BIOTIC INTERACTIONS OF THE COMMENSAL BACTERIUM *ELIZABETHKINGIA ANOPHELIS* (FLAVOBACTERIACEAE) WITH *ANOPHELES STEPHENSI* LISTON AND *ANOPHELES GAMBIAE SENSO STRICTO* GILES (DIPTERA: CULICIDAE)

By

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ABSTRACT

BIOTIC INTERACTIONS OF THE COMMENSAL BACTERIUM *ELIZABETHKINGIA ANOPHELIS* (FLAVOBACTERIACEAE) WITH *ANOPHELES STEPHENSI* LISTON AND *ANOPHELES GAMBIAE SENSO STRICTO* GILES (DIPTERA: CULICIDAE)

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Vector-microbe interactions can be viewed from the standpoint of mutualist and commensalist partnerships or as antagonistic parasitism. Investigating the roles of microbes living in association with arthropod vectors can provide insights into their influences, for example, on vectorial capacity. In this study the physiological effects on mosquito fecundity of the flavobacterium *Elizabethkingia anophelis* living in a gut-associated commensalism with two major malaria vector species, *An. gambiae* and *An. stephensi*, was investigated. Laboratory reared *An. stephensi* and *An. gambiae* were subjected to three treatments: (1) Adult, female mosquitoes were supplied with the antibiotic erythromycin through sugar feeding in order to clear gut bacteria. After 7 days of antibiotic feeding, the mosquitoes were then supplemented with bacteria *E. anophelis* sugar meal. (2) Adult, female mosquitoes were treated similarly to group 1 but with no bacterial feeding after antibiotic treatment. (3) Adult, female mosquitoes conventionally reared. Additionally, hemolysin activity of *E. anopheli*s was determined first *in vitro* using culture on blood agar plates, and *in vivo* by dissecting the midgut of blood fed mosquitoes from the three treatment groups and counting red blood cells. *E. anophelis* was found to augment fecundity in *An. stephensi* mosquitoes but not in *An. gambiae.* There was no significant effect of *E. anophelis* on egg viability, for both *An. stephensi* and *An. gambiae*. Hemolysin activity was demonstrated on blood agar as well as in *An. stephensi*.

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To my late parents, Mr. Luke Emmanuel Uzalili and Mrs. Theresa Malikebu Uzalili.

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KEY TO ABBREVIATIONS AND ACRONYMS

BP	Base pair
BTI	Bacillus thuringiensis isriaelensis
CDC	Center for Disease Control and prevention
CI	Cytoplasmic Incompatibility
CFU	Colony forming unit
CO	Control group
DDT	Dichlorodiphenyltrichloroethane
EA	*Elizabethkingia anophelis* treated group
EEE	Eastern equine encephalomyelitis
EM	Erythromycin treated group
H	Hour
IRS	Indoor Residual Spraying
ITN	Insecticide -treated net
LB	Luria – Bertani
LSM	Larval source management
PBS	Phosphate buffered saline
RBCs	Red blood cells
RDT	Rapid diagnostic test
TSA	Tryptone soya Agar
VBD	Vector borne disease
WHO	World Health Organization
%	Percent

^{O}C	Degrees Celsius
µl	Micro litter

CHAPTER 1. INTRODUCTION

1.1 Background of the study

Malaria is the third-most lethal human disease globally, as classified by World Health Organization (WHO, 2014) killing approximately 584,000 people in 2013 with 3200 million people being at risk in 97 countries mostly in Africa (WHO, 2015). At least 3.4 billion people live in malaria endemic areas of the tropical and subtropical regions (CDC, 2014). Malaria remains a challenge in most parts of the world and it is the leading cause of mortality and morbidity in developing countries throughout Africa, Asia and South America (Sachs, 2002.). Pregnant mothers and children under 5 years of age are particularly at high risk (CDC,2014). Malaria is caused by a protozoan called *Plasmodium* which is transmitted by female mosquitoes of the *Anopheles* genus.

Besides malaria, *Anopheles* mosquitoes are also known to transmit the nematode parasites that cause *lymphatic filariasis*. While filariasis tends not to be as fatal as malaria it incapacitates its victims. This disease is endemic to 80 countries and about 1.1 billion people are estimated to be at risk (WHO, 2002). Together, the burden of malaria and filariasis has contributed not only to mortality and morbidity but also to the poor economic growth of families and governments due to absenteeism from work(Sachs, 2002). Even school attendance decreases because sick pupils cannot go to school, individuals cannot go to work and those who care for the sick are forced to stay at home. An estimated 12 billion US dollars is spent every year on the purchase of medicine, transportation to health clinics, burial ceremonies and preventive measures such as bed nets and insecticides (CDC, 2014).

Anopheles gambiae Giles and *Anopheles stephensi* Liston are among the most important vectors of malaria in Africa and the Indian sub-continent respectively (Valenzuela et al., 2003). Malaria

vectors are truly polyphagous. Adult mosquitoes feed on nectar and blood while the immature stages are aquatic and detritivores thereby acquiring microorganisms orally. These microbes play different roles such as helping in food digestion, nutrition and blood meal digestion (Gaio et al., 2011). *Anopheles* mosquitoes require a blood meal for egg development, maturation and production (Clements, 1999) which facilitates vitellogenesis which is the yolk deposition into the oocytes. The red blood cells (RBCs) form a component of blood, and contain hemoglobin as well as a complement of amino acids, the building blocks of protein. In order for these proteins to be produced the RBCs have to be lysed, which has been shown to be facilitated by bacteria in *Aedes aegypti* mosquitoes (Gaio et al., 2011). The female *Anopheles* mosquito acquires and transmits the *Plasmodium* parasite in the course of obtaining a blood meal. The malaria parasite undergoes two phases; the intrinsic phase which is asexual reproduction inside its vertebrate host in close association with RBCs, and then sexual reproduction and extrinsic incubation in the mosquito host. The microbiota of the *Anopheles* mosquito gut is predominantly composed of *Flavobacteriaceae*, *Enterobacteraceae* and *Acetobacteraceae* bacteria (Ricci et al., 2012b).

The gut microbes in *Anopheles* have various functional roles, such as providing essential vitamins; and indeed the presence of some does increase mosquito fitness (Sharma et al., 2013) as evidenced by susceptibility increase to *Plasmodium falciparum* in antibiotic treated *An. gambiae* (Dong et al., 2009). *Elizabethkingia anophelis* is a gram-negative bacterium in a class of *Flavobacteriaceae* and is widely distributed in nature and can be found in fresh water, salt water and soil. Due to its close association with water, *E. anophelis* has been isolated from the midgut of several mosquito genera (Kämpfer et al., 2011)(Teo et al., 2014a). On the other hand, *E. anophelis* causes neonatal meningitis and nosocomial outbreaks in humans and can be passed from mother to infant (Lau et al., 2015)(Frank et al., 2013). However, *E. anophelis* infection has been shown to reduce oocyst

load of *Plasmodium* parasites even with a low bacterial dose (10 cells/µl)(Bahia et al., 2014). The presence of *Elizabethkingia* spp. in *Anopheles* and *Aedes*, vectors of malaria and dengue fever respectively (Wang et al., 2011)(Terenius et al., 2012) and its ability to be transferred transstadially in the mosquitoes provide a great hope for the manipulation and paratransgenesis of the bacteria which could be turned into an effective tool in vector control (Chen et al., 2015).

In efforts to control vector borne diseases such as malaria and lymphatic filariasis, several vector control measures are commonly used including: use of insecticide treated bed nets (ITNs), indoor residual spraying (IRS), where insecticides are sprayed on the wall surfaces, environmental management and larviciding. Most of these measures are chemical based and their prolonged use has resulted in selection for resistant strains leading to the development of insecticide resistance among malaria vector populations. For instance, populations to *An. stephensi* have shown resistance to organophosphates, dichlorodiphenyltrichloroethane (DDT) and pyrethroids insecticides (Soltan et al., 2015) (Fathian et al., 2015), while populations of *An. gambiae* are also known to be resistant to pyrethroids, organophosphates, carbamates and DDT (Ochomo et al., 2012), presenting a challenge for malaria control programs.

1.2 Rationale

Insecticide resistance development by the malaria vectors poses a threat to the fight against malaria, hence the urgent need for novel malaria vector control methods which aim at reducing vectorial capacity using manipulation of the mosquito microbiome (Yee et al., 2014). One potential method is the paratransgenesis of *Anopheles* gut microbe *E. anophelis*, but before this can be exploited further there is need to understand the physiological role of *E. anophelis* on the fitness of malaria *Anopheles* vectors hence the importance of this study.

1.3 Main objective

The primary objective of this thesis was to evaluate the physiological role of *E. anophelis* in modulating fecundity in adult females of the malaria vector species *An. gambiae* s.s. and *An. stephensi*, which it naturally infects.

1.3.1 Specific objectives

1. To assess the effectiveness of antibiotic treatment in clearing gut- microbes in *Anopheles* mosquitoes and whether the bacteria can be restored.
2. To assess the impact of *E. anophelis* on fecundity and egg viability of *An. stephensi* and *An. gambiae s.s.*
3. To assess the role of *E. anophelis* bacteria on blood digestion through hemolysin activity.

1.4 Hypothesis

- Antibiotic treated *An. stephensi* and *An. gambiae* mosquitoes will harbor less bacteria than untreated (control) *An. stephensi* and *An. gambiae* mosquitoes, and the flavobacteria *E. anophelis* administered post antibiotic treatment will be able to colonize the mosquito gut.
- Fecundity in *E. anophelis* treated mosquitoes will increase than the fecundity in antibiotic treated mosquitoes and control mosquitoes.

CHAPTER 2. LITERATURE REVIEW

2.1 Overview of vectors and diseases

Vector-borne diseases (VBDs) are diseases caused by pathogens and parasites, transmitted by arthropod vectors (WHO, 2014). VBDs account for one third of the persistent and emerging infectious diseases in humans (WHO, 2015) and according to one source caused more deaths in humans than all other causes combined in the early 20th century (Kalluri et al., 2007). VBDs are among the important diseases in the World Health Organization (WHO) disease ranking system, with malaria ranked third after human immunodeficiency virus/ acquired immune syndrome diseases (Threats, 2008) and tuberculosis, killing at least 2.8 million, 1.6million and 1.3 million people per year respectively (Hill et al., 2005).

Arthropods involved in the spread of these pathogens may act as mechanical carriers, intermediate hosts or the primary hosts. Pathogens may propagate, cyclo-developmentally or cyclo-preoperatively inside the bodies of individual vectors and then later be transmitted to humans through mechanical and biological modes of transmission. Pathogens can be transmitted from vector to vector either vertically through transovarial processes, or by transtadial means. Vector to vector transmission can also be horizontal by venereal transmission or so-called co-feeding, when an uninfected individual feeding on blood near an infected individual on a vertebrate host becomes infected from that infected individual. Pathogens are horizontally transmitted from vectors to humans by salivation, regurgitation, stercorarian excretion, and ingestion by the host. There are several arthropods that are of medical importance such as: mosquitoes, ticks, deer flies, tsetse flies, black flies, lice, muscoid flies, sand flies, fleas and reduviid bugs (Kalluri et al., 2007), (Stone et al,. 2012). Some of the VBD apart from malaria that are also of medical importance in the 21st century include dengue, plague, leishmaniasis,

African trypanosomiasis, relapsing fever, yellow fever, West Nile encephalitis, Japanese encephalitis, Rift Valley fever, and chikungunya (Gubler, 2008).

