Role of Salivary Gland Secretions in *Anopheles* Mosquito Midgut and *Plasmodium* Development

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DECLARATION

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

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DEDICATION

This treatise on mosquito physiology and malaria transmission joins with all of creation in giving credit to God the creator whose attributes in the endeavours of science accredited thus:

"It is the glory of God to conceal a thing: but the honour of kings is to search out a matter." Proverbs 25:2 (KJV)

"But there is a God in heaven who reveals mysteries…
Daniel 2:28 (Good News Bible)

"The heavens declare His glory. The skies and indeed all of nature proclaim the work of His hands, day after day pouring forth speech; night after night displaying knowledge. There is no speech or language where their voice is not heard. Their voice goes out into all the earth, their words to the ends of the world." Psalms 19:1-4

To God I give thanks and attribute all the glory.

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LIST OF ABBREVIATIONS

°C	Degrees Celsius
A	Acid production
ACT	Artemisinin based combination therapy
ACUC	KEMRI, Animal Care and Use Committee
ADP	Adenosine diphosphate
AFRO	WHO Regional Office for Africa
AG5	Antigen 5 protein family
AIDS	Acquired Immune Deficiency Syndrome
AMG	Anterior Midgut
AMP	Antimicrobial peptides
AMRO	WHO Regional Office for the Americas
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BAPNA	N-a-benzoyl-DL-arginine-p-nitroanilide
BHI	Brain Heart Infusion broth
CAP	Cysteine-rich antigen protein family
CBRD	Centre for Biotechnology Research and Development
CFU	Colony forming units
CI	Confidence interval
DDT	Dichloro-diphenyl-trichloroethane

Df/df	Degrees of freedom
dH_2O	Distilled water
DMF	2%*N*-dimethlyformamide
DNA	Deoxyribonucleic acid
EC	Nomenclature Committee of the International Union of Biochemistry and Molecular Biology Enzyme Commission
EIR	Entomological Inoculation Rates
EMRO	WHO Regional Office for the Eastern Mediterranean
EURO	WHO Regional Office for Europe
Fig.	Figure
g	Grams
G	Gas production
G6-PD	Glucose 6 phosphate dehydrogenase
GC	Gametocytes
H	Housekeeping
H_2O	Water
H_2S	Hydrogen sulfide/sulfur reduction
HIV	Human Immuno-deficiency virus
Hr	Hour
HR/hr	Hazard ratio
icipe	International Centre for Insect Physiology and

	Ecology
IMVC	Indole, Methyl Red, Voges-Proskauer and Citrate tests
INDO	Indole
IPT	Intermittent Preventive Treatment
IRS	Indoor Residual Spraying
ITNs	Insecticide Treated Nets
ITROMID	Institute of Tropical Medicine and Infectious Diseases
JKUAT	Jomo Kenyatta University of Agriculture and Technology
K	Alkaline reaction
KEMRI	Kenya Medical Research Institute
KM	Kaplan Meier
L:D	Light:darkness photoperiod ratio
LB	Luria-Bertani broth
LLITNs	Long Lasting Insecticide Treated Nets
MDG	Millennium Development Goals
N	Number per treatment
n	Number per replicate
NCBI	National Centre for Biotechnology Information (of United States of America)
OC	Oocyst

OK	Ookinete
ORN	Ornithone
OXD	Oxidase test
PBF	Post Blood Feeding
PBS	Phosphate buffered saline
pH	potentiometric hydrogen ion concentration
PM	Peritrophic membrane/matrix
PMG	Posterior Midgut
PR	Prevalance
PRP	Pattern recognition proteins
QC	Quality control
RBC	Red Blood Cells
RBM	Roll Back Malaria
RH	Relative humidity
RNA	Ribonucleic acid
rpm	Revolutions per minute
S	Secreted
s.l.	*sensu lato*
s.s.	*sensu strict*
SC	Simon citrate
SEARO	WHO Regional Office for South East Asia
Serpin/SRN	Serine protease inhibitors protein family
SG	Salivary gland

SGH	Salivary gland homogenate
Sig.	Significance
SIT	Sterile Insect Technique
SP	Sulfadoxine-pyrimethamine
Spp.	Species
SSA	sub-Saharan Africa
Std.	Standard
TB	Tuberculosis
TDR	WHO Special Programme for Research and Training in Tropical Diseases
TE	Transposable Elementss
TMOF	Trypsin-modulating oostatic factor
Tris-HCl	tris(hydroxymethyl)aminomethane-hydrochloric acid
TSI	Triple sugar iron test
U	Unknown
UNDP	United Nations Development Programme
UNICEF	The United Nations Children's Fund
UR	Urea
US	United States of America
WHO	World Health Organization
WPRO	WHO Regional Office for the Western Pacific

