosomal RNA operons in sequenced bacterial genomes. *FEMS Microbiology Letters*, ***228*(1), 45–49.**

Coetzee, M., Hunt, R. H., Wilkerson, R., Torre, A. D., Coulibaly, M. B., & Besansky, N. J. (2013). Anopheles coluzzii and Anopheles amharicus , new members of the *Anophelesgambiae* complex. *Zootaxa*, ***3619*(3), 246–274.**

Cook, P. E., & McGraw, E. A. (2010). *Wolbachia pipientis*: an expanding bag of tricks to explore for disease control. *Trends in Parasitology*, ***26*(8), 373–375.**

Curtis, T. P., Head, I. M., Lunn, M., Woodcock, S., Schloss, P. D., & Sloan, W. T. (2006). What is the extent of prokaryotic diversity? *Philosophical Transactions of The Royal Society in London B*, ***361*, 2023–37.**

Damiani, C., Ricci, I., Crotti, E., Rossi, P., Rizzi, A., Scuppa, P., & Favia, G. (2010). Mosquito-bacteria symbiosis: the case of *Anopheles gambiae* and Asaia. *Microbial Ecology*, ***60*(3), 644–654.**

Demaio, J., Pumpuni, C. B., Kent, M., & Beier, J. C. (1996). The midgut bacterial flora of wild *Aedes triseriatus*, *Culex pipiens*, and *Psorophora columbiae* mosquitoes. *The American Journal of Tropical Medicine and Hygiene*, ***54*(2), 219–223.**

DeSantis TZ., Brodie E.L., Moberg, J.P., Zubieta, I.X., Piceno, Y.M., & Andersen, G.L. (2007) High-density universal 16S rRNA microarray analysis reveals broader diversity than typical clone library when sampling the environment. *Microb Ecol***53:371–383**

Dillon, R. J., & Dillon, V. M. (2004). THE GUT BACTERIA OF INSECTS: Nonpathogenic Interactions. *Annual Review of Entomology*, ***49*(1), 71–92.**

Dimopoulos, G. (2003). Insect immunity and its implication in mosquito–malaria interactions. *Cellular Microbiology*, ***5*(1), 3–14.**

Dimopoulos, G., Richman, A., Müller, H. M., & Kafatos, F. C. (1997). Molecular immune responses of the mosquito *Anopheles gambiae* to bacteria and malaria parasites. *Proceedings of the National Academy of Sciences of the United States of America*, ***94*(21), 11508–11513.**

Dong, Y., Manfredini, F., & Dimopoulos, G. (2009). Implication of the Mosquito Midgut Microbiota in the Defense against Malaria Parasites. *PLoS Pathog*, ***5*(5), e1000423.**

Dowd, S. E., Delton Hanson, J., Rees, E., Wolcott, R. d., Zischau, A. m., Sun, Y., & Jones, C. E. (2011). Survey of fungi and yeast in polymicrobial infections in chronic wounds. *Journal of Wound Care*, ***20*(1), 40–47.**

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, ***32*(5), 1792–1797.**

Eiler, A., Heinrich, F., & Bertilsson, S. (2012). Coherent dynamics and association networks among lake bacterioplankton taxa. *The ISME Journal*, ***6*(2), 330–342.**

Elahi, E., & Ronaghi, M. (2004). Pyrosequencing: a tool for DNA sequencing analysis. *Methods in Molecular Biology (Clifton, N.J.)*, ***255*, 211–219.**

Eren, A. M., Zozaya, M., Taylor, C. M., Dowd, S. E., Martin, D. H., & Ferris, M. J. (2011). Exploring the Diversity of *Gardnerella vaginalis* in the Genitourinary Tract Microbiota of Monogamous Couples Through Subtle Nucleotide Variation. *PLoS ONE*, *6*(10).

Fanello, C., Santolamazza, F., & Della Torre, A. (2002). Simultaneous identification of species and molecular forms of the *Anopheles gambiae* complex by PCR-RFLP. *Medical and Veterinary Entomology*, ***16*(4), 461–464.**

Favia, G., Ricci, I., Damiani, C., Raddadi, N., Crotti, E., Marzorati, M., & Daffonchio, D. (2007). Bacteria of the genus Asaia stably associate with *Anopheles stephensi*, an Asian malarial mosquito vector. *Proceedings of the*

National Academy of Sciences of the United States of America, ***104*(21), 9047–9051.**

Fisher, M. M., & Triplett, E. W. (1999). Automated Apporach for Ribosomal Intergenic Spacer Analysis of microbial diversity and its application to freshwater bacterial communities. *Applied Environmental Microbiology*, ***65*(10), 4630–4636.**

Fontenille, D., & Simard, F. (2004). Unravelling complexities in human malaria transmission dynamics in Africa through a comprehensive knowledge of vector populations. *Comparative Immunology, Microbiology and Infectious Diseases*, ***27*(5), 357–375.**

Foster, W. A. (1995). Mosquito Sugar Feeding and Reproductive Energetics. *Annual Review of Entomology*, ***40*(1), 443–474.**

Frias-Lopez, J., Shi, Y., Tyson, G. W., Coleman, M. L., Schuster, S. C., Chisholm, S. W., & Delong, E. F. (2008). Microbial community gene expression in ocean surface waters. *Proceedings of the National Academy of Sciences of the United States of America*, ***105*(10), 3805–3810.**

Gaio, A. de O., Gusmão, D. S., Santos, A. V., Berbert-Molina, M. A., Pimenta, P. F. P., & Lemos, F. J. A. (2011). Contribution of midgut bacteria to blood digestion and egg production in aedes aegypti (diptera: culicidae) (L.). *Parasites & Vectors*, ***4*, 105.**

Gifford, S. M., Sharma, S., Rinta-Kanto, J. M., & Moran, M. A. (2011). Quantitative analysis of a deeply sequenced marine microbial metatranscriptome. *The ISME Journal*, ***5*(3), 461–472.**

Gillies, M. T., & De Meillon, B. (1968). The Anophelinae of Africa south of the Sahara (Ethiopian Zoogeographical Region). **343 pp.**

Gonzalez-Ceron, L., Santillan, F., Rodriguez, M. H., Mendez, D., & Hernandez-Avila, J. E. (2003). Bacteria in Midguts of Field-Collected Anopheles albimanus Block Plasmodium vivax Sporogonic Development. *Journal of Medical Entomology*, ***40*(3), 371–374.**

Gusmão, D. S., Santos, A. V., Marini, D. C., Bacci Jr., M., Berbert-Molina, M. A., & Lemos, F. J. A. (2010). Culture-dependent and culture-independent characterization of microorganisms associated with *Aedes aegypti* (Diptera: Culicidae) (L.) and dynamics of bacterial colonization in the midgut. *Acta Tropica*, ***115*(3), 275–281.**

Gusmão, D. S., Santos, A. V., Marini, D. C., Russo, E. de S., Peixoto, A. M. D., Bacci Júnior, M., & Lemos, F. J. A. (2007). First isolation of microorganisms from the gut diverticulum of Aedes aegypti (Diptera: Culicidae): new perspectives for an insect-bacteria association. *Memórias Do Instituto Oswaldo Cruz*, ***102*(8), 919–924.**

Hamady, M., Walker, J. J., Harris, J. K., Gold, N. J., & Knight, R. (2008). Error-correcting barcoded primers allow hundreds of samples to be pyrosequenced in multiplex. *Nature Methods*, ***5*(3), 235–237.**

Handelsman, J. (2004). Metagenomics: application of genomics to uncultured microorganisms. *Microbiology and Molecular Biology Reviews: MMBR*, ***68*(4), 669–685.**

Head, null, Saunders, null, & Pickup, null. (1998). Microbial Evolution, Diversity, and Ecology: A Decade of Ribosomal RNA Analysis of Uncultivated Microorganisms. *Microbial Ecology*, ***35*(1), 1–21.**

Herlemann, D. P., Labrenz, M., Jürgens, K., Bertilsson, S., Waniek, J. J., & Andersson, A. F. (2011). Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. *The ISME Journal*, ***5*(10), 1571–1579.**

http://www.ozanimals.com/Insect/Brown-House-Mosquito/*Culex*/*quinquefasciatus*.html: Brown House Mosquito (*Culex quinquefasciatus*).

Internews Data Dredger : Malaria by numbers: Why Kenya is not winning the fight.

Kampfer, P. (2006). *Janibacter anophelis* sp. nov., isolated from the midgut of *Anopheles arabiensis*. *INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY*, ***56*(2), 389–392.**

Kampfer, P. (2006). *Thorsellia anophelis* gen. nov., sp. nov., a new member of the Gammaproteobacteria. *INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY*, ***56*(2),335–338.**

Kämpfer, P., Matthews, H., Glaeser, S. P., Martin, K., Lodders, N., & Faye, I. (2011).*Elizabethkingia anophelis* sp. nov., isolated from the midgut of the mosquito *Anopheles gambiae*. *International Journal of Systematic and Evolutionary Microbiology*, ***61*(11), 2670–2675.**

Liu, W. T., Marsh, T. L., Cheng, H., & Forney, L. J. (1997). Characterization of microbial diversity by determining terminal restriction fragment length

polymorphisms of genes encoding 16S rRNA. *Applied and Environmental Microbiology*, ***63*****(11), 4516–4522.**

Manguin, S., Thuy, C., Tainchum, K., Juntarajumnong, W., Chareonviriyaphap, T., Laure Michon, A., & Jumas-Bilak, E. (2013). Bacterial Biodiversity in Midguts of *Anopheles* Mosquitoes, Malaria Vectors in Southeast Asia. In S. Manguin (Ed.), *Anopheles mosquitoes - New insights into malaria vectors*. InTech.

Maniatis, T., Fritsch, E. F., Sambrook, J., & others. (1982).*Molecular cloning: a laboratory manual***(Vol. 545).** Cold Spring Harbor Laboratory Cold Spring Harbor, NY.