2.2 Mosquitoes

Mosquitoes are considered important vectors because they transmit virulent pathogens causing such diseases as malaria (Y. Chen & Xu, 2015)(Chen et al., 2015) besides other diseases like dengue, chikungunya, lymphatic filariasis, West Nile virus, yellow fever, western equine encephalitis and Rift Valley fever (Kalluri et al., 2007). Different mosquito species are carries of different disease agents such as protozoa, helminthes and viruses, with genus *Aedes*, *Anopheles* and *Culex* being the main disease vectors. For instance, *Aedes aegypti* and *Ae. albopictus* are known to transmit dengue and chikungunya viruses while *Culex pipien* is the vector for West Nile virus. *Culiseta melanura* transmits eastern equine encephalomyelitis (EEE) virus, while helminths for lymphatic filariasis can be vectored by species in four mosquito genera, i.e., *Aedes*, *Culex*, *Mansonia* and *Anopheles* (Kalluri et al., 2007). The role of insects in disease transmission should not be undermined as evidenced by the efficiency in West Nile transmission which was found to be widely spread in the United States of America within four years after its discovery in 1999 (CDC, 2014).

Anopheles mosquitoes are the only known vectors of malaria and are commonly found in the tropical and subtropical regions hence the disease distribution. Malaria is still a huge disease burden in Africa - south of the Sahara, the Indian sub-continent and parts of South America. *An. gambiae* s.l., *An. funestus* and *An. stephensi* are among some of the most important disease vectors in these areas (Valenzuela et al., 2003)(Gillies & Coetzee, 1987)(Gimnig et al., 2001).

2.2.1 *An. stephensi*

The geographical distribution of *An. stephensi* ranges from the Middle East through the Indian subcontinent and western China (Vector base, 2011). Malaria is endemic to the Indian subcontinent where 70 – 100 million people are affected by this disease yearly (WHO, 2009) with *An. stephensi* being the major vector (Qi et al., 2012). *An. stephensi* breeds in stream margins and irrigation canals.

However, its larvae have also been found in peri-domestic water containers (Qi et al., 2012) and adults are highly anthropophilic in that setting. As such it is responsible for malaria transmission both in Indian urban and suburban areas (Marinotti et al., 2013). This endophilic and endophagic mosquito though present throughout the year is most abundant in the summer season which happens to be the peak season for malaria too. There are three forms of *An. stephensi* (Sharma, 2013) and these are the "typical form" which is the efficient urban malaria vector, the intermediate form responsible for rural and peri-urban transmission and the mysorensis form which is zoophilic that has poor vectorial capacity and is known to be confined to rural areas (Vector base, 2011).

2.2.2 *An. gambiae*

An. gambiae species complex constitutes formerly seven and now nine morphologically indistinguishable sibling species namely, *An. gambiae* s.s., *An. arabiensis, An. bwambae, An. merus, An. melas , An. qudriannulatus* A & B (Besansky et al., 1994) and *Anopheles coluzzi* Coetzee and Wilkerson and *Anopheles amharicus* Hunt, Wilkerson, and Coetzee (Coetzee et al., 2013). *Anopheles gambiae* s.s. Giles is one of the primary vectors of *P. falciparum* in sub-Saharan Africa. Its larvae inhabit sunlit shallow pools, puddles, hoof prints and temporary rain water pool (Gillies & Coetzee, 1987). *An. gambiae* is abundant during the wet season and decreases during the dry season but persists to the next wet season (Yaro et al., 2012).

2.3 Malaria

Malaria is caused by a protozoan of the genus *Plasmodia* in the family of Plasmodiidae , order Haemasporida (Roberts and Janovy, 2005) and it is transmitted by female *Anopheles* mosquitoes from an infected person to non-infected person in the course of blood feeding. Blood is essential for egg development in anautogenous *Anopheles* mosquitoes. There are five species of *Plasmodium* that are known to cause malaria in human beings and these are; *P. falciparum*, *P. vivax*, *P. ovale* , *P. malariae* and the recently discovered *P. knowlesi* which was considered to be a parasite for Old world monkeys (White, 2008) with P. *falcipalum* considered the most important causing >90% of malaria fatalities (Baird, J. K. 2007).

2.3.1 Malaria parasite- life cycle

Plasmodium parasites undergo an intrinsic and extrinsic incubation periods inside vertebrate hosts and *Anopheles* mosquito`s bodies respectively. Before the parasite can be transmitted to a vertebrate, it must complete a biological developmental process in the mosquito host (Sinden, 2002) called extrinsic period. The extrinsic form is the onset of gametogenesis where gametocytes are released from the blood (Attardo et al., 2005a) in the form of female and male gametocytes (Vlachou et al., 2006). The female *Anopheles* picks up gametocytes during blood feeding into the midgut where they undergo gametogony cycle which is sexual reproduction that involves fertilization of exflagellated microgametocyte (microgametes) and macrogamete producing a diploid zygote. The zygote then matures and cyclo-propagates into ooknite (Mullen, 2009). These ookinetes are then absorbed by the midgut epithelium cells by passing through a non-cuticle membrane known as a peritrophic matrix (PM). The PM is made up of a network of chitin microfibrils within a matrix of carbohydrate and protein (Klowden, 2010)(Vinetz, 2000), though

the midgut is not cuticle lined, still more they need to produce chitinase that help them to penetrate the PM and then develop into oocysts (Klowden, 2010).

The oocysts undergo somatic reduction to the haploid genetic condition, and later rupture to release thousands of motile, haploid sporozoites in the hemolymph which migrate to the median lobe of the salivary glands and penetrate them. Thereafter, when the mosquito salivates into human skin, the sporozoites are inoculated into the human being in the course of blood feeding again. Upon inoculation through subdermal capillaries into the human body, the sporozoites migrate to quickly infect the liver paranchymal cells in which they form primary tissue meront except for *P. ovale* and *P. vivax*, these will form hypnozoite (Mullen, 2009) and then mature into schizoites then meroizoites rapture and move into the blood stream to invade the red blood cells (RBCs) where they now undergo asexual multiplication into trophozoites which is the onset of malaria, this cycle also takes at least 7- 14 days (Eldridge & Edman, 2003). A person suffering from malaria will have fever and general body pains and which can be diagnosed by taking a thick or thin blood film and stained then analyze under a microscope. Additionally, malaria can also be detected using the rapid diagnostic test (RDT).

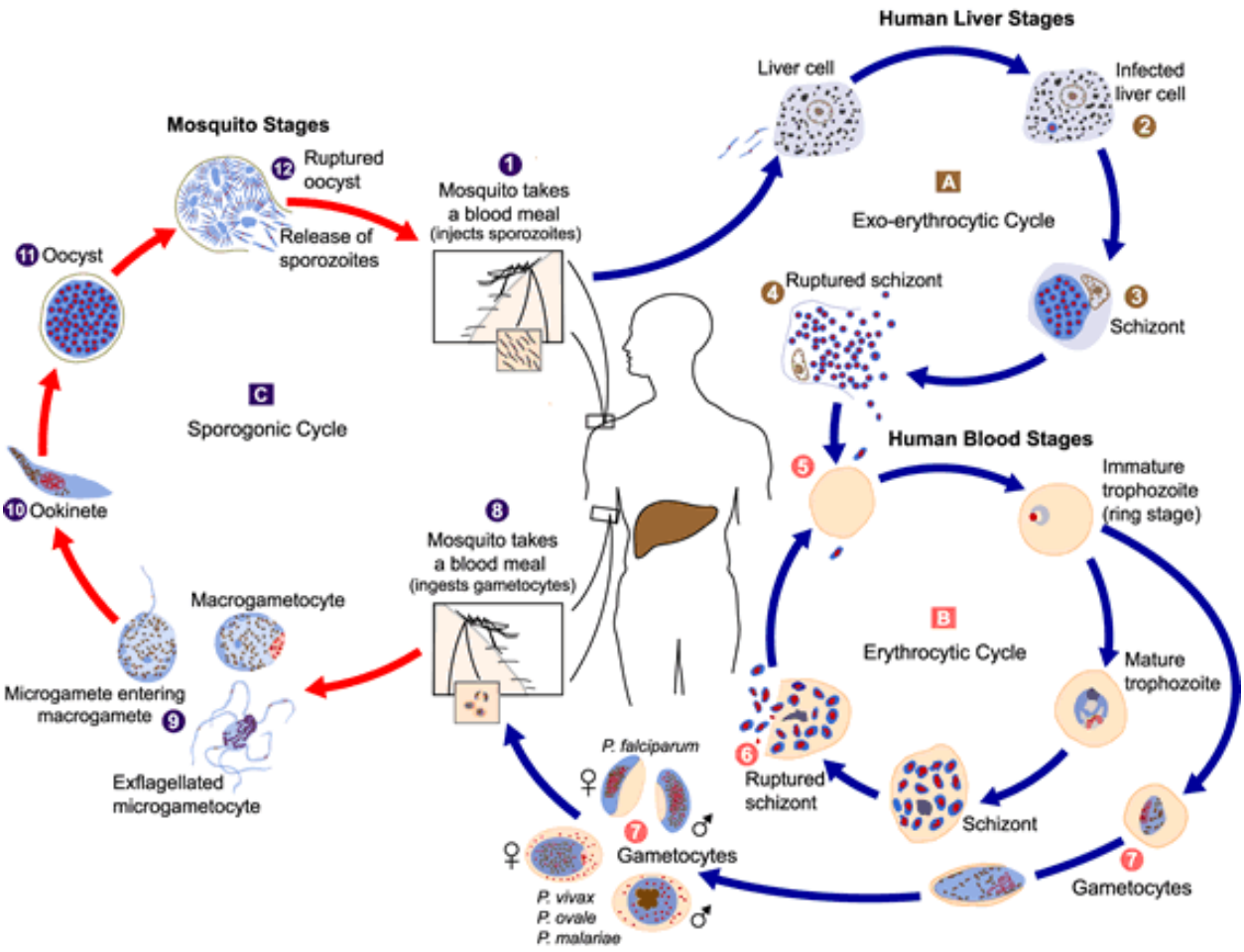

Figure 1. Malaria parasite life cycle inside the human body and in the *Anopheles* mosquito (www.cdc.gov)

2.4 *Anopheles* mosquito biology and ecology

Anopheles mosquitoes are in the order Diptera (two wings) and family Culicidae. They spend their life stages in two phases as immature and adults. They are known to be holometabolous that is they undergo a complete metamorphosis with the following stages ; egg, larva and pupa which is the immature stage and aquatic, lasting 5 - 14 days (CDC, 2012) and then they emerge into flying adults which are terrestrial. *Anopheles* mosquitoes lay their eggs singly which have lateral float (CDC, 2012) on water surface and wet surface. The larval stage is divided into instars, and a larva has to undergo 4 instars before turning into a pupa. The larva has to molt to attain a next instar and on fourth instar the larva needs to metamorphose into pupa. *Anopheles* larvae lie parallel to the water surface as they lack a syphon for drawing atmospheric air for respiration (Foster and Walker 2009) and they do feed on microorganisms such as fungi, algae, bacteria and detritus found in water, while pupae stage do not feed at all. After 2-3 days as pupae, adult *Anopheles* emerge and these do feed on sucrose and nectar from plants for their energy. Temperature and larvae nutrition are crucial factors in stage development and adult population of *Anopheles* mosquitoes (Moodle.digital-campus.org).