ABSTRACT

Mosquito saliva plays a central role in blood meal acquisition plus the development and transmission of the malaria parasite. Functional genomics and proteomics studies have characterized and predicted roles of many proteins in the sialotranscriptome of *An. gambiae* and their predicted roles. Whereas, this approach provides important insights or *a priori* on the role of saliva and its interaction with *Plasmodium* in the midgut, the large amounts of data often presents a problem in determining the best strategy for exploiting the knowledge in malaria control. For this reason, a series of experiments was designed to test and validate several *a priori* in this study: 1) Saliva contains antimicrobial proteins (AMPs) that may modulate bacteria population dynamics in the midgut thus affecting mosquito survival; 2) Saliva contains several catabolic enzymes and protease suppressors thus affecting *Plasmodium* development during blood meal digestion and 3) Saliva contains xanthurenic acid and other molecules whose role remains unknown that may directly or indirectly affect *Plasmodium* development in the midgut.

Surprisingly, mosquito salivary gland homogenate (SGH) of female mosquitoes did not exhibit any antimicrobial activity when tested against 8 bacteria species previously isolated from *An. gambiae* midgets indicating no role for saliva in modulation of endosymbiont bacteria in the midgut. Whereas bacteria was found to be crucial in the larval diet for survival and development in the immature stages, the role of endosymbiont bacteria in the adult mosquito proved ambivalent as it varied from beneficial to harmful under various experimental setups. However, the clear role in

larval survival and development indicates the employment of biological controls such as *Bti* and *Bs* is a winning strategy that should be promoted in integrated vector management. Lastly, SGH was demonstrated to have protease suppression properties that suppressed both the serine proteases and aminopeptidase. Interestingly, *P. falciparum* was also shown to modulate proteases by down-regulating serine proteases and up-regulating aminopeptidase, which was recently discovered as a *Plasmodium* receptor during midgut invasion. SGH suppression of aminopeptidase therefore suggests a possible role for SGH molecules in transmission blocking, however oocsyt counts in mosquitoes fed on infective blood meal + SGH did not differ significantly when compared to control group fed on infective blood meal only.

CHAPTER ONE

1.1 INTRODUCTION

Malaria is an acute and/or chronic condition caused by protozoans of the genus *Plasmodium* Machiafava & Celli, 1885 that is transmitted by female mosquitoes of the genus *Anopheles* (Diptera: Culicidae). Four species of *Plasmodium* are known to cause disease in humans, *P. malariae*, *P. vivax*, *P. ovale* and *P. falciparum*. More recently, *P. knowlesi* which causes malaria in monkeys has also been found in naturally occurring human infections in some regions such as Malaysia (Singh *et al.,* 2004). *P. falciparum* malaria is the most virulent and devastating with approximately 500 million new cases of malaria and 1 million mortalities annually (Fig 1.1) occurring mostly among children under 5 years old (Rowe *et al.,* 2006; Greenwood *et al.,* 2008) in sub-Saharan Africa (SSA); whilst significantly contributing to maternal and infant deaths resulting from malaria in pregnancy and low birth weight (Desai *et al.*, 2007). More recently, severe, life-threatening malaria resulting from *P. vivax* infections (conventionally viewed as mild or benign) has been reported as an emerging public health concern in a few Asian countries (Rogerson and Carter, 2008).

Globally, more than 2 billion people are at risk of malaria mainly consisting of poor populations living in tropical and subtropical regions with climates that are conducive for the development of the malaria parasite in *Anopheles* mosquitoes (Snow *et al.*, 2005). The disease has been successfully eradicated in Europe, the US, Japan, and many high and middle-income nations as a result of a combination of

several factors. These include economic development and public health measures (Zucker, 1996; Chareonviriyaphap *et al.,* 2000), and previous global eradication campaigns that focused primarily on vector control employing dichloro-diphenyl-trichloroethane (DDT), a residual insecticide (Attaran *et al.,* 2000). Today, the epidemiology of malaria is understood to be closely associated with poverty, both as a product and an enhancer of poverty (Obrist *et al.,* 2007; Castillo-Riquelme *et al.,* 2008; Packard, 2001). As such it is rightly regarded as among the top public health priorities in sub-Saharan Africa and globally together with HIV/AIDS and TB among the millennium development goals (MDGs).