Martinson, V.G., Moy J., & Moran, N.A. (2012) Establishment of characteristic gut bacteria during development of the honey bee worker. *Applied* and *Environmental Microbiology 78***(8): 2830–2840.**

McCaig, A.E., Glover, L.A., & Prosser, J.I. (2001) Numerical analysis of grassland bacterial community structure under different land management regimens by using 16S ribosomal DNA sequence data and denaturing gradient gel electrophoresis banding patterns. *Appl Environ Microbiol* **67:4554–4559**

Miller, L. H., Baruch, D. I., Marsh, K., & Doumbo, O. K. (2002). The pathogenic basis of malaria. *Nature*, ***415*****(6872), 673–679.**

Minard, G., Mavingui, P., & Moro, C. V. (2013). Diversity and function of bacterial microbiota in the mosquito holobiont. *Parasites & Vectors*, ***6*****, 146.**

Moll, R. M., Romoser, W. S., Modrzakowski, M. C., Moncayo, A. C., & Lerdthusnee, K. (2001). Meconial peritrophic membranes and the fate of

midgut bacteria during mosquito (Diptera: Culicidae) metamorphosis. *Journal of Medical Entomology*, ***38*(1), 29–32.**

Moncayo, A. C., Lerdthusnee, K., Leon, R., Robich, R. M., & Romoser, W. S. (2005). Meconial peritrophic matrix structure, formation, and meconial degeneration in mosquito pupae/pharate adults: histological and ultrastructural aspects. *Journal of Medical Entomology*, ***42*(6), 939–944.**

Mourya, D. T., Gokhale, M. D., Pidiyar, V., Barde, P. V., Patole, M., Mishra, A. C., & Shouche, Y. (2002). Study of the effect of the midgut bacterial flora of *Culex quinquefasciatus* on the susceptibility of mosquitoes to Japanese encephalitis virus. *Acta Virologica*, ***46*(4), 257–260.**

Njenga, S. M., Mwandawiro, C. S., Wamae, C. N., Mukoko, D. A., Omar, A. A., Shimada, M., & Molyneux, D. H. (2011). Sustained reduction in prevalence of lymphatic filariasis infection in spite of missed rounds of mass drug administration in an area under mosquito nets for malaria control. *Parasites & Vectors*, ***4*, 90.**

Oliveira, J. H. M., Gonçalves, R. L. S., Lara, F. A., Dias, F. A., Gandara, A. C. P., Menna-Barreto, R. F. S., & Oliveira, P. L. (2011). Blood Meal-Derived Heme Decreases ROS Levels in the Midgut of Aedes aegypti and Allows Proliferation of Intestinal Microbiota. *PLoS Pathog*, ***7*(3), e1001320.**

Osei-Poku, J., Mbogo, C. M., Palmer, W. J., & Jiggins, F. M. (2012). Deep sequencing reveals extensive variation in the gut microbiota of wild mosquitoes from Kenya. *Molecular Ecology*, ***21*(20), 5138–5150.**

Pace, N.R. (1996) New perspective on the natural microbial world: molecular microbial ecology. ASM News **62:463–470**

Patel, J. B. (2001). 16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory. *Molecular Diagnosis: A Journal Devoted to the Understanding of Human Disease Through the Clinical Application of Molecular Biology*, ***6*(4), 313–321.**

Pidiyar, V. J., Jangid, K., Patole, M. S., & Shouche, Y. S. (2004). Studies on Cultured and Uncultured Microbiota of Wild *Culex Quinquefasciatus* Mosquito Midgut Based on 16S Ribosomal Rna Gene Analysis. *The American Journal of Tropical Medicine and Hygiene*, ***70*(6), 597–603.**

Poretsky, R. S., Bano, N., Buchan, A., LeCleir, G., Kleikemper, J., Pickering, M., & Hollibaugh, J. T. (2005). Analysis of microbial gene transcripts in environmental samples. *Applied and Environmental Microbiology*, ***71*(7), 4121–4126.**

Pumpuni, C. B., Demaio, J., Kent, M., Davis, J. R., & Beier, J. C. (1996). Bacterial population dynamics in three anopheline species: the impact on Plasmodium sporogonic development. *The American Journal of Tropical Medicine and Hygiene*, ***54*(2), 214–218.**

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., & Glöckner, F. O. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. **41, D590–D596.**

Rani, A., Sharma, A., Rajagopal, R., Adak, T., & Bhatnagar, R. K. (2009). Bacterial diversity analysis of larvae and adult midgut microflora using culture-dependent and culture-independent methods in lab-reared and field-collected *Anopheles stephensi*-an Asian malarial vector. *BMC Microbiology*, ***9*, 96.**

Rawat, S., & Johri, B.N. (2014) Thermophilic fungi: diversity and significance in composting.***KAVAKA* 42:52–68.**

Reid, G., Younes, J. A., Van der Mei, H. C., Gloor, G. B., Knight, R., & Busscher, H. J. (2011). Microbiota restoration: natural and supplemented recovery of human microbial communities. *Nature Reviews Microbiology*, ***9*(1), 27–38.**

Ricci, I., Damiani, C., Capone, A., DeFreece, C., Rossi, P., & Favia, G. (2012). Mosquito/microbiota interactions: from complex relationships to biotechnological perspectives. *Current Opinion in Microbiology*, ***15*(3), 278–284.**

R development Core Team (2012). R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing: Vienna, Austria.

Riehle, M. A., Moreira, C. K., Lampe, D., Lauzon, C., & Jacobs-Lorena, M. (2007). Using bacteria to express and display anti-Plasmodium molecules in the mosquito midgut. *International Journal for Parasitology*, ***37*(6), 595–603.**

Rod Dillon, K. C., Dillon RJ, & Charnley, AK. (2002). Mutualism between the desert locust Schistocerca gregaria and its gut microbiota. Research In Microbiology. *Research in Microbiology*, ***153*(8), 503–9.**

Simon, C., & Daniel, R. (2009). Achievements and new knowledge unraveled by metagenomic approaches. *Applied Microbiology and Biotechnology*, *85*(2), 265–276.

Sogin, M. L., Morrison, H. G., Huber, J. A., Welch, D. M., Huse, S. M., Neal, P. R., & Herndl, G. J. (2006). Microbial Diversity in the Deep Sea and the Underexplored "Rare Biosphere." *Proceedings of the National Academy of Sciences of the United States of America*, ***103*(32), 12115–12120.**

Swofford, D.L., Olsen, G.J., Waddell, P.J., & Hillis, D.M. (1996) Phylogenetic inference. In: Hillis DM, Moritz C, Mable BK (eds)*Molecular systematics.* **Sinauer, Sunderland, pp 407–514.**

Tajedin, L., Hashemi, J., Abaei, M. R., Hosseinpour, L., Rafei, F., & Basseri, H. R. (2009). Study on Fungal Flora in the Midgut of the Larva and Adult of the Different Populations of the Malaria Vector Anopheles stephensi. *Iranian Journal of Arthropod-Borne Diseases*,***3*(1), 36.**

Takken, W., & Knols, B. G. (1999). Odor-mediated behavior of Afrotropical malaria mosquitoes. *Annual Review of Entomology*, ***44*, 131–157.**

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution*, ***30*(12), 2725–2729.**

Terenius, O., Lindh, J. M., Eriksson-Gonzales, K., Bussière, L., Laugen, A. T., Bergquist, H., & Faye, I. (2012). Midgut bacterial dynamics in *Aedes aegypti*. *FEMS Microbiology Ecology*,***80*(3), 556–565.**

Wang, Y., Gilbreath, T. M., III, Kukutla, P., Yan, G., & Xu, J. (2011). Dynamic Gut Microbiome across Life History of the Malaria Mosquito *Anopheles gambiae* in Kenya. *PLoS ONE*, ***6*(9), e24767.**

Weisburg, W. G., Barns, S. M., Pelletier, D. A., & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology*, ***173*(2), 697–703.**

Whitten, M. M. A., Shiao, S. H., & Levashina, E. A. (2006). Mosquito midguts and malaria: cell biology, compartmentalization and immunology. *Parasite Immunology*, ***28*(4), 121–130.**

Woese, C.R., (1987) Bacterial evolution**.** *Microbiol Rev* **51:221–271.**

World malaria report 2015 - 9789241565158

Yoshida, S., Ioka, D., Matsuoka, H., Endo, H., & Ishii, A. (2001). Bacteria expressing single-chain immunotoxin inhibit malaria parasite development in mosquitoes. *Molecular and Biochemical Parasitology*, ***113*(1), 89–96.**

Yu, K., & Zhang, T. (2012). Metagenomic and Metatranscriptomic Analysis of Microbial Community Structure and Gene Expression of Activated Sludge. *PLoS ONE*, *7*(5).

Zouache, K., Michelland, R. J., Failloux, A.-B., Grundmann, G. L., & Mavingui, P. (2012). Chikungunya virus impacts the diversity of symbiotic bacteria in mosquito vector. *Molecular Ecology*, ***21*(9), 2297–2309.**

Zouache, K., Raharimalala, F. N., Raquin, V., Tran-Van, V., Raveloson, L. H. R., Ravelonandro, P., & Mavingui, P. (2011). Bacterial diversity of field-caught

mosquitoes, *Aedes albopictus* and *Aedes aegypti*, from different geographic regions of Madagascar. *FEMS Microbiology Ecology*, ***75*(3), 377–389**

APPENDICES

Appendix 1: Midgut bacterial composition at species level and their abundance

Species level	FC.*Anopheles*	FC.*Culex*	LR.*Anopheles*	LR.*Culex*	Total
Phylum *Archeabacteria*					
Archaea, Euryarchaeota, Methanobacteria, Methanobacteriales, Methanobacteriaceae, *Methanobrevibacter*, uncultured archaeon	24	0	0	0	
Phylum *Acidobacteria*					
Bacteria, Acidobacteria, Acidobacteria, Subgroup 4, Unknown Family, Blastocatella, uncultured *Acidobacteria* bacterium	10	0	0	0	
Bacteria, Acidobacteria, Acidobacteria, Subgroup 6, uncultured *Acidobacteria* bacterium	32	0	0	0	
Phylum *Actinobacteria*					
Bacteria, Actinobacteria, Actinobacteria, Corynebacteriales, Corynebacteriaceae, *Corynebacterium* uncultured bacterium	72	10	0	0	
Bacteria, Actinobacteria, Actinobacteria, Corynebacteriales, Corynebacteriaceae, Corynebacterium, uncultured *Corynebacterium* sp.	6	6	0	0	
Bacteria, Actinobacteria, Actinobacteria, Corynebacteriales, Corynebacteriaceae, *Corynebacterium* unidentified marine bacterioplankton	10	3	0	0	
Bacteria, Actinobacteria, Actinobacteria, Corynebacteriales, Corynebacteriaceae, uncultured, uncultured bacterium	31	1	0	0	
Bacteria, Actinobacteria, Actinobacteria, Corynebacteriales, Dietziaceae, *Dietzia* uncultured bacterium	8	0	0	0	
Bacteria, Actinobacteria, Actinobacteria, Frankiales, Geodermatophilaceae, *Blastococcus* uncultured bacterium	7	0	0	0	
Bacteria, Actinobacteria, Actinobacteria, Micrococcales, Cellulomonadaceae, *Actinotalea* uncultured bacterium	17	0	0	0	
Bacteria, Actinobacteria, Actinobacteria, Micrococcales, Intrasporangiaceae, *Terrabacter* uncultured bacterium	38	1	0	0	
Bacteria, Actinobacteria, Actinobacteria, Micrococcales, Microbacteriaceae, *Curtobacterium* uncultured bacterium	24	0	0	0	
Bacteria, Actinobacteria, Actinobacteria, Micrococcales, Micrococcaceae, Arthrobacter, *Arthrobacter* sp. TSBY-23	7	0	0	0	
Bacteria, Actinobacteria, Actinobacteria, Micrococcales, Micrococcaceae, Enteractinococcus, *Yaniella* sp. YUAB-SO-24	7	1	0	0	
Bacteria, Actinobacteria, Actinobacteria, Micrococcales, Micrococcaceae, Kocuria, *Kocuria* sp. oral clone AW006	11	2	0	0	
Bacteria, Actinobacteria, Actinobacteria, Micrococcales, Micrococcaceae, *Micrococcus* uncultured bacterium	43	0	0	0	
Bacteria, Actinobacteria, Actinobacteria, Propionibacteriales, Nocardioidaceae, *Nocardioides* uncultured bacterium	4	2	0	0	
Bacteria, Actinobacteria, Actinobacteria, Streptomycetales, Streptomycetaceae, Streptomyces, *Streptomyces ferralitis*	12	0	0	0	
Bacteria, Actinobacteria, Thermoleophilia, Gaiellales, uncultured, uncultured bacterium	12	0	0	0	
Phylum *Bacteroidetes*					
Bacteria, Bacteroidetes, Bacteroidia, Bacteroidales, Rikenellaceae, RC9 gut group, uncultured bacterium	6	0	0	0	
Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Cryomorphaceae, *Fluviicola* uncultured bacterium	14	0	0	0	
Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, Chryseobacterium, *Chryseobacterium* sp. M13	5	0	113	0	
Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, Elizabethkingia, *Elizabethkingia meningoseptica*	1	0	0	291	
Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, uncultured, uncultured Bacteroidetes bacterium	0	21	0	0	
Phylum *Chloroflexi*					
Bacteria, Chloroflexi, Thermomicrobia, JG30-KF-CM45, uncultured soil bacterium	31	0	0	0	
Bacteria, Chloroflexi, Thermomicrobia, Sphaerobacterales, Sphaerobacteraceae, Nitrolancea, uncultured *Chloroflexi* bacterium	19	0	0	0	
Phylum *Cyanobacteria*					
Bacteria, Cyanobacteria, *Chloroplast* uncultured bacterium	6	0	0	0	
Phylum *Firmicutes*					
Bacteria, Firmicutes, Bacilli, Bacillales, Bacillaceae, *Anoxybacillus* uncultured bacterium	0	10	1	0	
Bacteria, Firmicutes, Bacilli, Bacillales, Bacillaceae, Bacillus, uncultured *Bacillus* sp.	183	31	1	0	