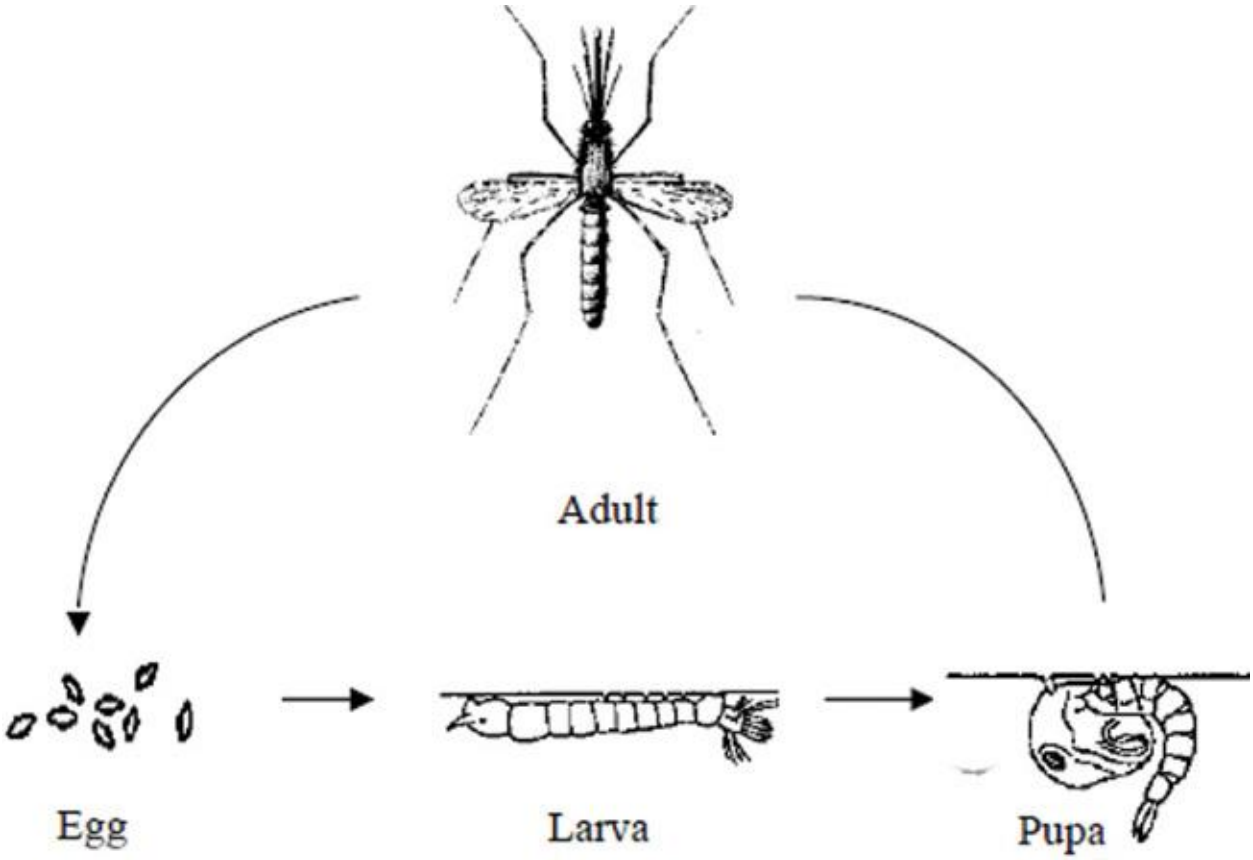

Figure 2. *Anopheles* mosquito life cycle showing all the life stages (moodle.digital-campus.org)

2.4.1 *Anopheles* mosquito – reproduction

Female *Anopheles* mosquitoes are known to be anautogenous, since they must obtain a blood meal for vitellogenesis to occur. Carbohydrates, protein and lipids are all essential for egg production(Chambers & Klowden, 1994) as such blood meal obtained from vertebrates is synthesized into proteins in the mid gut by gut microbes (Gaio et al., 2011) for protein provision to the mosquitoes. The proteins will then form yolk which serves as a vital resource for egg development (Attardo et al., 2005b). Olfactory cues play an important role in host seeking as the mosquitoes are attracted to body odors (Mboera et al., 1997). This anautogenous behavior of mosquitoes is what caused the female mosquitoes to be disease transmitters as they move from person to person in seeking for blood meal (Attardo et al., 2005b).

Anopheles mosquitoes usually form swarms where female and males mate, they are attracted to these swarms by the male-female flight tone matching (Gibson et al.,2006) produced by the wing beats by the females (Belton, 1994) (Clements, 1999), that acts as a form of species recognition too (Pennetier et al., 2010). In some anopheline mosquitoes such as *An. gambiae* this usually happens at dusk and mating lasts for about 15-20 seconds (Shaw et al., 2015). Mating is more successful for younger males than older males, with no respect to body sizes (Charlwood et a., 2002). Some males such as *An gambiae* species do secrete seminal transglutaminase AgTA3 which coagulates seminal fluids and forms a "mating plug" for the mating succession (Le et al., 2014). Fertilization is equally important in embryo development, hence the importance of mating in *Anopheles* egg oviposition, just like in most animals (Clements, 1999). On average *Anopheles* mosquitoes can lay 50 -200 eggs per oviposition (CDC, 2012), and this phenomenon of laying large number of eggs is what contributes the acceleration in vectorial capacity through vector density increase.

2.5 Mosquitoes and microbes

Microbes that include bacteria, protozoa, viruses and fungi are tiny organisms and are present everywhere, living and nonliving organism are known to harbor them. These microorganisms can either be symbionts, commensals or pathogens of the host (Kämpfer et al., 2015). Among arthropods there are several examples of mutual microbes - insects symbiotic relationships (Chavshin et al., 2015). For instance, termites have evolved together with its microbiota which help in cellulose digestion (Abe, 2000). Mosquitoes have not been spared from this microbe colonization. They do contain a diversity of microbiota (Gaio et al., 2011). There are several bacterial families that reside inside mosquitoes such as, *Enterobacteriaceae, Flavobacteriaceae, Acetobacteraceae, Rickettsiales* and *Proteobacterium.* Adult mosquitoes emerge with already established bacteria in their guts (Gusmão et al., 2010) which are acquired from their environment at larval stage, most gut bacteria do colonize the mosquito midgut, where also fertilization of malaria plasmodium microgametocytes and macrogametocytes take place to form a zygote known as ookinete (Bahia et al., 2014) . While in the midgut, the plasmodium do face challenges such as the natural midgut microbial flora (Cirimotich., 2011)which also contribute to the outcome of mosquito infections (Boissière et al., 2012). It has been observed that the more the microbial load in the malaria vectors, result in the low infection of the plasmodium (Cirimotich et al., 2011).

The alpha-*proteobacteria* *Wolbachia* and *Asaia* (Ricci et al., 2012a), have been discovered to reside inside some mosquitoes of the *Anopheles* genus (Rossi et al., 2015). Different microbes inhabit in different organs according to their functions. For examples, *Wolbachia*, an intracellular bacteria which is also known to be maternally transmitted (Joshi et al., 2014) has been isolated in the gut and reproductive tissue of *Culex* (Atyame et al., 2014) . *Wolbachia* causes some

reproduction manipulation such as cytoplasmic incompatibility (CI), male killing and pathogenesis when introduced into other mosquitoes (Bourtzis & Thomas, 2006).

Some bacteria such as *Serratia marcescens* which is also one of the gut microbiota, have shown to reduce mosquito lifespan and also inhibit malaria plasmodium development (Bahia et al., 2014). Another example is *Asaia* which is also an intracellular microbe localized in the reproductive tissue and can be transmitted trans-stadially (Favia et al., 2007). The importance of microbes to their hosts ranges from beneficial as evidenced by the negative effect in host fecundity in antibiotic treated mosquitoes (Sharma et al., 2013) helping in digestion, providing macro nutrients and protection from other pathogens, while some enhance susceptibility to pathogens such as malaria parasites (Gendrin et al., 2015), for example the remove of microbes such as *Chryseobacterium, Enterobacter* and *Serratia* species from vector gut by antibiotic treatment prior to infectious blood feed, enhances the susceptibility of *An. gambiae* to the plasmodium (Meister et al., 2009) . However there is still lack of knowledge on the biological role of various gut microbes such as *E. anophelis* that are found in mosquitoes(Sharma et al., 2013). Therefore, a need for more studies like this and proper documentation of roles of microbes in mosquitoes, that may aid in application of paratransgenesis for vector control.

2.5.1 *E. anophelis*

E. anophelis is a gram- negative extracellular bacterium in a family of *Flavobactericeae* of bacterioidetes and genus *Elizabethkingia*. Phylogenically it is closely related to *Chryseobacterium*. It has been found to predominantly reside in the midgut of *An. gambiae* (Kampfer et al 2011)(Wang et al., 2011)), *An. stephensi* (Rani et al., 2009) and *Aedes aegypti* mosquitoes. The presence of *E. anophelis* in these mosquitoes which are also of medical importance due to their efficiency in disease transmission, makes *E. anophelis* an important bacteria to study. Though *E.*

anophelis is also closely related (98.6%) RNA sequencing to *E. meningoseptica* bacteria (Kukutla et al., 2014a), which is in a family of *Flavobacteriaceae* as well, and has been isolated from human beings, their only difference is the R26T (Kukutla et al., 2014b). This difference in RNA sequencing placed *E. anophelis* as a novel bacterium species. *E. anophelis* has shown to be resistant to ampicillin, chloramphenicol, kanamycin, streptomycin and tetracycline (Kämpfer et al., 2011).