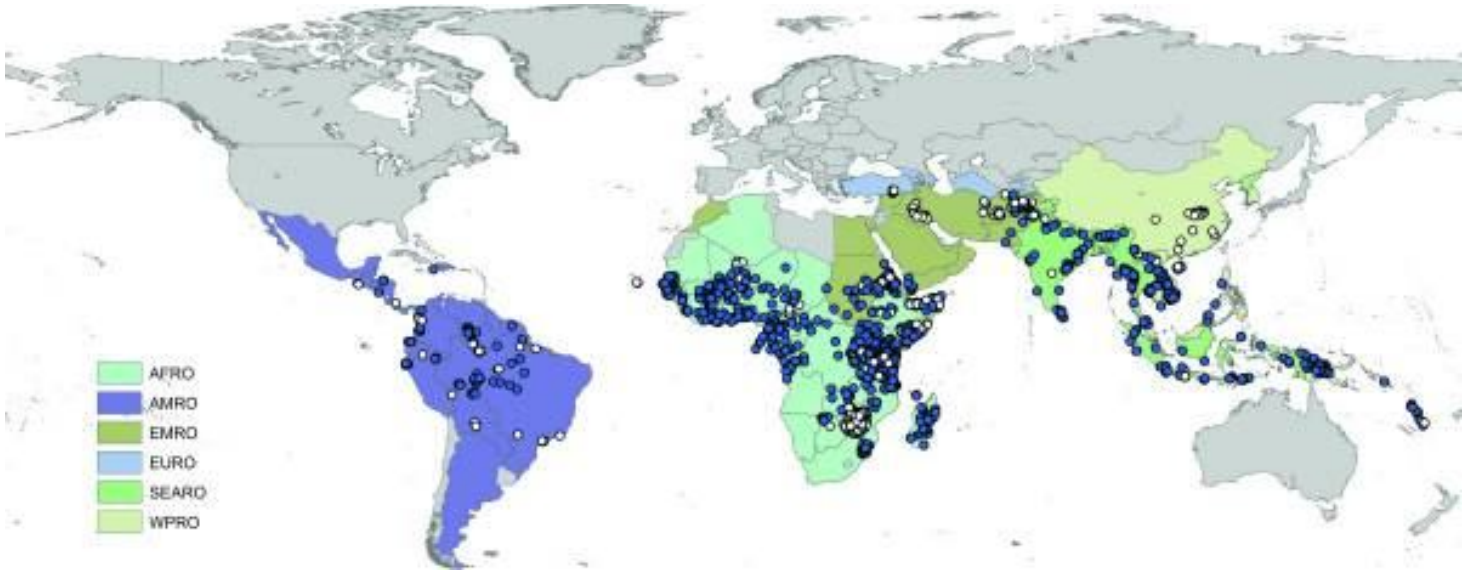

Fig 1.1: The global distribution of Plasmodium falciparum (Adapted from Guerra et al., 2007)

Key: Blue dots indicate presence (PR > 0) and white dots absence (PR = 0). Malaria endemic countries are coloured by the WHO regional office they belong to.

1.1.1 Malaria Control

Successful malaria control has traditionally focused on two aspects, namely: the control of *Anopheles* mosquitoes that transmit malaria and the treatment of infected persons that remain as the reservoir (Okie, 2008). The epidemiology of malaria intensity and patterns of transmission are primarily a function of the seasonality, abundance and feeding habits of the mosquito vector (Greenwood *et al.*, 2008). Vector control is therefore an essential component of any malaria control program as is evidenced by its successful exploitation in the past.

The use of dichlorodiphenyltrichloroethane (DDT) in indoor residual spraying (IRS) strategy facilitated the eradication of malaria in the temperate countries of the developed North, during the early global malaria eradication campaigns of the 1950s and 60s especially. However, due to DDT's inherent persistent nature in the environment and perceived health risks arising from its pesticide use in agriculture, the role of vector control in malaria control has floundered (Carson, 1962; Guimarães *et al.*, 2007). This led to an increase in malaria prevalence in spite of the ongoing Roll Back Malaria (RBM) campaign that was criticised for favouring insecticide treated nets (ITNs) over DDT use and IRS strategy in recent times (Attaran *et al.*, 2000; Yamey, 2004; Driessen, 2003). Consequently, the WHO has now approved the use of DDT in malaria control where the vector is still susceptible to the insecticide, giving much needed impetus to refocusing on vector control (WHO 2006).