Appendix 1: Contd

Bacteria, Firmicutes, Bacilli, Bacillales, Bacillaceae, *Bacillus* uncultured bacterium	5	1	0	0
Bacteria, Firmicutes, Bacilli, Bacillales, Planococcaceae, *Lysinibacillus* uncultured bacterium	22	5	0	0
Bacteria, Firmicutes, Bacilli, Bacillales, Staphylococcaceae, *Salinicoccus* uncultured bacterium	39	0	0	0
Bacteria, Firmicutes, Bacilli, Lactobacillales, Carnobacteriaceae, *Atopostipes* uncultured bacterium	32	1	0	0
Bacteria, Firmicutes, Bacilli, Lactobacillales, Carnobacteriaceae, uncultured, uncultured bacterium	6	0	0	0
Bacteria, Firmicutes, Bacilli, Lactobacillales, Streptococcaceae, *Streptococcus* uncultured bacterium	53	4	0	0
Bacteria, Firmicutes, Clostridia, Clostridiales, Peptostreptococcaceae, *Incertae Sedis* uncultured bacterium	26	4	1	0
Bacteria, Firmicutes, Clostridia, Clostridiales, Ruminococcaceae, uncultured, uncultured bacterium	15	0	0	0
Phylum *Gemmatimonadetes*				
Bacteria, Gemmatimonadetes, Gemmatimonadetes, AT425-EubC11 terrestrial group, uncultured bacterium	42	0	0	0
Phylum *Proteobacteria*				
Bacteria, Proteobacteria, Alphaproteobacteria, Caulobacterales, Caulobacteraceae, *Brevundimonas* uncultured bacterium	12	0	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Bartonellaceae, Bartonella, *Bartonella grahamii* as4aup	0	737	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Bradyrhizobiaceae, Bosea, uncultured *Bosea* sp.	37	0	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Methylobacteriaceae, Methylobacterium, *Methylobacterium lusitanum*	468	2	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Methylobacteriaceae, *Methylobacterium* uncultured bacterium	665	32	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhizobiaceae, Rhizobium, *Agrobacterium tumefaciens*	87	0	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhizobiaceae, Rhizobium, *Rhizobium* sp. JC140	32	0	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhizobiaceae, Rhizobium, *Rhizobium* sp. M51	106	0	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhizobiaceae, Rhizobium, uncultured *Agrobacterium* sp.	754	21	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhizobiaceae, *Rhizobium* uncultured bacterium	10	18	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhizobiaceae, Rhizobium, uncultured *Paracoccus* sp.	19	1	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, *Paracoccus* uncultured bacterium	9	0	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Acetobacteraceae, Acetobacter, uncultured *Acetobacter* sp.	0	0	8	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Acetobacteraceae, Asaia, *Asaia bogorensis*	0	0	116	1
Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Acetobacteraceae, *Asaia* uncultured bacterium	16	2	2223	55
Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Acetobacteraceae, *Asaia* uncultured bacterium	0	0	47	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Acetobacteraceae, *Asaia* uncultured bacterium	5	0	39	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Acetobacteraceae, Gluconacetobacter, *Gluconacetobacter liquefaciens*	370	0	37	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rickettsiales, Anaplasmataceae, *Wolbachia* Embioptera sp. UVienna-2012	0	125	0	261
Bacteria, Proteobacteria, Alphaproteobacteria, Rickettsiales, Anaplasmataceae, *Wolbachia* uncultured bacterium	0	5	0	43
Bacteria, Proteobacteria, Alphaproteobacteria, Rickettsiales, mitochondria, *Triticum aestivum*	17	0	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rickettsiales, Rickettsiaceae, Rickettsia, uncultured Rickettsia sp.	19	0	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae, *Sphingomonas* uncultured bacterium	21	2	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae, *Sphingomonas*, uncultured *Firmicutes* bacterium	24	0	0	0
Bacteria, Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae, *Delftia* uncultured bacterium	74	0	59	0
Bacteria, Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae, Delftia, uncultured *Delftia* sp.	46	0	7	0
Bacteria, Proteobacteria, Betaproteobacteria, Burkholderiales, Oxalobacteraceae, Oxalicibacterium, *Oxalicibacterium flavum*	30	0	0	0
Bacteria, Proteobacteria, Betaproteobacteria, Neisseriales, Neisseriaceae, uncultured, uncultured bacterium	7	1	0	0

Appendix 1: Contd

Bacteria, Proteobacteria, Epsilonproteobacteria, Campylobacterales, Campylobacteraceae, *Arcobacter* uncultured bacterium	0	1050	0	0
Bacteria, Proteobacteria, Epsilonproteobacteria, Campylobacterales, Helicobacteraceae, Helicobacter, *Helicobacter* sp. 'B52D Seymour'	0	204	0	0
Bacteria, Proteobacteria, Gammaproteobacteria, Aeromonadales, Aeromonadaceae, Aeromonas, *Aeromonas* sp. DMA1	0	125	588	37
Bacteria, Proteobacteria, Gammaproteobacteria, Aeromonadales, Aeromonadaceae, Aeromonas, uncultured *Aeromonas* sp.	0	0	12	2
Bacteria, Proteobacteria, Gammaproteobacteria, Aeromonadales, Aeromonadaceae, *Aeromonas* uncultured bacterium	0	64	254	18
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, *Enterobacter* uncultured bacterium	10	23	71	13
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Escherichia-Shigella, *Serratia marcescens*	0	0	1	4
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, *Rahnella* uncultured bacterium	364	7	399	1050
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, *Rahnella* uncultured bacterium	207	0	210	31
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, *Rahnella* uncultured bacterium	12	0	0	0
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Serratia, *Serratia marcescens*	168	48	365	2146
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Serratia, *Serratia marcescens*	157	14	232	1236
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Serratia, *Serratia marcescens*	0	0	15	98
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Serratia, *Serratia marcescens*	11	3	69	399
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Serratia, *Serratia marcescens*	1	0	7	23
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Serratia, *Serratia* sp. DR.Y5	0	0	1	13
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, *Serratia* uncultured bacterium	4	0	4	18
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, *Serratia* uncultured bacterium	28	0	0	1
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, *Serratia* uncultured bacterium	42	0	12	59
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, *Serratia* uncultured bacterium	0	0	11	91
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, *Serratia* uncultured bacterium	19	0	10	53
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Thorsellia, *Thorsellia anophelis*	0	0	81	0
Bacteria, Proteobacteria, Gammaproteobacteria, Orbales, Orbaceae, Gilliamella, uncultured *gamma proteobacterium*	0	69	0	0
Bacteria, Proteobacteria, Gammaproteobacteria, Orbales, Orbaceae, Gilliamella, uncultured *gamma proteobacterium*	0	65	0	0
Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae, Acinetobacter, *Acinetobacter* sp. B7_2TCO2	9	0	0	0
Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae, *Acinetobacter* uncultured bacterium	6	3	0	0
Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae, *Acinetobacter* uncultured bacterium	6	1	1	0
Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae, *Enhydrobacter* uncultured proteobacterium	13	4	0	0
Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae, *Psychrobacter* uncultured bacterium	11	0	0	0
Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae, *Pseudomonas* uncultured bacterium	21	14	26	6
Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae, *Pseudomonas* uncultured bacterium	28	0	0	0
Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae, *Pseudomonas* uncultured Pseudomonas sp.	1	0	177	0
Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, Xanthomonadaceae, *Stenotrophomonas* uncultured bacterium	196	0	5	0
Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, Xanthomonadaceae, *Stenotrophomonas*, uncultured bacterium	15	0	0	0
Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, Xanthomonadales Incertae Sedis, *Steroidobacter* uncultured bacterium	16	0	0	0
Phylum *Spirochaetae*				
Bacteria,Spirochaetae,Spirochaetes,Spirochaetales,Spirochaetaceae,uncultured,*Spironema culicis*	0	6	0	0
Phylum *Verrucomicrobia*				
Bacteria,Verrucomicrobia,Verrucomicrobiae,Verrucomicrobiales,Verrucomicrobiaceae,*Akkermansia*uncultured bacterium	47	0	0	0

Appendix 1: Contd

Bacteria,Verrucomicrobia,Verrucomicrobiae,Verrucomicrobiales,Verrucomicrobiaceae,*Akkermansia*uncultured bacterium	0	7	0	0	
Phylum *Eukaryota*					
Eukaryota, Opisthokonta, Nucletmycea, Fungi, Microsporidia, Incertae Sedis, Amblyosporidae, Parathelohania, *Parathelohania divulgata*	51	0	0	0	
Eukaryota, Opisthokonta, Nucletmycea, Fungi, Microsporidia, Incertae Sedis, Amblyosporidae, *Parathelohania* *Parathelohania obesa*	33	0	0	0	
Eukaryota, Opisthokonta, Nucletmycea, Fungi, Microsporidia, Incertae Sedis, Amblyosporidae, *Takaokaspora* *Takaokaspora* nipponicus	0	100	0	0	
No blast hit	1030	34	757	0	1721
Total abundance	6214	2890	5960	5949	21666

Appendix 2: Full Length Research paper

academicJournals

Vol. 11(29), pp. 1171-1183, 7 August, 2017
DOI: 10.5897/AJMR2016.8256
Article Number: 6648E3F65431
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Midgut bacterial diversity analysis of laboratory reared and wild *Anopheles gambiae* and *Culex quinquefasciatus* mosquitoes in Kenya

Erastus Mwaringa Mwadondo[1], Amanuel Ghilamicael[2], Amos Ematiti Alakonya[2,3] and Remmy Wekesa Kasili[2]*

[1]Department of Zoology, Jomo Kenyatta University of Agriculture and Technology, P. O. Box 62000-00200, Nairobi, Kenya.
[2]Institute for Biotechnology Research, Jomo Kenyatta University of Agriculture and Technology P. O. Box 62000-00200, Nairobi, Kenya.
[3]International Institute of Tropical Agriculture, Headquarters, PMB, 5320, Ibadan Oyo State, Nigeria.