This emerging bacterium has been associated with neonatal meningitis and nosocomial outbreaks in Central African Republic (Frank et al., 2013) and in Singapore (Teo et al., 2014b). *E. anophelis* is transmitted transstadially in mosquitoes and has been found to be transmitted from mother to child in humans like in one case in Thailand where the mother passed the bacterial to the baby. The combination of *E. anophelis* dominancy in *Anopheles* mosquitoes and its ability to be transmitted transstadially makes *E. anophelis* a good candidate for paratransgenic (S. Chen et al., 2015). *E. anophelis* is characterized by producing yellow colonies if plated on Columbia agar plate (Frank et al., 2013). Though these bacteria have been isolated from *Anopheles* mosquitoes, there are fears that they can cause life threatening infections in neonates, severely immunocompromised and post-operative patients (Li et al., 2015). The role that mosquitoes play in the transmission of this bacterial is not known. However, there is evidence that the *E. anophelis* is being acquired from the environment by *An. gambiae*. Studies have also revealed *E. anophelis* in the mosquito gut increases both *in vivo* and *in vitro* when vertebrate blood is provided indicating the possibility of *E. anophelis* being involved in erythrocytes lysing in order to acquire nutrients (Chen et al., 2015).

2.6 Bacteria hemolytic activity

The relationship that exists between bacteria and their hosts are based on different roles. Some bacteria produce hemolytic enzymes into the extracellular space which help in the lysis of blood cells (Vogl et al., 2008) in order for the proteinases to be released. Hemolysis is the destruction of the cell membrane of RBCs caused by lipids and proteins. These will then aid in amino acids production.

In anautogenous insect vectors such as *Anopheles* mosquitoes, the efficient of blood hemolysis is an important activity since their reproduction effectiveness is largely determined by this phenomenon. Bacteria present three types of hemolysin activity which are Gamma, Alpha and Beta (Buxton, 2013). This classification is based on color changes portrayed on the blood agar media where the bacteria have been cultured. No color change on the media represents Gamma hemolysin, a greenish or brownish color change represents alpha hemolysis while the transparent halos on the media indicate beta hemolysis (Buxton, 2013). Understanding how blood is digested and how can this be regulated is important in the fight against vector borne diseases**.**

2.7 Malaria vector control

There are several factors that affect malaria transmission such as, climate, host feeding preference and rainfall patterns. And in malaria control, all these factors need to be considered**.** As the war against mosquito- borne diseases such as malaria continues, there have been several measures which have been put in place. Malaria control interventions do focus on reducing vector density, reducing human- vector interaction and finally killing of the *Plasmodium*. Control measures such as use of insecticide treated bed nets (ITN`s), indoor residual spraying (IRS), larviciding and the general environmental management are the common methods employed in vector density reduction. In the event that vector control is not effective enough such that a person ends up being

infected with the plasmodium, the use of drugs to kill the parasite once it enters into the human body becomes the only option in order to cure malaria. Coartem (a combination of artemether and lumefantrine) is now the recommended malaria drug (WHO, 2006), as there is still no malaria vaccine up to date. However, though these measures have showed great achievements, morbidity and mortality of this disease is still high (Wilke & Marrelli, 2015). Vector control in fighting malaria is facing quite a big challenge due to insecticide resistance development in *Anopheles* (Diabate et al., 2002). Also some control measures such as IRS are expensive to maintain because of high cost resulting from low and short residual lifespan of the insecticides (Akogbéto et al., 2015).

The resistance may be as a result of gene modulation or vector behavior change, where the mosquitoes avoid resting on ITNs (Curtis et al., 2006)(Siegert et al., 2009). Further, malaria control programs are potentially affected by the development of drug resistance by parasites**.** For example, recently the most deadly malaria parasite *P. falciparum* has been reported to be resistant to artemisnin which is the current malaria front line drug in south East Asia (Noedl et al., 2010). On the other hand, the other common method of vector control is larviciding, where chemical and biological insecticides are applied on vector breeding sites. This method has an advantage over bed net use as bed nets mainly target adult mosquitoes which are highly mobile and this proves to be difficult than larviciding which target the immature stage that is less mobile and less behavioral responsiveness (Killeen et al., 2002). However the use of chemicals pose a threat to the environment as a result microbial application seems to be a better approach (Bhattacharya, 1998). Microbes such as *Bacillus thuringiensis* var *isriaelensis* and *B. sphaericus* which produce endotoxins once ingested by mosquitoes that destroys the gut lining of the insects, killing the insect eventually are used in larviciding (Suom et al., 2008).

Larval source management (LSM) which is the management of the vector breeding sites encompasses manipulation, modification and biological control of breeding sites(WHO, 2013). In habitat manipulation the water level can be manipulated by flushing streams which might also be applied in irrigation farming. In habitat modification, land can be reclaimed to ensure that breeding site are being controlled. While biological control involves the introduction of other species into the larval habitants which are predators of the immature vectors (WHO, 2013). However LSM is not an easy thing to do considering that most malaria endemic regions are in the tropics and are in developing regions, (www.malariaconsortium.org). Furthermore, *Anopheles* mosquitoes are affected by environmental factors such as temperature, environmental sanitation (Murdock et al., 2014) and rainfall. As such the high temperatures and lots of rainfall of the tropics and subtropical regions together create a conducive condition for mosquitoes. Also, impoundments are created for the *Anopheles* larvae which thrive well in stagnant 'fresh water' that become abundant after the rains (Vector base, 2011). As such this intervention faces challenge as well.

In view of all these, new control measures that look at manipulation of mosquito hosts and their microbial communities have been proposed (Ricci et al., 2012b). The microbes can be manipulated to produce anti-pathogen molecules in their localized parts (Cirimotich et al., 2011). These new measures will complement the already in use strategies to enhance the integrated malaria control measures.

CHAPTER 3. MATERIALS AND METHODS

3.1 Mosquito rearing

An. stephensi Liston Johns Hopkins strain and *An. gambiae* Giles Mbita strain were reared in the MSU insectary chambers in 60 X 60 X 60 cm mosquito cages. The temperature in the incubators was maintained at 28 ±1 ºC and relative humidity between 50% – 60% under a photo regime of 12 h light and 12 h darkness. Cotton wicks were soaked in 10% sucrose solution and placed inside the cages as a source of energy to the mosquitoes. After 3-5 days of sucrose feeding, mosquitoes were blood-fed with defibrinated bovine blood (Hemostat Lab, Dixon, CA) for their ovarian development by using an artificial feeder covered with a parafilm, presented to mosquitoes on tops of cages for 30 minutes and blood kept warm by circulating warm water (37ºC) from a water bath (Huang et al. 2005). After 3 days, Petri dishes containing soaked cotton balls and a filter paper on top were placed inside the cage for egg deposition. Two days later the filter paper with eggs was transferred into the plastic pans containing distilled water for them to hatch. For the first instar larvae, first bite fish food (Kyron, Himeji, Japan) was provided and later kitten food (Purina cat chow Nestle, Switzerland) was provided for the second, third and fourth instar until pupation.

3.2. Microbial analysis

3.2.1 Antibiotic treatment

The aim of this experiment was to clear the mosquito gut of microbiota and this was attempted using three independent set ups in cages (A, B and C)**.** Mosquito cages were wiped with 70% alcohol and then a pan containing pupae (n=100) placed in each cage. After mosquitoes emerged, sterilized 10% sugar solution mixed with erythromycin antibiotic (200 µg/ ml) was provided to the mosquitoes through water wicks for 7 days, with fresh antibiotic being administered daily.

Antibiotics were administered to the two cages (A and B) while mosquitoes in cage C were supplied 10% sucrose and no erythromycin as a control.

3.2.2 Bacterial clearance determination

From day 2 of antibiotic treatment, a total of 5 mosquitoes were randomly collected from each cage daily for up to seven days for dissection. Each mosquito was immersed in 70% ethanol for one minute and then washed in sterile phosphate buffer solution (PBS). Thereafter the mosquito was placed on a clean slide containing sterile PBS. The midgut was pulled out under a microscope with the aid of a sterile pin and forceps. The midgut was washed three times in PBS and then placed into a 1.5 ml eppendorf tube containing 200 µl PBS. Using a pestle, the midgut was crushed in 200 µl PBS and then washed down the pestle with another 200 µl PBS to ensure maximum collection of the sample, then the volume was increased to 1000 µl and serial dilutions made accordingly. Aliquots of 100 µl of the homogenate was then pipetted and plated onto agar media under sterile conditions (near a flame), using a grass rod. The results were inspected after 72h incubation of the plates at 28 ºC.

3.2.3 Agar medium preparation

Agar medium was prepared by suspending 10 g bacto-tryptone, 5 g yeast extract and 10 g sodium chloride in 1000 ml distilled water and split into two 500 ml bottles, then 10 g agar added into each bottle and autoclaved. About 20 ml of the agar was then poured into each petri dish and let it to solidify at room temperature.

3.2.4 Plate reading

After 72 h of incubation, the plates were removed from the incubator, colonies were counted and colony forming units (CFU) were calculated and the colony colors recorded. CFU was calculated using the following formula below:

CFU / ml=

$$\frac{total\ number\ of\ counted\ colonies\ x\ dilution\ factor}{volume\ inoculated}$$

3.3 Mosquito infection with *E. anophelis.*

E. anophelis isolated from mosquito colonies reared at the Michigan State University insectaries as described in (Chen et al., 2015) were cultured in Luria -Bertani **(**LB) broth.

3.3.1 Luria Bertani broth preparation

LB broth was prepared by adding 10 g bacto-tryptone, 5 g yeast extract and 10 g NaCl into 800 ml distilled water with pH adjusted to 7.5 by adding sodium hydroxide, and then autoclaved. *E. anophelis* (100 µl) was added into 10 ml LB broth and left on a shaker (200 rpm) at 30 ºC for overnight. The following day, mosquitoes in cage A treated with EM were provided with 10 ml of 10% sterile sucrose to which 2.4 X 10^{3} CFU/ml of cultured *E.anophelis* had been added. After 24 h, it was replaced with 10% sterile sucrose solution only. EM treated mosquitoes in cage B were supplied with 10% sterile sucrose for 2 days, post-antibiotic treatment. For the naïve cage C, 10 % sucrose was continuously supplied to the mosquitoes.

3.4 16s rDNA pyrosequencing and data analysis

All DNA extractions were performed in a sterile environment (Biosafety II hood) to avoid contamination. Mosquitoes were surface-disinfected with 70% of ethanol and next rinsed with sterile water. Each individual was crushed using a sterilized pestle in an Eppendorf tube and re-suspended in 200 µl of extraction buffer. Next, the genomic DNA was extracted by DNeasy Blood & Tissue Kit following the manufacturer's procedures. DNA integrity was confirmed by the polymerase chain reaction (PCR) amplification before the samples were submitted for pyrosequencing. The PCR was carried out with Failsafe enzyme system and the following cycles were used: 94 ºC for 2mins, 30 cycles of 94 ºC for 15s, 50 ºC for 15s, and 72 º C for 1.5mins and a final extension at 72 ºC for 7mins.

Amplicon tagging and pyrosequencing were carried out by Research Technology Support Facility (RTSF) at Michigan State University. A total of 12 rrs V4 amplicon libraries were prepared, barcoded, and sequenced by using the standard procedures (Reference). A typical PCR reaction (50µl) for library preparation is as the followings: 50 ng DNA, 41µl molecular biology grade water, 5 µl 10 x FastStart High Fidelity Reaction Buffer containing 18 mM MgCl2, 1µl dNTPs (10mM each), 1µl Fusion Primer A (10 mM), 1µl Fusion Primer B (10mM), and 1µl FastStart High Fidelity Enzyme Blend (5U/ml). The cycles are: 95°C 2 min, 30 cycles of 95°C 20s, 55°C 15s and 72°C 5 min, and a final extension at 72°C 10 min.