Nevertheless, the emergence of resistance to DDT and pyrethroids (synthetic derivatives of pyrethrum insecticides) used in ITNs and long lasting ITNs (LLITNs), presents a challenge to malaria vector control programs (Ranson *et al.*, 2002; Takken, 2002; WHO, 1992). Moreover, in sub-Saharan Africa vector control is especially challenging not only due to the abject poverty that creates conditions in which mosquitoes thrive alongside human habitats, but also due to the special characteristics of the *An. gambiae* as the most efficient mosquito vector species (Committee On The Economics Of Antimalarial Drugs, 2004; Muturi *et al.*, 2008). The *An. gambiae* are long-lived, homophilic mosquitoes with a widespread distribution in SSA. *An. gambiae* is a multiple feeding vector whereby host seeking behaviour is not inhibited by a blood-meal until after ovipositing, making it an aggressive vector (Klowden, 2007). Consequently, where the entomological inoculation rate (EIR), which measures the frequency a human is bitten by infective mosquito bites, of S. America or Asia rarely exceeds 5 per year, EIRs of more than 1,000 are not uncommon in SSA (Greenwood and Mutabingwa, 2002; Beier *et al.*, 1999).

The chemotherapy of malaria has a direct impact on disease burden and mortality, with the availability of cheap, safe and effective drugs being the ideal scenario for this strategy (Committee On The Economics Of Antimalarial Drugs, 2004). Additionally, chemotherapy can also offer the additional advantage of prophylaxis as a preventive measure applicable as both individual and public health intervention such as intermittent preventive treatment (IPT) of pregnant mothers based on Sulfadoxin-pyrimethamine drug combination (SP) as part of maternal

health (Briand *et al.,* 2007). However, this ideal scenario is fraught with challenges such as the emergence of drug resistance in the parasites as was the case in chloroquine and Sulphadoxine-pyrimethamine (SP) first line treatments (Committee on the Economics of Antimalarial Drugs, 2004; White, 2008). Adverse side effects are also known to arise from the antimalarial medication as is the case for primaquine in glucose 6 phosphate dehydrogenase (G6-PD) deficienct populations or mefloquine-associated neuro-psychiatric symptoms (Croft and Garner, 2008; Beutler *et al.,* 2007). Meanwhile, highly safe and efficacious drug such as artemisinin based combination therapies (ACTs) is faced by high costs of production that makes them unavailable especially to the poor without government subsidy, weighing down heavily on the sustainability of these drugs as a first line strategy (Committee On The Economics Of Antimalarial Drugs, 2004).

1.1.2 Biotechnological Breakthroughs and the Development of Novel Malaria Control Strategies

Biotechnological breakthroughs such as genetic engineering and the relentless advances in new fields such as genomics, proteomics and bioinformatics are providing new insights for developing of innovative tools and approaches for malaria control. Currently these approaches can be grouped into three main control strategies. The first category focuses on the development of vaccines and transmission blocking vaccines (Sutherland, 2009; Mitri *et al.,* 2009; Hirai and Mori, 2010). The second strategy seeks to develop genetically modified mosquitoes that express desired phenotypic traits that are desirable for disease control including for sterile insect technique or being refractory to the malaria parasite. These mosquitoes

can then be mass produced and released to compete favourably in order to displace the wild mosquito population that can transmit malaria (Catteruccia *et al.*, 2003).

The last strategy is similar to the second but focuses on the development of paratransgenic mosquitoes whereby endosymbiotic bacteria are exploited to deliver effector molecules that result in the desired phenotypic traits for disease control. Development of the paratransgenics strategy in transmission blocking has been reviewed comprehensively by Lindh (2007). Briefly, the technique was first developed, and proved successful in a laboratory setting for Chagas disease (Beard *et al.*, 1992; Durvasula *et al.*, 1997), utilizing a symbiotic bacterium (*Rhodococcus rhodnii*) isolated from the midgut of the vector *Rhodnius prolixus* (Hemiptera: Reduviidae). *R. prolixus* bugs lacking the symbiont fail to become sexually mature adults. The symbiont is spread between the bugs by copro-phagy (probing of fecal droplets). *R. rhodnii*, transformed with a shuttle plasmid expressing a cecropin A fusion protein, was reintroduced into the vector making it refractory to the parasite *Trypanosoma cruzi* (Durvasula *et al.*, 1997).

Paratransgenics as a strategy has also been examined in tsetse flies, the vectors of sleeping sickness (Cheng and Aksoy, 1999) and in mosquitoes where *Wolbachia pipientis* strain *w*MelPop, an endosymbiont originally isolated from the fruit fly, *Drosophila melanogaster*, has been shown to reduce mosquito longevity and inhibit the development of filarial nematodes in *Ae. aegypti* by up-regulation of the innate immune system (McMeniman *et al.*, 2009; Kambris *et al.*, 2009).