Received 5 August, 2016; Accepted 21 June, 2017

Midgut symbiotic bacteria are known to play fundamental roles in the biology of mosquitoes, however knowledge of midgut bacterial communities associated with mosquitoes is scanty due to limitation of the isolation techniques based on culturing. In this study, the composition and diversity of midgut bacteria in field collected and lab reared adult female *Anopheles gambiae* and *Culex quinquefasciatus* mosquitoes was explored using the Illumina sequencing. Deoxyribonucleic acid was isolated from the pooled midgut extracts and their 16S rRNA gene sequenced using Illumina sequencing platform. Operational taxonomic units (OTUs) were analyzed using QIIME 1.8.0; taxonomy was assigned using BLASTn against SILVA 119 and hierarchical clustering was done using R program software. Out of the total number of sequence reads obtained, 145 OTUs were realized at 3% genetic distance. The 145 OTUs spanned 12 phyla; *Proteobacteria*, *Firmicutes*, *Cyanobacteria*, *Euryarchaeota*, *Gemmatimonadetes*, *Spirochaetae*, *Archeabacteria* *Verrucomicrobia*, *Chloroflexi*, *Bacteriodetes*, *Acidobacteria* and *Actinobacteria*. Microbial community composition based on OTUs showed significant difference between field collected and lab reared mosquitoes (χ^2 = 45.0799, p = 3.2 × 10^{-5}). Similarly, there was a significant difference in community composition at OTU level between *Anopheles gambiae* and *Culex quinquefasciatus* (χ^2 = 31.2257, p = 7.7 × 10^{-4}). The bacterial composition and diversity appeared to be influenced by the environment and the species of the mosquitoes.

Key words: *Anopheles gambiae*, *Culex quinquefasciatus*, midgut, DNA, diversity.

INTRODUCTION

Mosquitoes transmit diseases like malaria, dengue, lymphatic filariasis, yellow fever among others. Among these diseases, malaria is the most important mosquito borne disease with an estimated 214 million new cases of malaria worldwide (World Health Organization (WHO), 2015). The African region accounted for most of the global cases of malaria (88%), followed by South-East Asia region (10%) and Eastern Mediterranean region (2%) (WHO, 2015). In Kenya, there were an estimated 6.7 million new clinical cases and 4,000 deaths each year

and those living in Western Kenya have an especially high risk of malaria (Centers for Disease Control and Prevention (CDC), 2015). Most of the deaths are caused by the parasite *Plasmodium falciparum* whose major vector in Africa is the mosquito species *Anopheles gambiae* that is widely distributed throughout the Afro-tropical belt (Boissière et al., 2012).

Another mosquito species *Culex quinquefasciatus* is the principal vector for *Wuchereria bancrofti*, the filarial worm that causes filariasis and Japanese encephalitis (Agrawal and Sashindran, 2006). Lymphatic filariasis is a major public health problem worldwide. It is estimated that 1.3 billion people from 83 countries are living with the disease or are at risk of infection (Agrawal and Sashindran, 2006). Lymphatic filariasis is present on the East African coast especially in Kenya (Njenga et al., 2011).

Current mosquito vector control strategies include insecticide treatment delivered through spraying houses and insecticide-impregnated mosquito nets. While these methods are effective at decreasing mosquito vector numbers, they have also contributed to the rise in insecticide resistant mosquitoes (Bando et al., 2013).

Various alternative approaches are being tried to reduce malaria cases in the world, and one such approach is paratransgenesis. Paratransgenesis is a method where by a symbiotic bacteria is used to express effector molecules inside a targeted vector. The symbiotic bacteria are genetically modified to produce effector molecules and then reintroduced into the mosquito to produce the required effect (Chavshin et al., 2012). Understanding the microbial community structure of the mosquito midgut is therefore necessary in order to identify possible bacterial candidates for paratransgenesis. The mosquito midgut plays a critical role to the survival and development of the parasites and is therefore, the most attractive site to target malaria parasites (Whitten et al., 2006). The midgut microbiota of mosquitoes is still not well investigated and a few studies have been carried out on microflora of wild caught malaria vectors (Wang et al., 2011). The available conventional culture techniques limit the scope in determination of the microbial diversity since it sometimes misses out on non-culturable microbes (Pidiyar et al., 2004).

In laboratory-raised mosquitoes, the midgut bacteria can be acquired through contaminated sugar solutions, blood meals and transmitted transstadially. However, in wild mosquitoes, the origin of the midgut bacteria, is still unknown (Riehle and Jacobs-Lorena, 2005). In the current study the bacterial composition and diversity in the midgut of lab reared and field collected *A. gambiae* and *C. quinquefasciatus* mosquitoes were determined using the illumina sequencing.

MATERIALS AND METHODS

Study site

The study area for field collected mosquitoes was Ahero, Kenya, which is a malaria endemic region. It is located at latitude 0º 11'S and longitude 34º 55'E and is approximately 1153 m above sea level. The area has a tropical climate with significant rainfall throughout the year and with an average temperature of 23.0°C.

Collection of field *A. gambiae* and *C. quinquefasciatus* mosquitoes

Adult *A. gambiae* and *C. quinquefasciatus* mosquitoes were captured from pit shelters by use of Centre for Disease Control and Prevention (CDC) light traps. The CDC light traps were hung at least one meter above the ground on a tree or pole between 6:00 and 7:00 pm in the evening and left overnight. The collection bags containing the mosquitoes were picked between 6.00 and 6.30 am in the morning. The mosquitoes were then put into vial/jars from the collection bags using mouth aspirators and stored at 4°C. One hundred and thirty eight adult female *Anopheles gambiae* and *Culex quinquefasciatus* mosquitoes were identified to species level using a standard morphological key according to Gillies and De Meillon, (1968). The specimens were then transferred to the laboratory at the Institute for Biotechnology Research, Jomo Kenyatta University of Agriculture and Technology (JKUAT).

Acquisition of laboratory-reared *A. gambiae* and *C. quinquefasciatus*

One hundred and thirty eight laboratory reared adult female *A. gambiae* and *C. quinquefasciatus* mosquitoes were acquired from the International Centre of Insect Physiology and Ecology (ICIPE) Kasarani, Nairobi. They were transferred live to the laboratory at the Institute for Biotechnology Research, (JKUAT) and maintained in a mosquitarium at 28°C and 70 to 80% humidity until dissection. The mosquitoes were offered resins and 1% glucose solution as a source of energy and were not fed on blood.

Dissection of mosquitoes and isolation of DNA

Dissection of mosquitoes was done according to Rani et al. (2009). Before dissecting, the mosquitoes were chilled to death and sterilized with 70% ethanol then transferred into sterile distilled water in a sterile hood. The mosquitoes were dissected individually under sterile conditions. The midguts were mashed and suspended in 100 µl of sterile phosphate buffered solution (PBS). The mashed midguts were ground to homogeneity. Each midgut extract consisted of 20 pooled midguts of adult female mosquitoes. Field collected and lab reared mosquitoes had seven pooled midgut extracts each. The midgut extracts were stored at -80ºC until further analysis.

The total microbial genomic DNA was extracted separately from each group of mosquito midgut extracts using purelink genomic DNA mini kit (Invitrogen), following the manufacturer's

*Corresponding author: E-mail: rkasili@gmail.com. Tel: +254713800972.

instructions (CAT number, K1820-02 Life technologies, California, USA). Genomic DNA concentration was quantified using a nano drop spectrophotometer and the DNA stored at -20°C until further analysis.

Polymerase chain reaction amplification

Polymerase chain reaction (PCR) amplification of the 16S rRNA gene V4 variable region was carried out on the extracted DNA using primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) that had a barcode (Caporaso et al., 2010). PCR amplification was carried out in 30 cycles using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 min of intitial heating, followed by 30 cycles of 94°C for 30 s, 53°C for 40 s and 72°C for 1 min, followed by a final elongation step at 72°C for 5 min. PCR products were visualized on 2% agarose gel to determine the success of amplification and the relative intensity of bands. Multiple samples were pooled together in equal proportions based on their DNA concentrations from the gel images. Pooled samples were purified using calibrated Ampure XP beads (Agencort Bioscience Corporation, MA, USA).

Amplicon library preparation

The pooled and purified PCR products were used to prepare DNA library by following Illumina TruSeq DNA library protocol (Yu and Zhang, 2012). Sequencing was performed at Molecular Research DNA (www.mrdnalab.com, Shallowater, TX, USA) on a MiSeq platform following the manufacturer's guidelines. The resulting raw sequences were submitted to NCBI (Sequence Read Archive) with the following study accession numbers; sequences for field collected *A. gambiae* SAMN04386463; field collected *C. quinquefasciatus* SAMN04386464; lab reared *A. gambiae* SAMN04386465 and lab reared *C. quinquefasciatus* SAMN04386466.

Sequence analysis and taxonomic classification

Sequences obtained from the Illumina sequencing platform were depleted of barcodes and primers using a proprietary pipeline (www.mrdnalab.com, MR DNA, Shallowater, TX) developed at the service provider's laboratory. Short sequences < 200 bp, sequences with ambiguous base calls, and those with homopolymer runs exceeding 6 bp were removed. The sequences were denoised, chimeras and singleton sequences removed (Capone et al., 2011; Dowd et al., 2011; Eren et al., 2011). De novo OTU clustering was done with standard UCLUST method using the default settings as implemented in QIIME Version 1.8.0 at 97% similarity level (Caporaso et al., 2010). Taxonomy was assigned to each OTU using BLASTn against SILVA SSU Reference 119 database at default e-value threshold of 0.001 in QIIME (Quast et al., 2013).

Diversity indices

Diversity indices (Shannon, Inverse Simpson, Evenness), rarefaction, Venn diagram (to compare the shared OTUs between the samples of mosquitoes) and hierarchical clustering were computed, using Vegan package version 1.16 to 32 in R software (R development Core Team (2012). Kruskal-Wallis rank sum test was used to compare the relative abundance of gut microflora among *A. gambiae* and *C. quinquefasciatus* from lab reared and field collected samples using R programming language (R development Core Team (2012). Significance was tested at 95% confidence interval (p = 0.05). To support OTU-based analysis, taxonomic groups were derived from the number of reads assigned to each taxon at all ranks from domain to species using the taxa_summary.txt output from QIIME pipeline Version 1.8.0.

RESULTS

Assemblage and diversity of the microbial communities

After removing chimeras, denoising and demutiplexing, a total of 24,025 sequence reads greater than 200 bp were attained from the 16S rRNA data. Total OTU richness at 3% distance amounted to 145. The OTUs per data set ranged between 26 and 102. OTUs comprised 87% bacteria, 0.7% Archaea, 2% Fungi, 1.4% Eukarya and 8% no blast hit (sequences reads that were not assigned). Rarefaction curve was plotted in order to evaluate if all the diversity within the samples had been exhaustively recovered (Figure 1).