The samples were purified and loaded to Illumina Miseq sequencer for sequencing. Sequencing reads were processed and analyzed using mothur v.1.35.1 (3/31/2015 version) on the mothur wiki webpage (http://www.mothur.org). After denoising by using the PyroNoise, Uchime, and preclustering methods, the high quality sequences (> 250 bp) without sequencing errors or chimeras were used for assigning OTUs using an average neighbor algorithm (97% similarity

cutoff). OTUs were classified at the genus level using the Bayesian method. Data were further analyzed by first trimming sequence quality with different cutoffs using standard filtering tools (Schloss et al., 2009). Rarefaction curves were built to estimate sample coverage. Richness, α diversity and β diversity were calculated to compare samples with respectively richness estimators (Chao, Jackknife, and Abundance Coverage Estimator), diversity indices (Simpson, Shannon) and Bray–Curtis dissimilarity**.**

3.5 Fecundity determination

3.5.1 Blood feeding

Mosquitoes were reared for 2 days after *E. anophelis* supplement in order to allow establishment of bacteria in the mosquito gut. On the third day female mosquitoes were starved for 12 h and then blood fed using bovine blood as described above. The unfed and partial fed mosquitoes were removed from the cages.

3.5.2 Egg laying and collection

Three days post blood feeding, each gravid mosquito was transferred into respective 50 ml sterile tubes (n = 30) per treatment, covered with a netting material for easy access of sucrose. Each mosquito was provided with an autoclaved wet filter paper and cotton wool for oviposition. Female mosquitoes were allotted 2 days for oviposition and then the filter papers were removed and number of eggs counted under a stereoscopic microscope.

Both mosquitoes which laid eggs and those that did not lay eggs but were still gravid, were dissected and mature eggs were removed and counted.

3.5.3 Egg viability

For the determination of egg viability**,** each filter paper containing eggs was transferred into plastic pan with distilled water for the eggs to hatch and made sure that no eggs were stuck on the sides of the pan to avoid drying up. Larval counts were then determined using the stereoscopic microscope after 24 h.

3.5 ***E. anophelis*** **hemolytic activity**

3.5.3 ***In vitro*** **experiment**

For determination of hemolysin activity, *E. anopheles* was cultured in LB broth overnight and the following day a 100 µl aliquot was spread on blood agar- Thermo scientific Remel blood agar (5% sheep blood Tryptone Soya Agar or TSA) – MacConkey, which was purchased from Fisher Scientific and incubated at 28 ºC for at least 48 h. Color change was used as an indicator for hemolysin activity by the bacteria. Positive and negative controls were also cultured in the same way.

3.6 2 ***In vivo*** **experiment**

Hemolysin activity of *E. anophelis* was tested in vitro by first establishing the total number of RBC`s in Bovine blood (Hemostat Lab, Dixon, CA). Blood was diluted, 1:200 blood: isotonic solution (0.9% normal saline). 10 µl was placed on a hemocytometer covered with a clean glass slide. RBCs on the center square of the chamber where smaller squares have been drawn were counted (in five small squares) under a microscope. The total number of RBCs was deduced by using the formula below:

Number of RBC`s =

$$\frac{number\ of\ cells\ counted\ x\ dilution\ factor}{volume}$$

Further, antibiotic treated mosquitoes were blood fed on bovine blood together with the control mosquitoes. Then RBC lysis was determined by pulling out midgut and diluting the blood meal into 0.9% normal saline (1:200) and the number of RBC`s counted using a hemocytometer and compared the total number of RBC in the midgut to the total number of RBC of the bovine blood, at 24 h, 36 h and 48 h after blood feeding (Gaio et al., 2011).

3.7 Statistical methods

Data were entered into Microsoft Excel spread sheets, and later analyzed using SAS 9.3 for normality, then analysis of variance was used to compare means in fecundity, hatching rate and hemolysin activity; means were separated using Least significant difference (LSD) test ($\alpha = 0.05$). Graphs were made in SAS 9.3, SIGMA and Excel.

CHAPTER 4. RESULTS, DISCUSSION AND CONCLUSION

The following abbreviations have been used in reporting the results: EA = *E. anophelis*, EM = erythromycin, and CO = control.

4.1 Microbial clearance

The microbial density from the EM and control group was determined by pulling the mosquito midgut and plating them on agar media. A total of 30 mid guts from each group were used with 5 mid guts being pulled per day for 6 days and plated. The total CFU/ ml was calculated from each group and the control group had a total of 6.7 x 10^5 CFU/ml which was higher as compared to EM treated group which had 2.2 x 10^5.

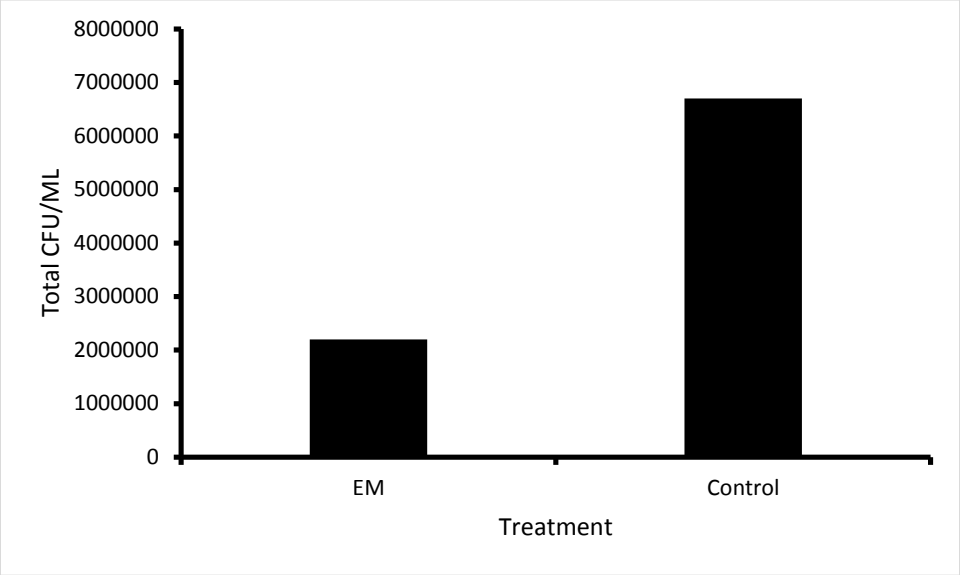

Figure 3. Total CFU/ml in *An. stephensi* mid guts pulled from control and EM treated groups.

Microbial diversity of *An. stephensi* was determined using 16s rDNA sequencing, Amplicons were obtained from 12 mosquitoes and these were sampled from all three treatments (EA, EM and CO). The most dominant bacterial family was the gram-negative *Enterobacteriaceae* with the highest frequencies of OTU (275,296) and percentage of all OTUs (87.9%). *Acetobacteraceae* was the second most dominant family with a frequency of 32,376 OTUs, representing 10.3%; while *Flavobactericeae* was the third with 2,812 OTUs, representing 0.89% of all OTUs. The fourth most common family was the *Comamonadaceae* which had a frequency of 554 OTUs (0.17%). Table 1 provides a complete list of the OTUs found in the analysis.

Table 1: The microbial diversity of the *An. stephensi* with OTU and their frequency percentages

Order	Family	Genus	OTU Sums	Percentage
Enterobacteriales	Enterobacteriaceae	unclassified	275296	87.9
Rhodospirillales	Acetobacteraceae	Asaia	32376	10.3
Flavobacteriales	Flavobacteriaceae	Flavobacterium	2812	0.89
Burkholderiales	Comamonadaceae	unclassified	554	0.17
unclassified	unclassified	unclassified	456	0.14
Acidobacteria_Gp2_(	Acidobacteria_Gp2_	Gp2	421	0.13
Bacillales	Staphylococcaceae	Staphylococcus	253	0.08
Sphingobacteriales	Cytophagaceae	Flectobacillus	220	0.07
unclassified	unclassified	unclassified	134	0.04
Clostridiales	Ruminococcaceae	unclassified	107	0.03
OD1_order_incertae	OD1_family_incerta	OD1_genus_incert	105	0.03
Bacteroidales	Porphyromonadace	unclassified	96	0.03
unclassified	unclassified	unclassified	78	0.02
Desulfuromonadale	Geobacteraceae	Geobacter	76	0.02
Campylobacterales	Campylobacterace	Arcobacter	26	0.008
unclassified	unclassified	unclassified	25	0.007
Fusobacteriales	Fusobacteriaceae	Fusobacterium	21	0.006
Planctomycetales	Planctomycetaceae	Singulisphaera	21	0.006
Subdivision3_order_	Subdivision3_family	3_genus_incertae_	20	0.006
Erysipelotrichales	Erysipelotrichaceae	Erysipelotrichace	9	0.002
Chlamydiales	Parachlamydiaceae	unclassified	7	0.002
unclassified	unclassified	unclassified	6	0.001
Anaerolineales	Anaerolineaceae	unclassified		

4.1.1 Bacterial communities per treatment

The composition of the bacterial community revealed in *An. stephensi* varied among the three treatment groups, when considering the top ten most abundant OTUs based on ranked frequency (figure 4). At least nine bacteria genera were found in samples from the control groups, with *Enterobacteraceae* being the most dominant family with a percentage mean of 78.3% while the remaining 27.7% is distributed amongst 9 genera. Sequencing for EM antibiotic treated mosquitoes showed less variation in terms of bacterial species abundancy. *Enterobacteraceae* was the most dominant with a percentage mean 97.1% and the remaining 2.9% comprised of at least three different bacterial genera of *Pseudomonas*, *Actinetobacter* and *Flavobacterium*. In the EA treatment group, 91.2% mean percentage was the Enterobacteraceae and the rest which was 8.8%included the following genera *Shigella*, *Asaia*, *Pseudomonas Flavobacterium* and *Acinetobacter.*