1.2 STUDY JUSTIFICATION

History has demonstrated that the role of the mosquito in malaria transmission is a critical point of intervention in the successful control and eradication of the disease during the 1950s global eradication effort and consequent elimination programmes (Muturi *et al.,* 2008). Mathematical models have indicated that only the substantive reduction of EIRs to levels less than 1 infective bite per person per year is likely to achieve the substantial reductions in malaria prevalence targeted in the millennium development goals (Smith *et al.*, 2005; Beier *et al.*, 1999). However, the super efficient transmission of malaria by the *An. gambiae* in SSA presents a special challenge to effective malaria control on the continent demonstrated in EIRs exceeding 1,000 per person per year (Greenwood and Mutabingwa, 2002; Yamey, 2004). Current WHO targets to reduce global malaria burden by at least 50% by 2010 and 75% by 2015 would cost an estimated $3.8 billion to $4.5 billion annually between now and 2015 (Kiszewski, 2007). The high costs make the prospects for a multi-decade global eradication campaign highly unlikely, especially due to inevitable waning of political will (Okie, 2008).

The Kenya government recently announced the anticipated elimination of malaria in Kenya by the year 2017 at an estimated cost of 100 million USD (GoK, 2009). This bold goal is based on the use of conventional methods and donor funding. While celebrating and commending Kenya's remarkable progress and effort towards achieving this millennium development goal, the hard won gains nevertheless remain under ominous threat of ever looming drug resistance, pesticide resistance and donor fatigue. Moreover, as the goal changes from focusing on

control to the more ambitious elimination and eradication programmes the need to adopt new tools and stratagems cannot be gainsaid. These new stratagems should not be dependent on centralized health authorities and high budgetary allocations to ensure uniform individual and community level implementation such as recommended drug and dose compliance and adherence to insecticide treated nets re-treatment.

The adoption of new biological control/biotechnology based tools and stratagems provide the much needed arsenal for elimination and maintenance of zero malaria prevalence. This is because they are well suited for the more ideal "area-wide vector" control method whose deployment is not dependent on individual compliance or community mobilization and is already being used extensively in agricultural pest control with great success (Robinson *et al.*, 2009; Catteruccia *et al.*, 2009). Sound knowledge of vector biology and its interactions with the parasite and vertebrate host is fundamental for the development of "area-wide vector control" such as sterile insect technique (SIT), transgenic, paratransgenic and other transmission blocking mechanisms that exploit this knowledge.

1.3 RESEARCH QUESTION

Mosquito saliva and salivary glands are central to parasite-vector-host interactions with previous studies indicating that it can be exploited to impede malaria transmission in line with the "area-wide vector control" paradigm. Saliva contains pattern recognition proteins (PRPs) and potent antimicrobial proteins (AMPs) that may play a role in the regulation of midgut microbiota (Rosinski-Chupin *et al.*, 2007); serine proteases and protease inhibitors that may play a role in

blood meal digestion in the midgut (Arcà *et al.,* 2005); and xanthurenic acid that has been shown to stimulate male gametocyte exflagellation *in vitro* (Hirai *et al.,* 2001). In addition, functional genomics and proteomics have availed a vast and verifiable platform of knowledge on the molecular components of mosquito saliva and their putative roles (Arcà *et al.,* 2005; Calvo *et al.,* 2004; 2006; 2007; 2009; Francischetti *et al.,* 2002; Valenzuela *et al.,* 2002; 2003). However, this wealth of information remains untapped due to the lack of empirical data that validates the potential of exploiting the predicted properties of proteins found in saliva.

This study therefore contributes to the basic understanding of *Anopheles gambiae* physiology, particularly by evaluating *a priori* derived from functional genomics and proteomics of anopheline mosquito saliva. In this regard, the putative role of salivary gland secretions in the mosquito midgut environment and parasite development has been tested in salivary gland homogenates.

1.4 PROBLEM STATEMENT

To evaluate the transmission blocking potential of saliva whose components are characterized in the sialotrancriptome of mosquito with predicted properties that may influence the midgut environment and/or *Plasmodium* development.

1.5 STUDY HYPOTHESES

This study was designed to test the following alternative hypotheses:

1. Mosquito-midgut microbiota interaction is symbiotic and/or beneficial to the mosquito

2. *An. gambiae* salivary gland homogenate has an indirect effect on *Plasmodium* development in the mosquito via bacteria population dynamics in the midgut

3. *An. gambiae* salivary gland homogenate has an indirect effect on *Plasmodium* development in the mosquito via regulation of blood-meal digestion

4. *An. gambiae* salivary gland homogenate has a direct effect on *Plasmodium* development in the mosquito

1.6 STUDY OBJECTIVES

1.6.1 Overall Objective

To determine the role of salivary gland homogenate on the mosquito midgut environment and the development of *Plasmodium falciparum* in *An. gambiae*.