The slopes of the curves flatten out in cases where full diversity has been detected. This indicates that even if more sequences were obtained, the number of OTUs detected in the samples would not increase. However, more sequences would be required to exhaust the full diversity within the samples if the slopes did not flatten out (Chao et al., 2014). The sequencing depth as shown by the rarefaction curve was exhaustive enough to ensure the inclusion of the entire diversity of the microbes in the midgut of the two species of mosquitoes collected from field and lab reared.

The distribution of shared OTUs across the two species of mosquitoes and the sample source (lab reared and field collected) is shown in (Figure 2). Seven OTUs were common in all the samples, fifty four (54) OTUs were only found in field collected *A. gambiae* while 18 OTUs were detected only from the field collected *C. quinquefasciatus* samples. Lab reared *C. quinquefasciatus* and *A. gambiae* samples had one and 10 unshared OTUs respectively.

A diversity index is a quantitative measure that reflects how many different types of species there are in a community and simultaneously takes into account how evenly the individuals are distributed among them.The estimated Shannon diversity index varied between (3.54) for field collected *A. gambiae* and (1.93) for lab reared *C. quinquefasciatus* (Table 1). The Shannon diversity index for field collected *C. quinquefasciatus* (2.73) was higher than lab reared *A. gambiae* (2.52) and lab reared *C. quinquefasciatus* (1.93). The Shannon index is a representation of species abundance and evenness, when either of these two factors increases, the diversity index also increases. Evenness index was used to estimate how well the species are evenly distributed in a community. The highest evenness was recorded from field collected *A. gambiae* (0.767) indicating that OTUs were evenly distributed as compared to other samples. The lowest evenness was recorded from lab reared *C.*

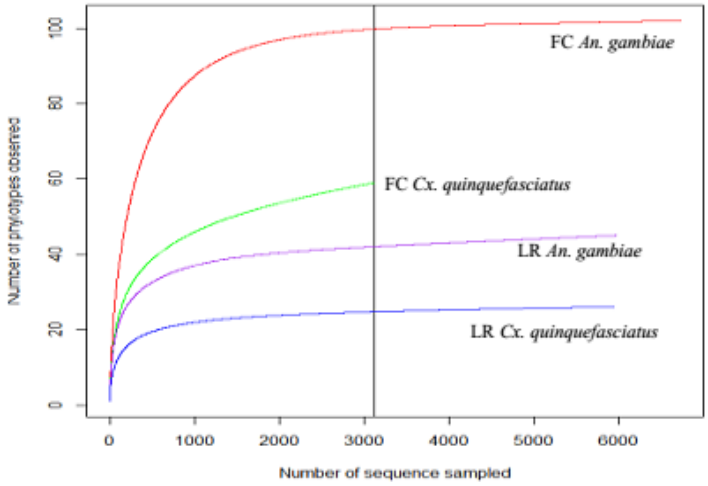

Figure 1. Rarefaction curve analysis in field collected (FC) *Cx.* (*Culex*) and lab reared (LR) *An.* (*Anopheles*) samples.

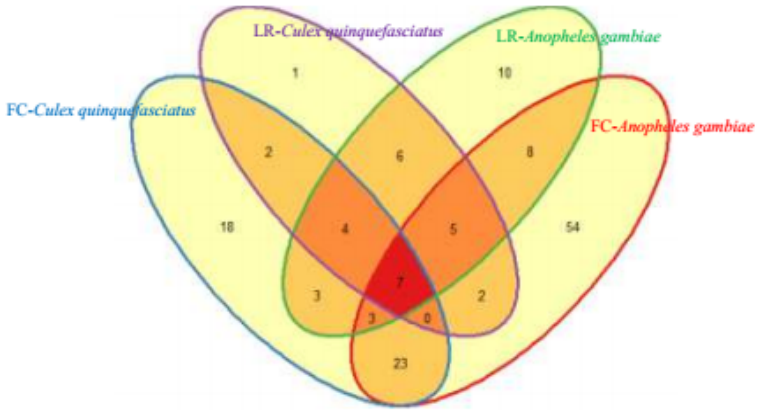

Figure 2. Venn diagram showing the distribution of shared OTUs across the 4 samples. Numbers indicate OTUs enumerated in field collected (FC) and lab reared (LR) samples.

Table 1. Diversity indices computed at OTU-based bacterial taxonomic units obtained from samples of the Field collected (FC) and Lab reared (LR) mosquitoes.

Sample	No. of sequences after filtering	Richness (OTUs)	Shannon (H)	Inverse Simpson	Evenness (J)	Effective no. of sp.
FC *Anopheles gambiae*	7516	102	3.54	19.98	0.767	34.47
FC *Culex quinquefasciatus*	3465	59	2.73	8.72	0.67	15.33
LR *Anopheles gambiae*	6669	45	2.52	5.98	0.661	12.43
LR *Culex quinquefasciatus*	6375	26	1.93	4.65	0.593	6.89
Total	**24,505**	**145**				

The microbial community composition, based on Kruskal-Wallis test, at OTU level showed significant difference between field collected and lab reared mosquitoes (χ^2= 45.0799, p = 3.2 × 10^{-8}). Similarly, there was significant difference in microbial community composition at OTU level between *Anopheles gambiae* and *Culex quinquefasciatus* (χ^2= 31.2257, p = 7.7 × 10^{-4}).

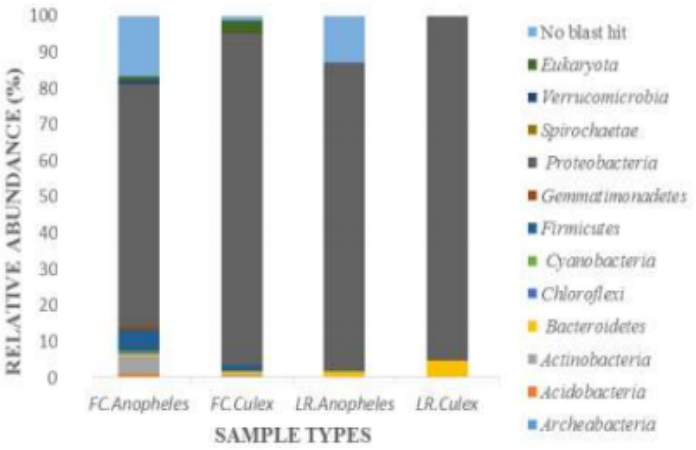

Figure 3. Relative abundance at phylum level from the field collected (FC) and lab reared (LR) samples.

quinquefasciatus (0.593) indicating that bacterial species are less evenly distributed and some species are more dominant than the others. The value of Inverse Simpson index ranged from 4.65 for lab reared *C. quinquefasciatus* to 19.98 for field collected *A. gambiae*. The value of Inverse Simpson index was observed to increase with increase in diversity.

Microbial taxonomic community composition

The SILVA SSU Reference 119 database (Quast et al., 2013) was used to assign reads to appropriate taxonomic ranks. The OTUs spanned 12 phyla (Figure 3); *Proteobacteria* (62.04-95.11 %), *Firmicutes* (0.00–6.13 %), *Bacteriodetes* (0.42–4.89 %), *Actinobacteria* (0.00-4.97%), *Eukaryota* (0.00-3.46%), *Gemmatimonadetes* (0.00-0.86%), *Spirochaetae* (0.00-0.21%), *Verrucomicrobia* (0.00-0.76%), *Chloroflexi* (0.00-0.80%), *Acidobacteria* (0.00-0.68%), *Archeabacteria* (0.00-0.39%) and *Cyanobacteria* (0.00-0.10%). The no blast hits had relative abundance ranging from 0.00 to 16.58%.

OTUs belonging to the Phylum Proteobacteria were the most abundant and were represented by the most genera as shown in Figure 4. In lab reared *C. quinquefasciatus* sample the OTUs were affiliated to following genera; *Aeromonas*, *Asaia*, *Elizabethkingia*, *Enterobacter*, *Pseudomonas*, *Rahnella*, *Serratia* and *Wolbachia*. *Serratia marcescens* was the most abundant species in this sample with a relative abundance of 64.29%. Other species present in higher abundance were *Rahnella* uncultured bacterium 18.13%, *Serratia* uncultured bacterium 5.08%, *Wolbachia Embioptera* sp. 4.37% and *Elizabethkingia meningoseptica* 4.88% (Figure 4). However, in field collected *Culex quinquefasciatus* sample genera represented were, *Wolbachia*, *Sphingomonas*, *Streptococcus*, *Serratia*, *Rhizobium*, *Rahnella*, *Pseudomonas*, *Methylobacterium*, *Ixodes*, *Helicobacter*, *Gamma proteobacterium*, *Enterobacter*, *Corynebacterium*, *Bartonella*, *Bacteroidetes*, *Bacillus*, *Asaia*, *Arcobacter*, *Akkermansia*, *Agrobacterium*, and *Aeromonas*. The most abundant species in field collected *C. quinquefasciatus* sample were *Arcobacter* uncultured bacterium with relative abundance of 34.83%, while *Bartonella grahamii* as4aup had 24.45% (Figure 4). *Arcobacter* uncultured bacterium, *Bacteroidetes* uncultured bacterium, *B. grahamii* as4up, *Gamma Proteobacteria* uncultured bacterium, *Helicobacter* sp. B52Seymour and *Ixodes scapularis* were unique species in the field collected *C. quinquefasciatus* sample.

In lab reared *A. gambiae* sample, *Asaia* uncultured bacterium was the most abundant species with 39.30% relative abundance. Other taxa represented in the sample include *Aeromonas* sp. DMA1, *Rahnella* uncultured bacterium and *Serratia marcescens* each scoring a relative abundance of 10%. The genera found in lab reared *Anopheles gambiae* sample include; *Aeromonas, Serratia, Bacillus, Asaia, Chryseobacterium, Gluconacetobacter, Delftia, Pseudomonas, Rahnella, Thorsellia, Enterobacter* and *Stenotrophomonas*. *Thorsellia anophelis* was unique to lab reared *A. gambiae* sample (Figure 5). The field collected *A. gambiae* sample was found to harbor a higher diversity of bacterial species. The most abundant species were *Agrobacterium* sp. 12.63% and *Methylobacterium* uncultured bacterium at 11.14% relative abundance. The most predominant genera found in field collected include; *Serratia, Bacillus, Agrobacterium Stenotrophomonas, Gluconacetobacter, Methylobacterium. Rahnella* (Figure 5). The unique species in field collected *A. gambiae* sample include