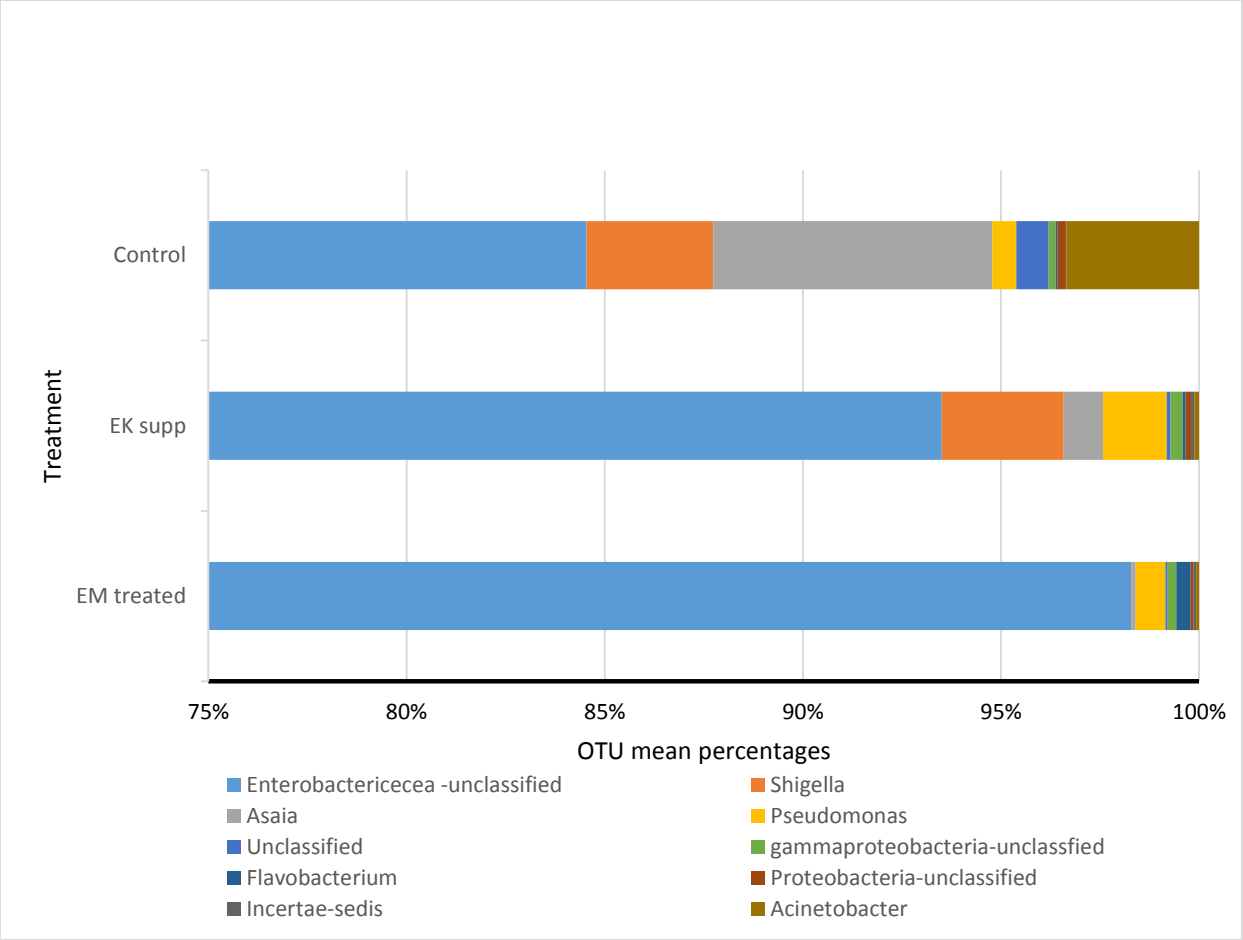

Figure 4. OTU percentages and bacterial community variability at genus level with respect to treatment.

4.2 Fecundity determination

4.2.1 Effect of *E. anophelis* on fecundity of *An. stephensi*

In this experiment, a total of 90 gravid *An. stephensi* were used, with 30 mosquitoes assigned to each treatment (EA, EM and CO). The mean number of eggs was highest in mosquitoes which had been cleared of gut microbiota and the EA supplemented back to them, lowest in mosquitoes treated with EM, and intermediate in mosquitoes treated as unmanipulated controls (figure 5). Analysis of variance indicated that there was a significant effect of experimental treatments on the mean number of eggs ($F = 15.93$, $df = 87$, $P = < 0.0001$). Furthermore, the *a posteriori* comparison of means by the LSD mean separation method showed that all the treatment groups were significantly different from each other.

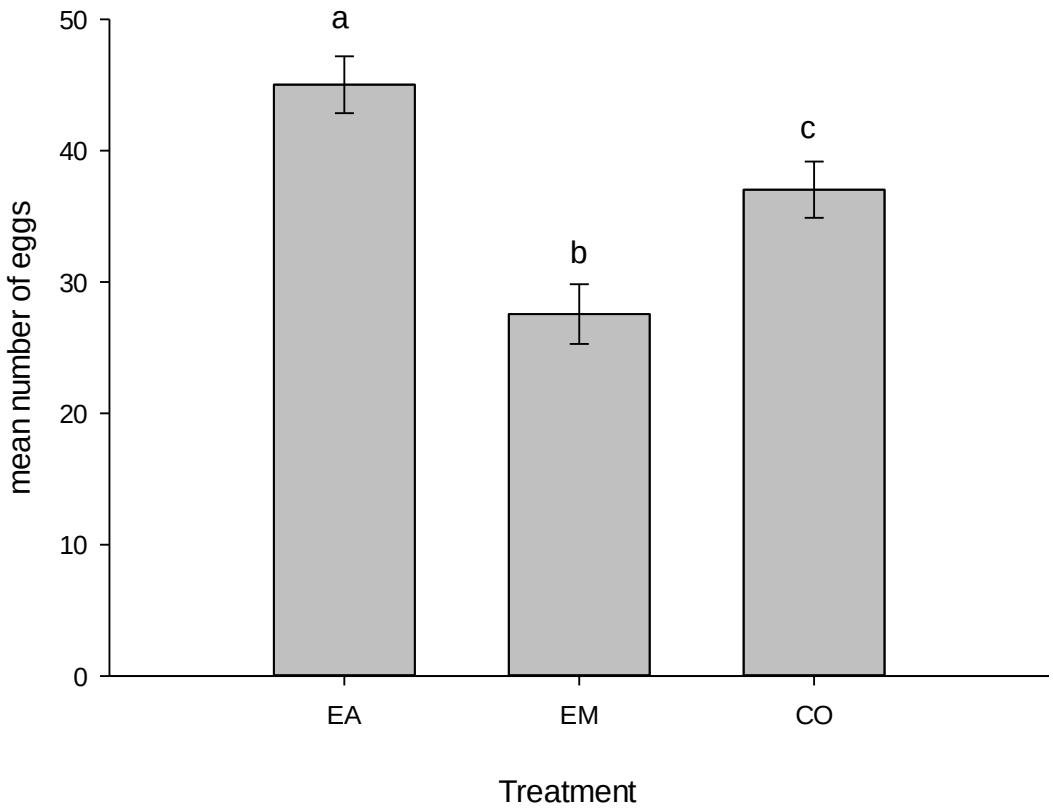

Figure 5. Mean number of eggs of *An. stephensi* when supplemented with *E. anophelis* bacteria (EA), erythromycin (EM), or unmanipulated as controls (CO).

Table 2 LSD statistical table on EA, EM and CO treatment groups.

Differences of Least Squares Means

Effect	trt	_ trt	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	CO	EA	-7.999	3.0982	87	-2.58	0.0115
Treatment	CO	EM	9.468	3.0982	87	3.06	0.003
Treatment	EA	EM	17.467	3.0982	87	5.64	<.0001

4.2.2 *An. stephensi* egg viability results

The viability of the eggs laid by the *An. stephensi* mosquitoes subjected to different treatments (EA, EM and CO) was determined by submerging a total of 3,289 eggs into water and allowing them to hatch. A total of 2269 larvae hatched representing 69%. Thereafter hatching rate per mosquito was calculated and one way ANOVA was conducted on the hatching rate, expressed as number of eggs hatched. The results were not statistically significant df =87 ($F = 0.08$, $P= 0.9204$), and the mean hatching rate was as follows; EA = 70.8%, EM = 69.9 % and CO = 69.9 %.

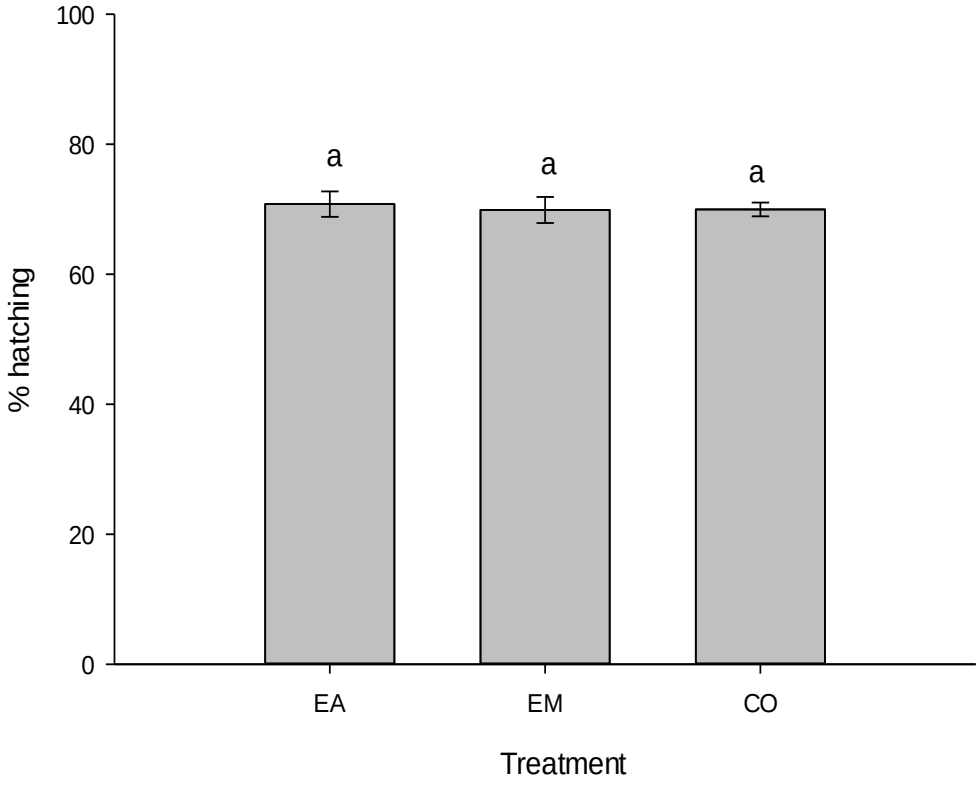

Figure 6. Mean percentage hatch rate of *An. stephensi* eggs laid from females supplemented with *E. anophelis* bacteria (EA), erythromycin (EM), or unmanipulated as controls (CO). Error bars denote the standard errors from the percentage number eggs hatched.

4.2.3 Effect of *E. anophelis* on *An. gambiae* fecundity

In this experiment *E. anophelis* effectiveness in contributing to *An. gambiae* egg production was tested. A total of 84 mosquitoes were used in this experiment, with 25 in EA group, 32 EM group and 27 CO. In this regard, the results had no significant effect in the mean number of eggs (laid and dissected) per treatment df = 81 (F = 2.22, P = 0.1151). Though no significant effect, the following mean number of eggs were observed EA =39, EM = 33 and CO = 40, with the CO having a highest mean, EM the lowest and EA the intermediate.