1.6.2 Specific Objectives

1. To determine the effects of salivary gland homogenate on the mosquito midgut proteases and on bacterial population dynamics

2. To determine the effect of bacteria resident in the mosquito midgut on vector survival

3. To determine the effect of salivary gland homogenate on *P. falciparum* development in experimentally infected *An. gambiae* mosquito

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The Anopheles Mosquitoes

Approximately 3,500 species of mosquitoes, all belonging to the monophyletic family Culicidae, are known to exist. These are categorized into three subfamilies Culicinae, Anophelinae and Toxorhinchitinae. Many of the generalizations made about mosquito behaviour and physiology are based on the extensive research on yellow fever mosquito, *Aedes aegypti* Linnaeus 1762 because it is easy to rear, feeds indiscriminately on a wide variety of vertebrate and invertebrate hosts for blood-meals, and unlike the anophelines, eggs of this mosquito can be stored for long periods and hatched when required for experimentation (Klowden, 2007). However, the reality is very different in that there is no shared/common physiology of 'the mosquito' since each group and even each species has its own set of metabolic adaptations necessary to thrive in its respective ecological niche (Briegel, 2003).

The Anophelinae is theorized to represent the ancestral group that radiated in Africa and south America approximately 100 million years ago (Krzywinski and Besansky, 2003), and possesses a relatively large number of species complexes whose sibling species are morphologically indistinguishable but genetically distinct (Collins and Paskewitz, 1996; White, 1974). It is only the female mosquitoes of the genus *Anopheles* that can transmit the malaria parasite, *Plasmodium*. All male

mosquitoes feed exclusively on sugar sources and therefore do not transmit the disease (Theobald, 1901). Female mosquitoes also feed on sugar from different sources but are anautogenic, in that they need blood for the development of their eggs (Knab, 1907). The eggs are laid on water and develop into larvae within approximately 48 hours. *Anopheles* larvae go through four instars before they develop into pupae. The larvae feed on organic matter and microorganisms at the surface layer of the water puddles they live in (Walker *et al.,* 1988; Briegel, 2003; Wotton *et al.,* 1997; Merritt *et al.,* 1992). The pupa does not have a mouth and hence does not feed. From the pupal stage the mosquito transforms through complete metamorphosis into an adult mosquito.

Anopheles gambiae sensu stricto (Giles) 1902, *Anopheles arabiensis* (Patton) 1905, and *Anopheles funestus* (Giles) 1900, are the main vectors of malaria in Africa. The *An. gambiae* species complex contains sibling species whose vector capabilities range from non-vectors to minor vectors to primary vectors, with a wide range of ecological habits as larvae and adults (Klowden, 2007). This ranges from *An. gambiae s.s.*, which is anthropophilic, endophagic and endophilic (prefer to feed on humans, and to feed and rest indoors); to *An. arabiensis* which tends to be less strictly anthropophilic with a wider range for zoonotic host feeding and a peridomestic to exophilic preference in different areas; to the non-vector *An. quadriannulatus*, which is exophilic and largely zoophagic (prefer to rest outdoors and to feed on animals). *An. funestus* is also anthropophilic, endophagic and endophilic but less susceptible to *Plasmodium* than *An. gambiae s.s.* Whereas *An. gambiae s.l.* prefers to breed in small, usually seasonal habitats such as ponds and

tree holes, *An. funestus s.l.* prefer to breed in large, permanent water reservoirs such as lakes (Takken and Knols, 1999).

2.2 Mosquito and Plasmodium Co-Evolution

With more than 2 billion people at risk globally, malaria boasts a wide distribution that prior to the introduction of DDT, spanned the entire globe and affected virtually all of humanity (Snow *et al.,* 2005; Guimarães *et al.,* 2007). It therefore may seem contrary to conventional wisdom that mosquitoes present a severe bottleneck in the transmission of malaria, whereas this is clearly the case. Few *Anopheles* mosquitoes (approx 70 out of 422 spp. worldwide) are able to support the development of *Plasmodium* – the majority being refractory to the parasite. Of the few *Anopheles* vectors available (approx 40 out of 70 considered of importance), a high parasite attrition rate is further evidenced with the elimination of most of the input parasites (Blandin and Levashina, 2004). The successful transmission of the malaria parasite therefore hinges on a combination of physiological factors determining refractoriness to *Plasmodium* and mosquito host-seeking behaviour. Indeed, recent studies indicate that sympatric *Anopheles*/*Plasmodium* species combinations have co-evolved where mosquito refractoriness to *Plasmodium* infection is not significant and malaria transmission can be effectively supported (Mendes *et al.,* 2008; Lambrechts *et al.,* 2005).