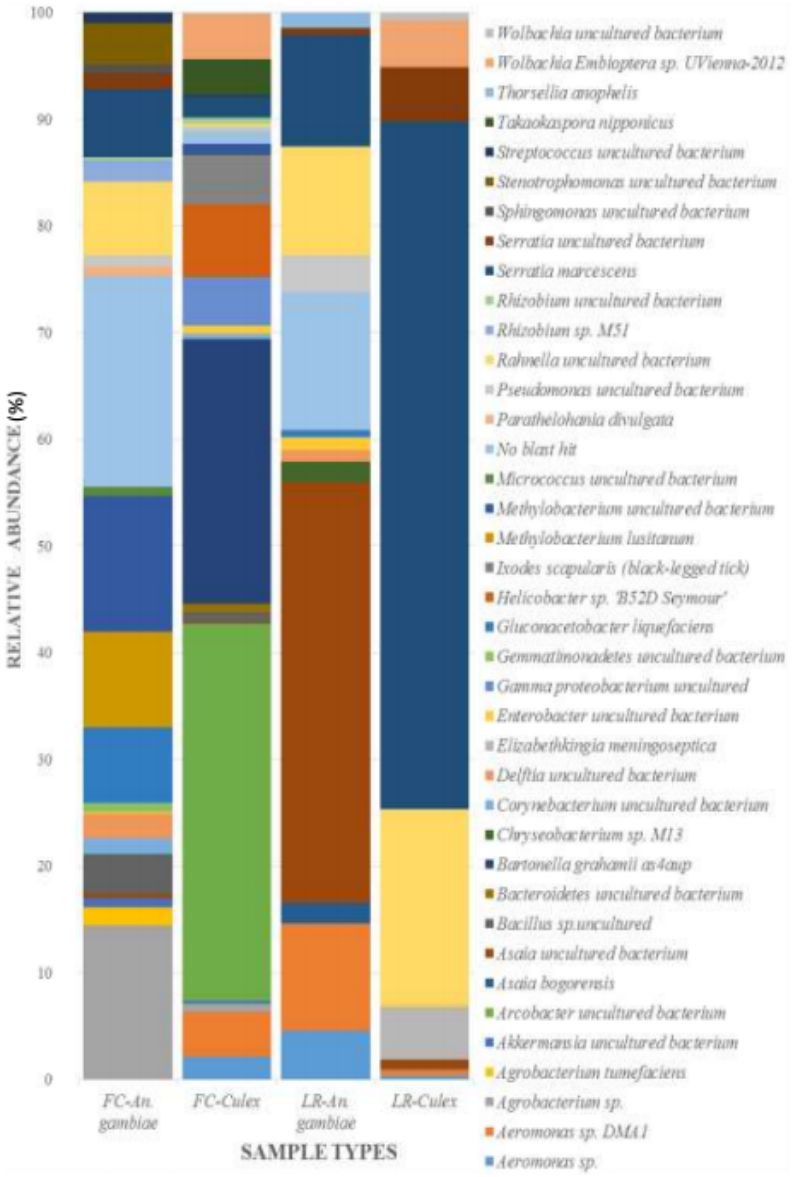

Figure 4. Relative abundance of the most predominant bacterial species from the field collected (FC) and lab reared (LR) samples.

Agrobacterium tumefaciens, *Gemmatimonadetes* uncultured bacterium, *Micrococcus* uncultured bacterium and *Rhizobium* sp. M51 (Figure 5).

Bacterial species which were recovered from all the four samples include, *Serratia marcescens*, *Asaia* uncultured bacterium, *Enterobacter* uncultured bacterium, *Pseudomonas* uncultured bacterium and *Rahnella* uncultured bacterium. *Parathelohania divulgata* and *Takaokaspora nipponicus* are fungal species recovered from the field collected *A. gambiae* and *C.quinquefasciatus* respectively (Figure 5). Detailed information on all the bacterial species recovered in this study is given in additional file 1 Table S1.

Hierarchical clustering, based on Bray-Curtis

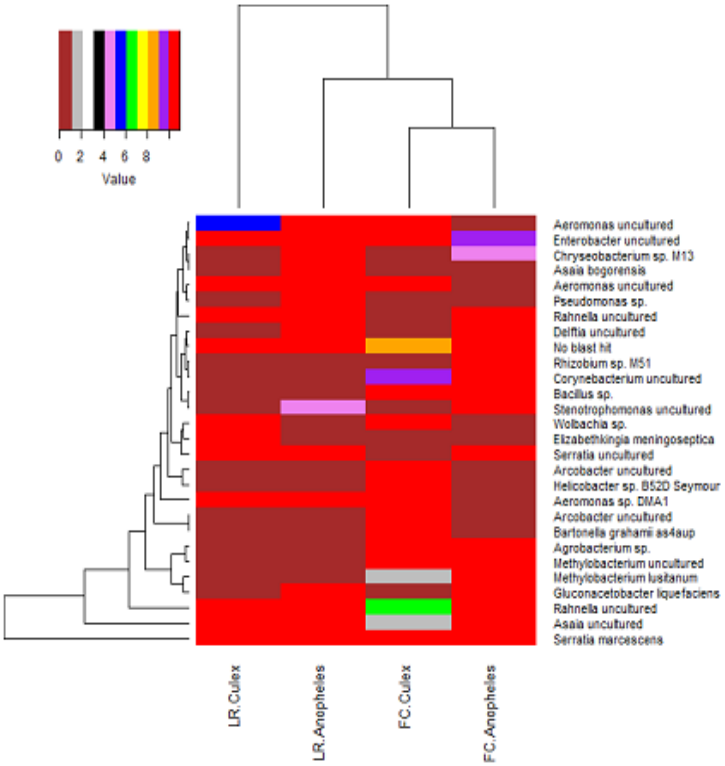

Figure 5. Hierarchical clustering of most abundant midgut bacterial species of the field collected (FC) and lab reared (LR) mosquitoes. Species level was chosen to be used in hierarchical clustering to assess the relationship between sample and taxa.

dissimilarity, showed two clusters (Figure 5). The dendogram shown on the top signify the relationship between the four samples. The bacteria composition of lab reared *A.gambiae*, field collected *A. gambiae* and field collected *C. quinquefasciatus* samples were clustered together. Within this cluster the field collected *A. gambiae* and field collected *C. quinquefasciatus* samples were more closely related to each other. The bacterial community recovered from the lab reared *C. quinquefasciatus* samples was observed to form a distinct cluster.

DISCUSSION

The microbes inhabiting mosquito midgut have drawn special attention in the recent past due to their interactions with both the mosquito host as well as disease causing parasites. The present study sought to investigate the composition and diversity of microbes in midguts of lab reared and field collected *A. gambiae* and *C. quinquefasciatus* mosquitoes. The field collected mosquitoes showed more midgut bacterial composition and diversity than the lab reared mosquitoes. A similar observation was reported by Rani et al. (2009) in their study involving the analysis of bacterial diversity in larvae and adult midgut microflora in lab reared and field collected *Anopheles stephensi* mosquito vectors. The higher bacterial diversity in field collected mosquitoes could probably be due to the fact that wild mosquitoes are exposed to the natural environment where they feed on various natural foods that could be the source of the diverse microbes, whereas the lab reared mosquitoes are fed on artificial diet of glucose and resins. Furthermore, adult female mosquitoes require a blood meal for their egg development and the blood acquired in the field could also be a source of various bacterial flora. On the other hand, the blood given to the adult female lab reared mosquitoes is from infection-free rabbits/mice (Rani et al., 2009). In the present study, the highest number of bacterial species was detected from field collected *A. gambiae* mosquitoes followed by field collected *C. quinquefasciatus* and lab reared *A. gambiae*. The least number of bacterial species were detected from lab

reared *C. quinquefasciatus*.

Diversity indices analysis at OTU level from field collected and laboratory reared mosquitoes revealed a significant difference in microbial community composition. Field collected *A. gambiae* had the highest value of Inverse Simpson index and while the lowest was lab reared *C. quinquefasciatus* samples. The value of Inverse Simpson increases with diversity (Chandel et al., 2013). The Shannon index is another widely used index for comparing diversity between various habitats (Chandel et al., 2013). The Shannon index is a representation of both species abundance and evenness, when either of these two factors increase, the diversity index increases. Evenness was used for the estimating how well the species are evenly distributed among the samples. The lowest evenness was recorded from laboratory reared *C. quinquefasciatus* sample indicating that the bacterial species in this sample are less evenly distributed, that is, some species are more dominant than others. Comparative diversity was visualized using heatmap based on Bray-Curtis dissimilarities at species level. The microbial composition of the field collected samples at species level, were more similar compared to the laboratory reared. However, the laboratory reared samples the bacterial composition seemed to differ between *A. gambiae* and *C. quinquefasciatus*.

Members of the phylum Proteobacteria, were predominantly recovered from both the field collected and lab reared samples than those of phylum *Firmicutes*, *Actinobacteria* and *Bacteriodetes*. *Proteobacteria* were also shown to be dominant in a previous study conducted in Kenya on *A. gambiae* mosquitoes (Wang et al., 2011). Proteobacteria was the largest phylum represented by 43 species belonging to 26 genera. Some of the groups of bacteria recovered in the present study are similar to those recovered from previous culture dependent and culture-independent studies (Rani et al., 2009). Phylum *Firmicutes* consisted of ten species which were affiliated to nine genera. *Actinobacteria* represented fifteen species belonging to thirteen genera whereas *Bacteriodetes* consisted of five species belonging to five genera. Phylum *Cyanobacteria*, *Gemmatimonadetes*, *Spirochaetae*, *Verrucomicrobia*, *Chloroflexi*, *Archeabacteria* and *Acidobacteria* represented only a small portion of the mosquito midgut communities.

The dominant genera recovered in the present study belong to *Serratia, Asaia*, *Arcobacter*, *Rahnella*, *Bartonella*, *Aeromonas*, *Agrobacterium*, *Methylobacterium* and *Wolbachia*. The results of the study are consistent with earlier reports which have shown that that above genera are dominant (Pidiyar et al., 2004; Demaio et al., 1996; Favia et al., 2007; Dong et al., 2009). This suggests that at least a fraction of the mosquito midgut inhabitants could be common for different mosquito species inhabiting similar environments and may represent evolutionary conservation of association between bacteria and mosquito gut. Members of the genera *Acinetobacter*, *Aeromonas*, *Bacillus*, *Enterobacter*, *Pseudomonas*, *Serratia*, *Asaia*, *Rahnella*, and *Stenotrophomonas* have been frequently reported in mosquito gut in previous studies (Pidiyar et al., 2004; Boissière et al., 2012; Chandel et al., 2013). Sequences belonging to genera *Asaia*, *Enterobacter*, *Pseudomonas*, *Rahnella* and *Serratia* were recovered from all the samples and comprise a major part of microbiota of *A. gambiae* and *C. quinquefasciatus* mosquitoes in the present study.

Serratia marcescens appeared to be the core species (23.6%) in the present study, as it was observed in both lab reared and field collected samples, suggesting that it possesses a competitive advantage over other bacterial species. *S. marcescens* is abundant in nature, and especially in the artificial foods given to the lab reared mosquitoes. Similar results were reported in five generations of lab reared *A. gambiae* (Dong et al., 2009).

Asaia uncultured bacterium species was recovered at 39.30% was more abundant in lab reared *A.gambiae*. *Asaia* has been associated with *Anopheles* species, in particular field collected *Anopheles funestus*, *Anopheles Maculipennis, Anopheles gambiae* and *Anopheles coustani*, and *Anopheles stephensi* in which *Asaia* bacteria was dominant and stably associated (Favia et al., 2007). The presence of *Asaia* species in *Anopheles* mosquito could be a target for malaria control it produces antiparasite molecules in mosquitoes that could be exploited in paratransgenic control of malaria (Damiani et al., 2010; Favia et al., 2007).