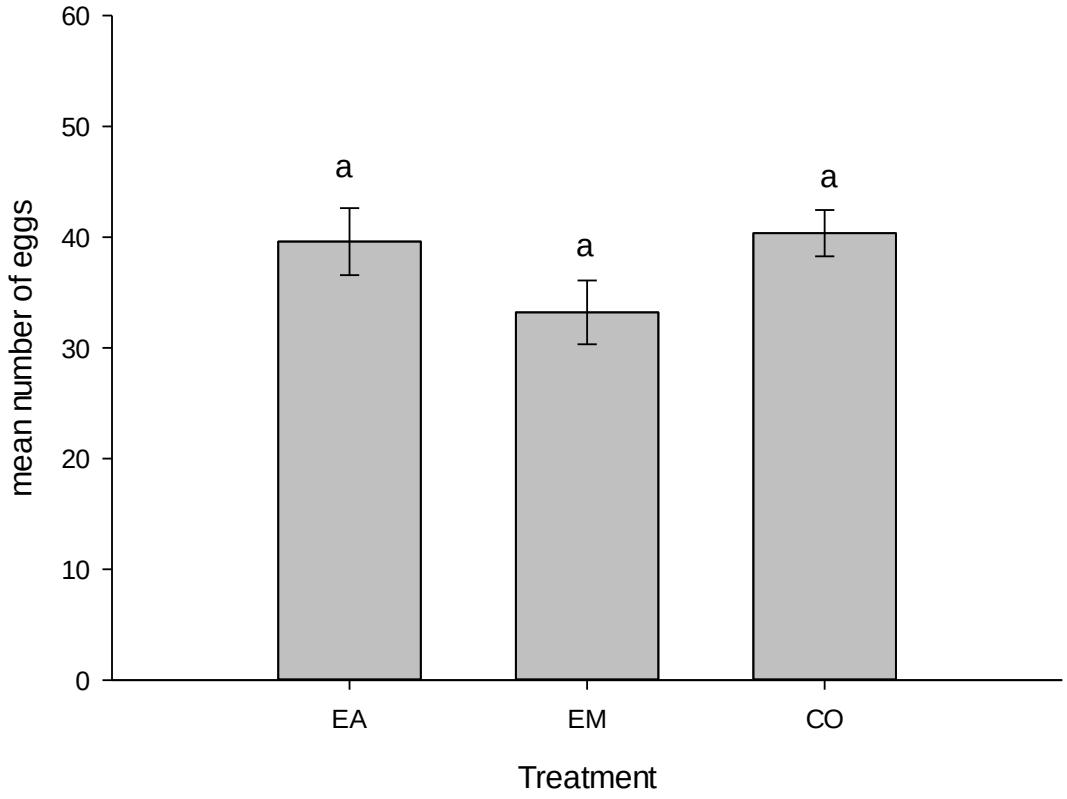

Figure7. Effects of *E. anophelis* on *An. gambiae* mean number of eggs produced. Each bar represents the mean number of eggs per treatment. Error bars denote the standard errors from the mean number of eggs.

In the *An.gambiae* fecundity experiment, there was variability in the percentage of mosquitoes that laid eggs per each treatment, however ,despite mosquitoes in the CO group having the highest mean number of eggs produced (40) , it had the least percentage which was 14% of number of mosquitoes that laid eggs while the treatment (EM) with the lowest mean number of eggs (33) , had the highest percentage (34 %) of the number of mosquitoes that laid eggs amongst the group and 28% of mosquitoes in EA treatment laid eggs.

Table 3**:** Number and percentage of eggs laid and dissected, number in parenthesis denote the total number of eggs (laid and dissected)

Treatment	Total number of eggs laid	Total number of eggs dissected	Percentage of eggs laid	Percentage of eggs dissected
EA	333(990)	657(990)	33.63%	66.36%
EM	359(1063)	704(1063)	33.77%	66.22%
CO	114(1090)	976(1090)	10.45%	89.54%

4.2.4 *An. gambiae* egg viability

A total number of 23 mosquitoes that laid eggs out of 84 mosquitoes representing 23.4%, were used in this experiment. The hatching rate of the *An. gambiae* eggs that were laid per treatment was deduced and 71.18% (n = 8) was the mean highest hatching rate in EA group, 56.21% in the EM group while CO had the least mean hatching rate of 51% .The egg viability was analyzed statistically and the results were no statistically significant df 20, (F=0.83, P= 0.4505).

Table 4 *An. gambiae* egg viability statistical results.

Treatment	Mean % hatching rate	Std error	P. value
EA	71.18	9.4	0.4505
EM	56.21	8.6	
CO	51	19.63	

4.3 *E. anophelis* hemolysin activity

4.3.1 *In vitro*

In determination of *E. anophelis* hemolysin activity on blood agar after a period of 48 h incubation, the results were indicated by change in color of the blood agar media. The 3 samples, negative control, positive control and test sample which was *E. anophelis* were plated on different sections of the blood agar plate. The test sample, in this regard *E. anophelis*, presented a greenish/brownish color change from original color of media which was red, indicating an alpha hemolysis. The positive control also turned the media red color into greenish/brownish indicating alpha hemolysis as well while for the negative control, gamma hemolysis was observed, which was represented by no color change (Figure 8).

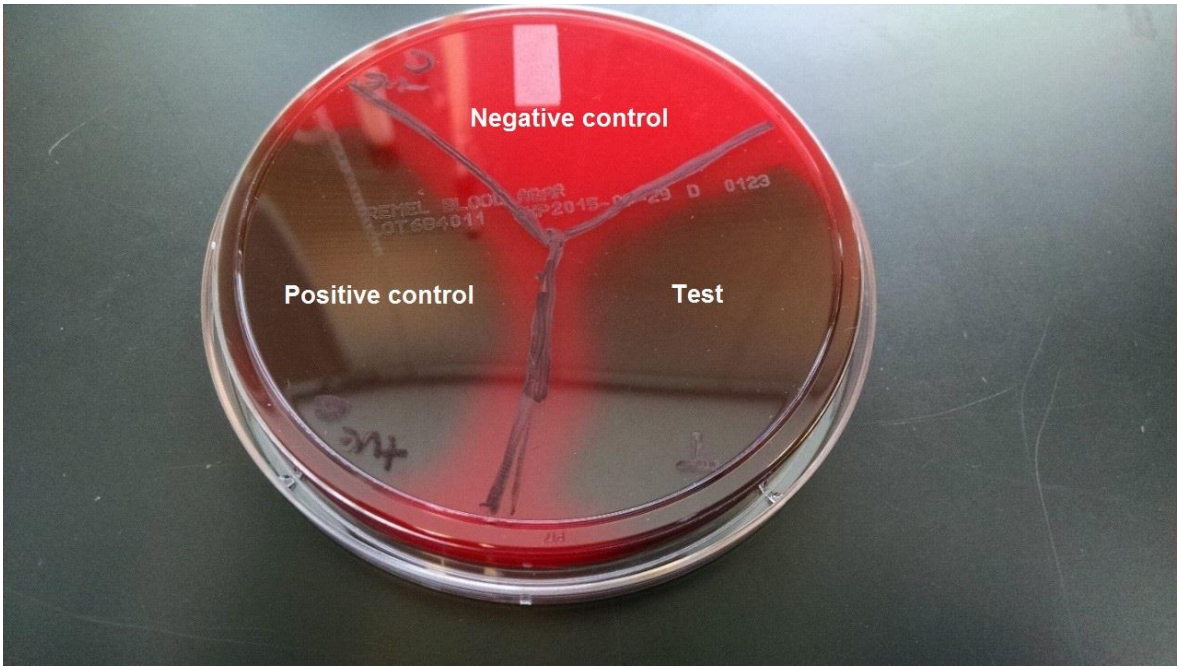

Figure 8. *In vitro* - MacConkey blood agar showing hemolytic activity, with alpha hemolysis activity on *E. anophelis* and positive control, and gamma hemolysis on the control on blood media.

4.3.2 *In vivo* hemolysin activity – *An. stephensi*

In order to assess the effect of *E. anopheles* on RBCs lysing, a total of 45 *An. stephensi* midgut were dissected (with 5 midguts assigned per treatment per time interval), and RBC`s number counted at three different time intervals (24 h, 36 h 1nd 48 h) post blood feeding in the three treatments . The RBCs mean per treatment were analyzed using one way ANOVA to compare the means. The results obtained after 24 h showed no significant effect in all the three groups df = 12 (F= 1.79, P = 0.2093). However at 36 h post blood feeding, the number of RBCs differed significantly among the three treatments after carrying out ANOVA, with df =12 (F=14.29, P = 0.0009). Furthermore post hoc test showed that all the three treatments were different from each other (P=0.0270, P=0.0217 and P = 0.0002). On the other hand, the results after 48 h were not different statistically, though the mean number of RBCs amongst the treatment groups differed, with EM =1,202,000 having the highest, EA= 846,000 having the intermediate while CO= 769,600 having the least (Figure 9).

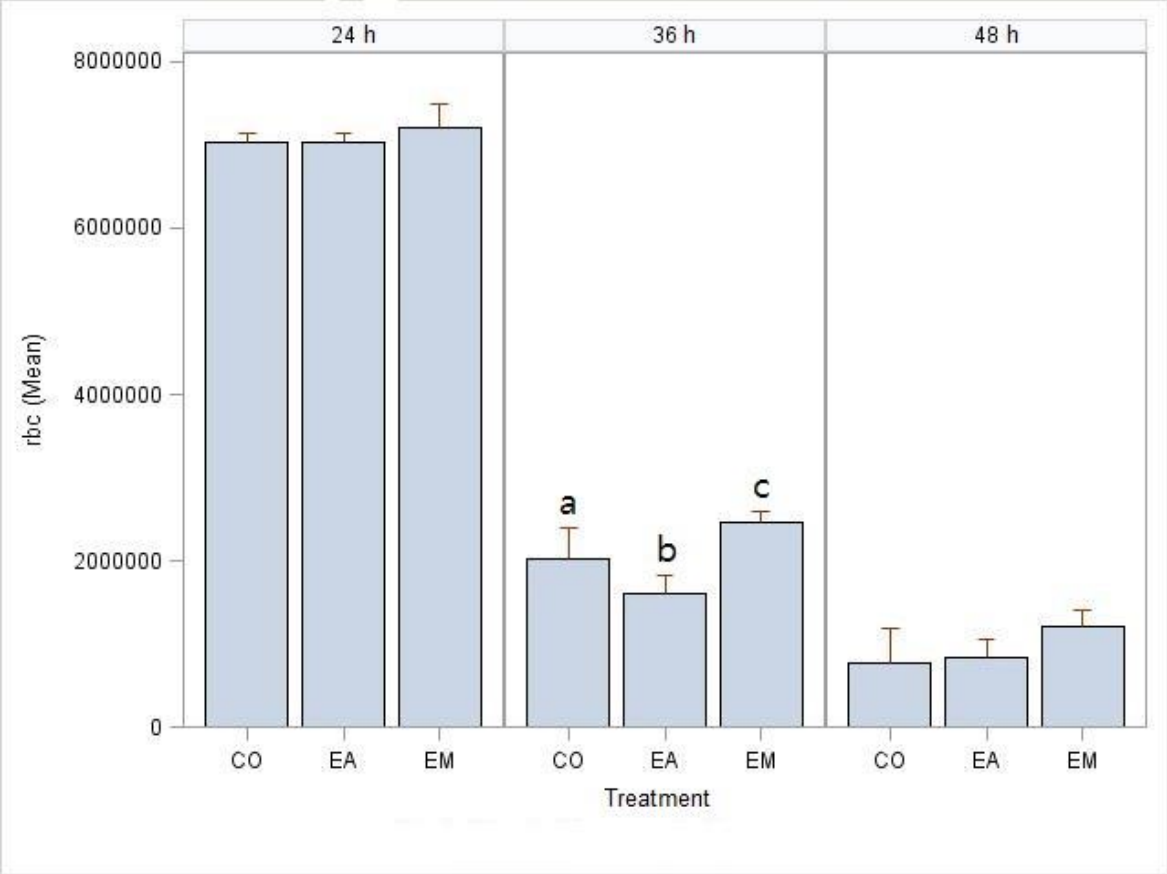

Figure 9. Mean number of RBCs in *An. stephensi* female blood fed midgut at 24 h, 36 h and 48 h time intervals. The bars with letters denote the treatment groups which were statistically different while the bars without letters denote the treatment groups that were statistically not different.