2.2.1 Life Cycle of Plasmodium falciparum

Plasmodium is an obligate parasite of two hosts: the vertebrate definitive host and the mosquito vector. The parasite undergoes many morphological changes

during its complex life cycle that alternates between invasive and replicative stages both in the human host and anopheline mosquito (Fig 2.1). Replicative stages can be divided into two main parts consisting of the sexual and asexual development stages, with the sexual stages occurring in the mosquito and asexual in the vertebrate host.

Human infection starts with the bite of an infected *Anopheles* mosquito that injects *Plasmodium* sporozoites together with its saliva as it takes a blood-meal, necessary for its eggs development (Frischknecht *et al.,* 2004). The number of sporozoites injected by the mosquito with saliva has been shown to vary between 1 and 420 in wild-caught anopheline mosquitoes from western Kenya (Beier *et al.,* 1991). The majority of sporozoites reside at the site of bite for hours where a few are slowly released into circulation with some ending up in lymph nodes, whilst others make their way to the liver (Yamauchi *et al.,* 2007; Sinnis and Zavala, 2008). Of those that make it to the liver, the sporozoites lose their motility and evade macrophages and dendritic cells by aggregating in vacuole structures within hepatocytes called merosomes (Sturm *et al.,* 2006).

The sporozoites then enter the phase known as pre-erythrocytic schizogony with each dividing into 10,000-30,000 merozoites. This phase lasts about 6 days and ends with the budding off of merosomes to release merozoites directly into the blood stream, rupturing hepatocytes in the process (Greenwood *et al.,* 2008). In the blood stages, merozoites invade the host red blood cells (RBC) where they grow and reproduce, dividing into 8-20 new merozoites every 48 hours whereupon they rupture the RBCs, invade new RBCs to start the cycle over again. It is this rhythmic cycle of RBC invasion and erythrocytic schizogony that is responsible for the tertiary

febrile symptoms associated with *P. falciparum* infection. Unlike other malaria causing *Plasmodium* species that can only invade young RBCs, *P. falciparum* is capable of invading RBC of all ages; resulting in a greater intensity of disease for *P. falciparum* due to the higher parasite burden (Tuteja, 2007).

Following several cycles of multiplication, some merozoites differentiate into non-pathogenic male and female sexual forms known as gametocytes that necessitate ingestion by a mosquito in order to develop further. The female mosquito ingests approx 2-3µl during a blood-meal. Upon ingestion the gametocytes mature to sexual gametes that pair up in the mosquito midgut setting in motion the sporogonic cycle (Beier, 1998). The male gametocyte undergoes exflagellation, a process whereby "whip-like" protrusions emerge that serve to transfer genetic material into the female gametocyte for fertilization. The resulting fusion forms the diploid zygote stage that develops into the invasive ookinetes (Billker *et al.*, 2004). These ookinetes are motile and within 24 hours after blood ingestion are able to invade and traverse the midgut epithelium. Diploid ookinetes undergo meiosis and upon reaching the basal side of the midgut, transform into oocysts that further undergo several rounds of mitosis as they mature for 3 to 12 days. Thousands of haploid sporozoites from each oocyst are then released directly into the mosquito's blood, the haemolymph, in which they migrate to invade the salivary gland from which they are injected into a new host during the next blood-meal.