Elizabethkingia meningoseptica and *Wolbachia* sp. have repeatedly been detected in both lab reared and wild caught mosquitoes (Pumpuni et al., 1996) indicating their prevalent symbiotic association with mosquitoes. In the present study, *Wolbacha* was detected in both field collected and lab reared *C. quinquefasciatus*, previously it has been reported in several other mosquito vectors including, *Aedes*, *Coquillettida* and *Masonia* (Ricci et al., 2012).

Bacillus sp., *Stenotrophomonas*, *Micrococcus Acinetobacter*, and *Rhizobium* frequently isolated from soil and environmental samples were recovered at significantly greater numbers from the field collected mosquitoes. This suggests that the local soil and water environment plays an important role in colonization of the mosquito midgut at breeding sites or during nectar/blood feeding (Chandel et al., 2013). *Parathelohania divulgata*, *Parathelohania obesa* and *Takaokaspora nipponicus* fungal species were recovered from the field collected *A. gambiae* and *C. quinquefasciatus* but were absent in lab reared mosquitoes.

From the foregoing, the mosquito midgut has a rich diversity of symbiotic bacteria. The parasite mosquito relationship is believed to have been in existence for many years and it is likely that the acquired microflora permit the maintenance of pathogenic parasites in mosquitoes. The microbes could be benefiting the

mosquitoes by protecting them against harmful bacteria and the mosquitoes could be benefiting the parasites by lowering the mosquito immunity against the parasites.

Conclusion

The results obtained present an analysis of the composition and bacterial diversity of lab reared and wild mosquitoes using Illumina sequencing technology. The bacterial flora of adult female *A. gambiae* and *C. quinquefansciatus* midgut is diverse and is dominated by bacterial species *S. marcescens*. In future, understanding the tripartite mosquito-microbes-parasite interaction will enable us gain more insight that may be useful in the development of novel approaches for the control of malaria and other mosquito borne diseases like filariasis, dengue, Zika and Chikungunya.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Agrawal V, Sashindran V (2006). Lymphatic Filariasis in India : Problems, Challenges and New Initiatives. Med. J. Armed Forces India 62:359-362.

Bando H, Okado K, Guelbeogo WM, Badolo A, Aonuma H, Nelson B, Fukumoto S, Xuan X, Sagnon NF, Kanuka H (2013). Intra-specific diversity of *Serratia marcescens* in *Anopheles* mosquito midgut defines Plasmodium transmission capacity. Sci. Rep. 3:1641.

Boissière A, Tchioffo MT, Bachar D, Abate L, Marie A, Nsango SE, Shahbazkia HR, Awono-Ambene PH, Levashina EA, Christen R, Morlais I (2012). Midgut Microbiota of the Malaria Mosquito Vector *Anopheles gambiae* and Interactions with *Plasmodium falciparum* Infection. PLoS Pathog. 8(5):e1002742.

Capone KA, Dowd SE, Stamatas GN, Nikolovski J (2011). Diversity of the Human Skin Microbiome Early in Life. J. Invest. Dermatol. 131:2026-2032.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R (2010). QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7:335-336.

Chandel K, Mendki MJ, Parikh RY, Kulkarni G, Tikar SN, Sukumaran D, Prakash S, Parashar BD, Shouche YS, Veer V (2013). Midgut Microbial Community of *Culex quinquefasciatus* Mosquito Populations from India. PLoS One 8(11):e80453.

Chao A, Gotelli NJ, Hsieh TC, Sander EL, Ma KH, Colwell RK, Ellison AM (2014). Rarefaction and extrapolation with Hill numbers: A framework for sampling and estimation in species diversity studies. Ecol. Monogr. 84:45-67.

Chavshin AR, Oshaghi MA, Vatandoost H, Pourmand MR, Raeisi A, Enayati AA, Mardani N, Ghoorchian S (2012). Identification of bacterial microflora in the midgut of the larvae and adult of wild caught *Anopheles stephensi*: a step toward finding suitable paratransgenesis candidates. Acta Trop. 121:129-134.

Damiani C, Ricci I, Crotti E, Rossi P, Rizzi A, Scuppa P, Capone A, Ulissi U, Epis S, Genchi M, Sagnon N, Faye I, Kang A, Chouaia B, Whitehorn C, Moussa GW, Mandrioli M, Esposito F, Sacchi L, Bandi C, Daffonchio D, Favia G (2010). Mosquito-bacteria symbiosis: the case of *Anopheles gambiae* and *Asaia*. Microb. Ecol. 60:644-654.

Demaio J, Pumpuni CB, Kent M, Beier JC (1996). The midgut bacterial flora of wild *Aedes triseriatus*, *Culex pipiens*, and *Psorophora columbiae* mosquitoes. Am. J. Trop. Med. Hyg. 54:219-223.

Dong Y, Manfredini F, Dimopoulos G (2009). Implication of the Mosquito Midgut Microbiota in the Defense against Malaria Parasites. PLoS Pathog. 5:e1000423.

Dowd SE, Delton Hanson J, Rees E, Wolcott RD, Zischau AM, Sun Y, White J, Smith DM, Kennedy J, Jones CE (2011). Survey of fungi and yeast in polymicrobial infections in chronic wounds. J. Wound Care 20:40-47.

Eren AM, Zozaya M, Taylor CM, Dowd SE, Martin DH, Ferris MJ (2011). Exploring the Diversity of *Gardnerella vaginalis* in the Genitourinary Tract Microbiota of Monogamous Couples Through Subtle Nucleotide Variation. PLoS One 6(10):e26732.

Favia G, Ricci I, Damiani C, Raddadi N, Crotti E, Marzorati M, Rizzi A, Urso R, Brusetti L, Borin S, Mora D, Scuppa P, Pasqualini L, Clementi E, Genchi M, Corona S, Negri I, Grandi G, Alma A, Kramer L, Esposito F, Bandi C, Sacchi L, Daffonchio D (2007). Bacteria of the genus *Asaia* stably associate with *Anopheles stephensi*, an *Asian* malarial mosquito vector. Proc. Natl. Acad. Sci. U. S. A. 104:9047-9051.

Gillies MT, DE Meillon B (1968). The Anophelinae of Africa south of the Sahara (Ethiopian Zoogeographical Region). 343 p.

Njenga SM, Mwandawiro CS, Wamae CN, Mukoko DA, Omar AA, Shimada M, Bockarie MJ, Molyneux DH (2011). Sustained reduction in prevalence of lymphatic filariasis infection in spite of missed rounds of mass drug administration in an area under mosquito nets for malaria control. Parasit. Vectors 4(1):90.

Pidiyar VJ, Jangid K, Patole MS, Shouche YS (2004). Studies on Cultured and Uncultured Microbiota of Wild *Culex quinquefasciatus* Mosquito Midgut Based on 16s Ribosomal Rna Gene Analysis. Am. J. Trop. Med. Hyg. 70:597-603.

Prevention, CDC - Malaria - Malaria Worldwide - CDC's Global Malaria Activities - Kenya http://www.cdc.gov/malaria/malaria_worldwide/cdc_activities/kenya.html.

Pumpuni CB, Demaio J, Kent M, Davis JR, Beier JC (1996). Bacterial population dynamics in three anopheline species: the impact on *Plasmodium* sporogonic development. Am. J. Trop. Med. Hyg. 54:214-218.

Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner F O (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41:D590-D596.

R development Core Team (2012). R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing: Vienna, Austria.

Rani A, Sharma A, Rajagopal R, Adak T, Bhatnagar RK (2009). Bacterial diversity analysis of larvae and adult midgut microflora using culture-dependent and culture-independent methods in lab-reared and field-collected *Anopheles stephensi*-an Asian malarial vector. BMC Microbiol. 9(1):96.

Ricci I, Damiani C, Capone A, DeFreece C, Rossi P, Favia G (2012). Mosquito/microbiota interactions: from complex relationships to biotechnological perspectives. Curr. Opin. Microbiol. 15:278-284.

Riehle MA, Jacobs-Lorena M (2005). Using bacteria to express and display anti-parasite molecules in mosquitoes: current and future strategies. Insect Biochem. Mol. Biol. 35:699-707.

Wang Y, Gilbreath TM, Kukutla P, Yan G, Xu J (2011). Dynamic Gut Microbiome across Life History of the Malaria Mosquito *Anopheles gambiae* in Kenya. PLoS One 6:e24767.

Whitten MMA, Shiao SH, Levashina EA (2006). Mosquito midguts and malaria: cell biology, compartmentalization and immunology. Parasite Immunol. 28:121-130.

WHO- World Health Organisation (2015). World malaria report. http://www.who.int/malaria/publications/world-malaria-report-2015/report/en/.

Yu K, Zhang T (2012). Metagenomic and Metatranscriptomic Analysis of Microbial Community Structure and Gene Expression of Activated Sludge. PLoS One 7(5):e38183.

Additional file 1

Table S1. Midgut bacterial composition at species level and their abundance.

Species level	FC.*Anopheles*	FC.*Culex*	LR.*Anopheles*	LR.*Culex*	Total
Phylum *Archeabacteria*					
Archaea, Euryarchaeota, Methanobacteria, Methanobacteriales, Methanobacteriaceae, *Methanobrevibacter*, uncultured archaeon	24	0	0	0	
Phylum *Acidobacteria*					
Bacteria, Acidobacteria, Acidobacteria, Subgroup 4, Unknown Family, Blastocatella, uncultured *Acidobacteria* bacterium	10	0	0	0	
Bacteria, Acidobacteria, Acidobacteria, Subgroup 6, uncultured *Acidobacteria* bacterium	32	0	0	0	
Phylum *Actinobacteria*					
Bacteria, Actinobacteria, Actinobacteria, Corynebacteriales, Corynebacteriaceae, *Corynebacterium* uncultured bacterium	72	10	0	0	
Bacteria, Actinobacteria, Actinobacteria, Corynebacteriales, Corynebacteriaceae, Corynebacterium, uncultured *Corynebacterium* sp.	6	6	0	0	
Bacteria, Actinobacteria, Actinobacteria, Corynebacteriales, Corynebacteriaceae, *Corynebacterium* unidentified marine bacterioplankton	10	3	0	0	
Bacteria, Actinobacteria, Actinobacteria, Corynebacteriales, Corynebacteriaceae, uncultured, uncultured bacterium	31	1	0	0	
Bacteria, Actinobacteria, Actinobacteria, Corynebacteriales, Dietziaceae, *Dietzia* uncultured bacterium	8	0	0	0	
Bacteria, Actinobacteria, Actinobacteria, Frankiales, Geodermatophilaceae, *Blastococcus* uncultured bacterium	7	0	0	0	
Bacteria, Actinobacteria, Actinobacteria, Micrococcales, Cellulomonadaceae, *Actinotalea* uncultured bacterium	17	0	0	0	
Bacteria, Actinobacteria, Actinobacteria, Micrococcales, Intrasporangiaceae, *Terrabacter* uncultured bacterium	38	1	0	0	
Bacteria, Actinobacteria, Actinobacteria, Micrococcales, Microbacteriaceae, *Curtobacterium* uncultured bacterium	24	0	0	0	
Bacteria, Actinobacteria, Actinobacteria, Micrococcales, Micrococcaceae, Arthrobacter, *Arthrobacter* sp. TSBY-23	7	0	0	0	
Bacteria, Actinobacteria, Actinobacteria, Micrococcales, Micrococcaceae, Enteractinococcus, *Yaniella* sp. YUAB-SO-24	7	1	0	0	
Bacteria, Actinobacteria, Actinobacteria, Micrococcales, Micrococcaceae, Kocuria, *Kocuria* sp. oral clone AW006	11	2	0	0	
Bacteria, Actinobacteria, Actinobacteria, Micrococcales, Micrococcaceae, *Micrococcus* uncultured bacterium	43	0	0	0	
Bacteria, Actinobacteria, Actinobacteria, Propionibacteriales, Nocardioidaceae, *Nocardioides* uncultured bacterium	4	2	0	0	
Bacteria, Actinobacteria, Actinobacteria, Streptomycetales, Streptomycetaceae, Streptomyces, *Streptomyces ferralitis*	12	0	0	0	
Bacteria, Actinobacteria, Thermoleophilia, Gaiellales, uncultured, uncultured bacterium	12	0	0	0	
Phylum *Bacteroidetes*					
Bacteria, Bacteroidetes, Bacteroidia, Bacteroidales, Rikenellaceae, RC9 gut group, uncultured bacterium	6	0	0	0	
Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Cryomorphaceae, *Fluviicola* uncultured bacterium	14	0	0	0	
Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, Chryseobacterium, *Chryseobacterium* sp. M13	5	0	113	0	
Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, Elizabethkingia, *Elizabethkingia meningoseptica*	1	0	0	291	
Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, uncultured, uncultured Bacteroidetes bacterium	0	21	0	0	
Phylum *Chloroflexi*					
Bacteria, Chloroflexi, Thermomicrobia, JG30-KF-CM45, uncultured soil bacterium	31	0	0	0	
Bacteria, Chloroflexi, Thermomicrobia, Sphaerobacterales, Sphaerobacteraceae, Nitrolancea, uncultured *Chloroflexi* bacterium	19	0	0	0	
Phylum *Cyanobacteria*					
Bacteria, Cyanobacteria, *Chloroplast* uncultured bacterium	6	0	0	0	
Phylum *Firmicutes*					
Bacteria, Firmicutes, Bacilli, Bacillales, Bacillaceae, *Anoxybacillus* uncultured bacterium	0	10	1	0	
Bacteria, Firmicutes, Bacilli, Bacillales, Bacillaceae, Bacillus, uncultured *Bacillus* sp.	183	31	1	0	