4.3.3 *In vivo* hemolysin activity – *An. gambiae*

Hemolysin activity of *E. anophelis* was ascertained in *An. gambiae* blood fed mosquitoes by comparing the number of RBCs as blood was being digested in mosquito midgut in the three treated groups EA, EM and CO. A total of 45 mosquitoes were sampled, with 5 samples per treatment per time interval. One way ANOVA on the mean number of RBC at three different time intervals (24 h, 36 h and 48 h) was conducted and there was no significant difference during all the time intervals P = 0.0764 , P = 0. 0574 and P = 0.1366 respectively. On the other hand the RBC means per treatment for each time interval were slightly different (Table 5).

Table 5 *An. gambiae* RBCs means after 24,36 and 48h post blood feeding.

Time (h)	Treatment	RBC mean	P- value
24	EA	5,052,000	0.07644
	EM	4,488,000	
	CO	4,214,000	
36	EA	2,834,000	0.0574
	EM	3,076,000	
	CO	2,748,000	
48	EA	1,490,000	0.1366
	EM	1,726,000	
	CO	1,402,000	

4.4 Discussion

In this study, a series of experiments was carried out to test several objectives. In a microbial clearance study, antibiotic erythromycin was used to clear the bacteria in mosquito guts. Plating of *An. stephensi* mosquito mid guts aliquoted from EM treated and control groups, on agar medium, showed a reduction in total number of CFU/ml in EM treated mosquitoes by almost two-third (Figure 3) as compared to the control group. The reduction in the number of CFU/ ml in EM treated mosquito mid guts show the effectiveness of the antibacterial erythromycin in reducing the bacterial density. However some microbes can be resistant to antibiotics, but also the selective nature of the agar medium which might still allow some microbes to grow can contribute to the persistent growth of some bacteria even after treated with erythromycin.

In microbial composition determination, 16s rDNA sequencing using the pyrosequencing method with mosquitoes from the three groups (EA, EM and CO) showed a diversity of bacteria present in the mosquitoes. The microbiota of the *An. stephensi* tested was dominated by gamma *proteobacteria* class (87%) as also observed in a previous study (Djadid et al., 2011). Furthermore, the following bacteria families were also found to be abundant; *Enterobacteriaceae*, *Acetobacteraceae*, *Flavobacteriaceae* and *Comamonadaceae* according to the OTU-reads percentage frequency (Table 1). Even though gamma *proteobacteria* was the most predominant in all the samples, the control had at least four genera the *Pseudomonas*, *Enterobacteria*, *Flavobacteria* and *Asaia* (Figure 4). The association of *Asaia* bacteria was also reported by (Favia et al., 2007), who found them to be stably associated with *An.stephensi* mosquitoes. When *E. anophelis* was supplemented to EM treated mosquitoes, the sequence from amplicons of these mosquitoes showed an increase in the microbial density as compared to EM treated group but still more not as much diversity as compared to the control group, (Figure 4).

The persistence of *Enterobacteraceae* in all the three groups though with different densities (with EM treated having the highest density) might be due to the bacteria being resistant to the antibiotic erythromycin.

In regard to the fecundity study, *An. stephensi* mosquitoes which were fed with *E. anophelis* laid significantly more eggs as compared to other two treatments which were not supplemented with *E. anophelis,* the conventionally reared (CO) and aseptic mosquitoes (EM). EM treated had the lowest number of eggs, while CO group was the intermediated. Supplement of *E. anophelis* a *Flavobacteria* to *An. stephensi*, increased egg production with almost double when the other bacteria communities were suppressed as compared to erythromycin treated mosquitoes which were not supplemented with *E. anophelis* (Figure 5). Erythromycin treatment had a negative effect in *An. stephensi* fecundity. Furthermore, even though *E. anophelis* does increase fecundity and has been found to be transmitted transstadially (Akhouayri et al., 2013) it seems to have no effect on hatching rate of the eggs, as evidenced by the non-significant effect observed in the egg viability in the three treatments. These results support observations from some previous studies which have reported antibiotic administration decreasing fecundity in mosquitoes but having no effect on egg viability(Gaio et al., 2011).

Contrary to this observation and to our hypothesis, are the results on the effect of *E. anophelis* on *An. gambiae* fecundity where the increase in egg production is not statistically significant ($p = 0.1151$) in all the three groups. Though there was a difference in the mean number of eggs in EA, EM and CO groups with CO producing a lot of eggs (40) followed by EA group with 39 eggs and the least being EM group with 33 eggs , statistically there was no difference in the mean number of eggs (Figure7). The trend of having least mean number of eggs in erythromycin treated mosquitoes was also observed in *An. gambiae* results (eggs laid and those that were

dissected). Since EM was aseptic and EA was solely *E.anophelis* supplemented, therefore the difference in the mean number of eggs between the EA and EM groups can be attributed to *E. anophelis* bacteria activity which resulted in increasing egg production in *E. anophelis* supplemented mosquitoes as opposed to the aseptic mosquitoes.

This similar observation was previously reported by (Gendrin et al., 2015) where he stated that antibiotic augments fecundity in *An. gambiae* mosquitoes. This was true for only the number of eggs that were laid by *An.gambiae* mosquitoes (EA 33.6%, EM 33.7% and CO 10.4%), but when the ovaries were dissected and the number of eggs in the ovaries were included this was not the case, the results showed antibiotic treated mosquitoes (EM) having the lowest mean number of eggs. This difference might be due to difference in the type of antibiotic used as some known bacteria are resistant to certain antibiotics. For instance, some flavobacteria have been found to be resistant to penicillin, streptomycin and tetracycline (Gendrin et al., 2015)(Kampfer et al., 2011).

A difference in the number of mosquitoes that laid eggs was observed in *An. gambiae* and *An. stephensi* fecundity experiment. At least 99% of *An. stephensi* mosquitoes laid eggs as compared to *An. gambiae* where only 27.7% of the mosquitoes laid eggs while 72.2 % of the mosquitoes did not lay eggs but had eggs in there ovaries when dissected. On egg viability study, non-significant effect was observed in the hatching rate of the two treatments plus the control. This implies that *E. anophelis* had no effect on egg hatching, even though it still has a positive effect on fecundity. On the other hand erythromycin had no effect on egg viability in both *An. stephensi* and *An. gambiae* but it has a negative effect on mosquito fitness, of reducing egg production. Though *E. anophelis* has been found to be transmitted vertically in human beings (Lau et al.,

2015) and transstadially in *An. gambiae* (Akhouayri et al., 2013), the transovarial transmission route seems to be lacking for mosquitoes.

In addition fecundity augmentation by *E.anophelis* provided a platform for further investigation in the hemolysin activity of the bacteria in mosquito. In this regard *E. anophelis* proves to aid in RBC lysis both *in vitro* as also observed by (Kukutla et al., 2014a) and *in vivo* as shown in this study. This bacteria when inoculated on blood agar media it lysis the RBC there by producing an alpha hemolysin and in the mosquito midgut, 36 h post blood feeding results showed number of RBC being significantly low in *An. stephensi* - *E. anophelis* treated group. Since in anautogenous mosquito`s egg production, blood is an essential element where the RBC need to be lysed for vitellogenesis to take place. This involves massive production of yolk protein precursors forming amino acids which are the building block of proteins and are then carried by the receptor mediated pathway and then deposited into the ovaries where they are required for the development and maturation of the oocytes (Attardo et al., 2005) (Klowden, 2010). Therefore this *E. anophelis* hemolysin activity aids in the speeding up of RBC lysing as such accelerating vitellogenesis thereby enhancing the egg development. This observation explains the mutual symbiotic relationship that exists between *An. stephensi* and its gut microbe *E. anophelis,* while this bacteria *E. anophelis* is aiding in lysing of RBC`s with its hemolysin activity in the mosquito midgut which increases mosquito fitness by increasing its fecundity*,* the mosquitoes do provide habitat for the bacteria. At starting point which was 24 h after blood feeding there was no significant effect in all three treatments. However, there was a drastic decrease of RBCs (70%) which was also statistically significant in *An. stephensi* between 24 and 36 hours (Figure 6) when the digestion was underway, which might imply that most of the blood processing/ RBC lysing occurs during this time. While 48 h post blood feeding there was no significant effect but

in all these results the RBCs in EM treated group remained the highest which indicate the slowness in blood lysing caused by lack of *E. anophelis* bacteria.

This phenomenon was not really different in *An. gambiae*, though there was no statistical significant effect in the number of RBCs in mosquito midgut post blood feeding, less number of RBCs were observed in CO groups throughout the experiment which coincides with the higher number of eggs in the control group.

4.5 Conclusion and recommendation

This study demonstrated that antibiotics such as erythromycin which was used in this study, is able to reduce the bacterial diversity and community sizes in mosquitoes, thereby affecting the roles played by the microbes which have been reduced or cleared as shown by the reduction in the amount of eggs produced in aseptic mosquito. Gut microbes such as the bacterium *E. anophelis* is involved in hemolysin activity which aid in RBC lysing, therefore augmenting fecundity in the malaria vector *An. stephensi* but not in *An. gambiae* mosquitoes. Antibiotic erythromycin was found to decrease the number of egg development in both *An. stephensi* and *An. gambiae*. Furthermore, hatching rate is not influenced by the presence or absence of both *E. anophelis* and erythromycin. As such the knocking down /manipulation of hemolysin genes in *E. anophelis* can be of help in reducing fecundity, thereby decreasing vector density which is one of the components of vectorial capacity hence reduction in vectorial capacity too.

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