Table S1. Contd.

Bacteria, Firmicutes, Bacilli, Bacillales, Bacillaceae, *Bacillus* uncultured bacterium	5	1	0	0
Bacteria, Firmicutes, Bacilli, Bacillales, Planococcaceae, *Lysinibacillus* uncultured bacterium	22	5	0	0
Bacteria, Firmicutes, Bacilli, Bacillales, Staphylococcaceae, *Salinicoccus* uncultured bacterium	39	0	0	0
Bacteria, Firmicutes, Bacilli, Lactobacillales, Carnobacteriaceae, *Atopostipes* uncultured bacterium	32	1	0	0
Bacteria, Firmicutes, Bacilli, Lactobacillales, Carnobacteriaceae, uncultured, uncultured bacterium	6	0	0	0
Bacteria, Firmicutes, Bacilli, Lactobacillales, Streptococcaceae, *Streptococcus* uncultured bacterium	53	4	0	0
Bacteria, Firmicutes, Clostridia, Clostridiales, Peptostreptococcaceae, *Incertae Sedis* uncultured bacterium	26	4	1	0
Bacteria, Firmicutes, Clostridia, Clostridiales, Ruminococcaceae, uncultured, uncultured bacterium	15	0	0	0
Phylum *Gemmatimonadetes*				
Bacteria, Gemmatimonadetes, Gemmatimonadetes, AT425-EubC11 terrestrial group, uncultured bacterium	42	0	0	0
Phylum *Proteobacteria*				
Bacteria, Proteobacteria, Alphaproteobacteria, Caulobacterales, Caulobacteraceae, *Brevundimonas* uncultured bacterium	12	0	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Bartonellaceae, Bartonella, *Bartonella grahamii* as4aup	0	737	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Bradyrhizobiaceae, Bosea, uncultured *Bosea* sp.	37	0	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Methylobacteriaceae, Methylobacterium, *Methylobacterium lusitanum*	468	2	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Methylobacteriaceae, *Methylobacterium* uncultured bacterium	665	32	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhizobiaceae, Rhizobium, *Agrobacterium tumefaciens*	87	0	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhizobiaceae, Rhizobium, *Rhizobium* sp. JC140	32	0	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhizobiaceae, Rhizobium, *Rhizobium* sp. M51	106	0	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhizobiaceae, Rhizobium, uncultured *Agrobacterium* sp.	754	21	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhizobiaceae, *Rhizobium* uncultured bacterium	10	18	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhizobiaceae, Rhizobium, uncultured *Paracoccus* sp.	19	1	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, *Paracoccus* uncultured bacterium	9	0	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Acetobacteraceae, Acetobacter, uncultured *Acetobacter* sp.	0	0	8	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Acetobacteraceae, Asaia, *Asaia bogorensis*	0	0	116	1
Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Acetobacteraceae, *Asaia* uncultured bacterium	16	2	2223	55
Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Acetobacteraceae, *Asaia* uncultured bacterium	0	0	47	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Acetobacteraceae, *Asaia* uncultured bacterium	5	0	39	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Acetobacteraceae, Gluconacetobacter, *Gluconacetobacter liquefaciens*	370	0	37	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rickettsiales, Anaplasmataceae, *Wolbachia* Embioptera sp. UVienna-2012	0	125	0	261
Bacteria, Proteobacteria, Alphaproteobacteria, Rickettsiales, Anaplasmataceae, *Wolbachia* uncultured bacterium	0	5	0	43
Bacteria, Proteobacteria, Alphaproteobacteria, Rickettsiales, mitochondria, *Triticum aestivum*	17	0	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rickettsiales, Rickettsiaceae, Rickettsia, uncultured Rickettsia sp.	19	0	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae, *Sphingomonas* uncultured bacterium	21	2	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae, *Sphingomonas*, uncultured *Firmicutes* bacterium	24	0	0	0
Bacteria, Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae, *Delftia* uncultured bacterium	74	0	59	0
Bacteria, Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae, Delftia, uncultured *Delftia* sp.	46	0	7	0
Bacteria, Proteobacteria, Betaproteobacteria, Burkholderiales, Oxalobacteraceae, Oxalicibacterium, *Oxalicibacterium flavum*	30	0	0	0
Bacteria, Proteobacteria, Betaproteobacteria, Neisseriales, Neisseriaceae, uncultured, uncultured bacterium	7	1	0	0

Table S1. Contd.

Bacteria, Proteobacteria, Epsilonproteobacteria, Campylobacterales, Campylobacteraceae, *Arcobacter* uncultured bacterium	0	1050	0	0
Bacteria, Proteobacteria, Epsilonproteobacteria, Campylobacterales, Helicobacteraceae, Helicobacter, *Helicobacter* sp. 'B52D Seymour'	0	204	0	0
Bacteria, Proteobacteria, Gammaproteobacteria, Aeromonadales, Aeromonadaceae, Aeromonas, *Aeromonas* sp. DMA1	0	125	588	37
Bacteria, Proteobacteria, Gammaproteobacteria, Aeromonadales, Aeromonadaceae, Aeromonas, uncultured *Aeromonas* sp.	0	0	12	2
Bacteria, Proteobacteria, Gammaproteobacteria, Aeromonadales, Aeromonadaceae, *Aeromonas* uncultured bacterium	0	64	254	18
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, *Enterobacter* uncultured bacterium	10	23	71	13
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Escherichia-Shigella, *Serratia marcescens*	0	0	1	4
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, *Rahnella* uncultured bacterium	364	7	399	1050
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, *Rahnella* uncultured bacterium	207	0	210	31
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, *Rahnella* uncultured bacterium	12	0	0	0
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Serratia, *Serratia marcescens*	168	48	365	2146
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Serratia, *Serratia marcescens*	157	14	232	1236
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Serratia, *Serratia marcescens*	0	0	15	98
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Serratia, *Serratia marcescens*	11	3	69	399
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Serratia, *Serratia marcescens*	1	0	7	23
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Serratia, *Serratia* sp. DR.Y5	0	0	1	13
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, *Serratia* uncultured bacterium	4	0	4	18
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, *Serratia* uncultured bacterium	28	0	0	1
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, *Serratia* uncultured bacterium	42	0	12	59
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, *Serratia* uncultured bacterium	0	0	11	91
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, *Serratia* uncultured bacterium	19	0	10	53
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Thorsellia, *Thorsellia anophelis*	0	0	81	0
Bacteria, Proteobacteria, Gammaproteobacteria, Orbales, Orbaceae, Gilliamella, uncultured *gamma proteobacterium*	0	69	0	0
Bacteria, Proteobacteria, Gammaproteobacteria, Orbales, Orbaceae, Gilliamella, uncultured *gamma proteobacterium*	0	65	0	0
Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae, Acinetobacter, *Acinetobacter* sp. B7_2TCO2	9	0	0	0
Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae, *Acinetobacter* uncultured bacterium	6	3	0	0
Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae, *Acinetobacter* uncultured bacterium	6	1	1	0
Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae, *Enhydrobacter* uncultured proteobacterium	13	4	0	0
Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae, *Psychrobacter* uncultured bacterium	11	0	0	0
Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae, *Pseudomonas* uncultured bacterium	21	14	26	6
Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae, *Pseudomonas* uncultured bacterium	28	0	0	0
Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae, *Pseudomonas* uncultured Pseudomonas sp.	1	0	177	0
Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, Xanthomonadaceae, *Stenotrophomonas* uncultured bacterium	196	0	5	0
Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, Xanthomonadaceae, *Stenotrophomonas*, uncultured bacterium	15	0	0	0
Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, Xanthomonadales Incertae Sedis, *Steroidobacter* uncultured bacterium	16	0	0	0
Phylum *Spirochaetae*				
Bacteria,Spirochaetae,Spirochaetes,Spirochaetales,Spirochaetaceae,uncultured,*Spironema culicis*	0	6	0	0
Phylum *Verrucomicrobia*				
Bacteria,Verrucomicrobia,Verrucomicrobiae,Verrucomicrobiales,Verrucomicrobiaceae,*Akkermansia*uncultured bacterium	47	0	0	0

Table S1. Contd.

Bacteria,Verrucomicrobia,Verrucomicrobiae,Verrucomicrobiales,Verrucomicrobiaceae,*Akkermansia*uncultured bacterium	0	7	0	0	
Phylum *Eukaryota*					
Eukaryota, Opisthokonta, Nucletmycea, Fungi, Microsporidia, Incertae Sedis, Amblyosporidae, Parathelohania, *Parathelohania divulgata*	51	0	0	0	
Eukaryota, Opisthokonta, Nucletmycea, Fungi, Microsporidia, Incertae Sedis, Amblyosporidae, *Parathelohania Parathelohania obesa*	33	0	0	0	
Eukaryota, Opisthokonta, Nucletmycea, Fungi, Microsporidia, Incertae Sedis, Amblyosporidae, *Takaokaspora Takaokaspora* nipponicus	0	100	0	0	
No blast hit	1030	34	757	0	1721
Total abundance	6214	2890	5960	5949	21666