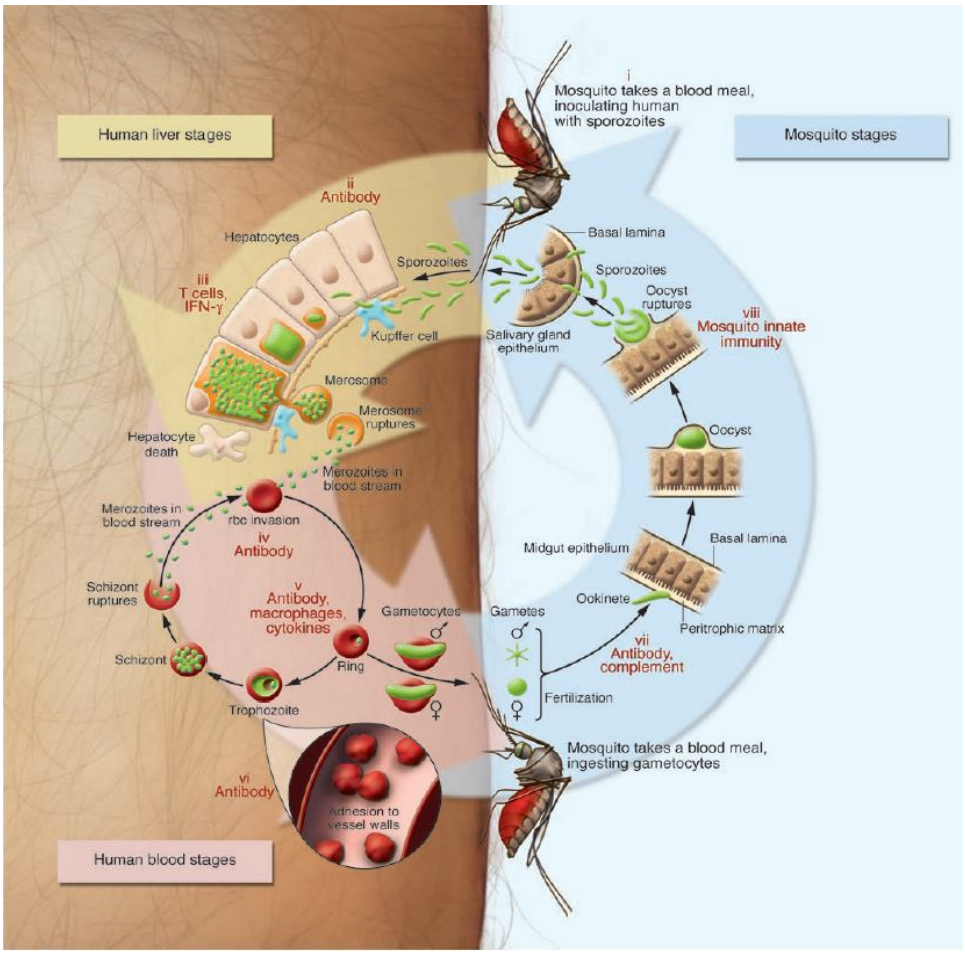

Fig 2.1: Life cycle of Plasmodium falciparum (Adapted from Greenwood et al., 2008)

2.3 Mosquito Salivary Glands and Saliva

Mosquitoes obtain carbohydrates from plant nectars and honeydew. Sugar meals provide energy for somatic functions, flight and reproduction (Holiday-Hanson *et al.*, 1997; Foster, 1995); whereas female mosquitoes are anautogenic meaning they require blood-meals for egg development (Clements, 1992). Salivary glands secretions lubricate mosquito mouthparts and contain a variety of compounds that help to obtain and digest sugars and blood. Both sexes have salivary activities related to lubrication such as mucin, sugar feeding such as maltase and antimicrobial activity such as lysozyme, which prevents microbial growth in the sugar meals stored in the insect crop (Marinotti *et al.*, 1990; Rossignol *et al.*, 1984; Rodriguez and Hernandez-Hernandez, 2004; Calvo *et al.*, 2006).

Additionally, cannulated feeding of blood from the vertebrate host by a female mosquito takes several minutes (Ribeiro *et al.*, 1984) and requires the repeated probing of host skin with its mouthparts until it locates and pierces a blood vessel, allowing the blood to be removed as though being drawn through a needle. The secretion of saliva by a mosquito during feeding is important for the successful location of host blood vessels and manipulation of host haemostatic and immune responses (Ribeiro, 1987; Ribeiro, 2000; Ribeiro *et al.*, 1984). The female mosquito introduces into the vertebrate host a cocktail of salivary proteins containing at least one vasodilatory, one antiplatelet, and one anticlotting agent/molecule (many of which are antigenic) although in some cases more than one of each is present (Calvo *et al.*, 2006; Francischetti *et al.*, 2002; Ribeiro, 2000).

2.3.1 The Salivary Gland Structure

Salivary glands of adult mosquitoes are located in the thorax where they occur as paired structures and are sexually dimorphic with the structural and functional differences between the male and female organs reflecting the ability of the latter to engage successfully in blood feeding (James, 1994; Stark and James, 1996). Each gland consists of three lobes that are attached to a common salivary duct. In culicine mosquitoes this duct extends the length of each lobe, whereas in anophelines, it extends only part-way along the lobe.

The three lobes of male salivary glands appear similar to one another and likely all three lobes have the same secretory capabilities (James, 1994); whereas female glands are larger than male glands and are differentiated into two lateral and one medial lobe. A salivary gland lobe comprises of a secretory epithelium surrounding a duct into which saliva is released. The cells in each lobe are organized into a single layer epithelium with characteristic basal and apical surfaces. The basal ends of the epithelial cells form the outside surface of the glands and are in contact with a basement membrane that provides the cohesiveness of the glands. Large secretory cells separated by a narrow region formed by non-secretory cells form the medial lobe. Two regions can be identified in the lateral lobes of female salivary glands, proximal and distal, separated by a narrow transitional region (Fig 2.2).

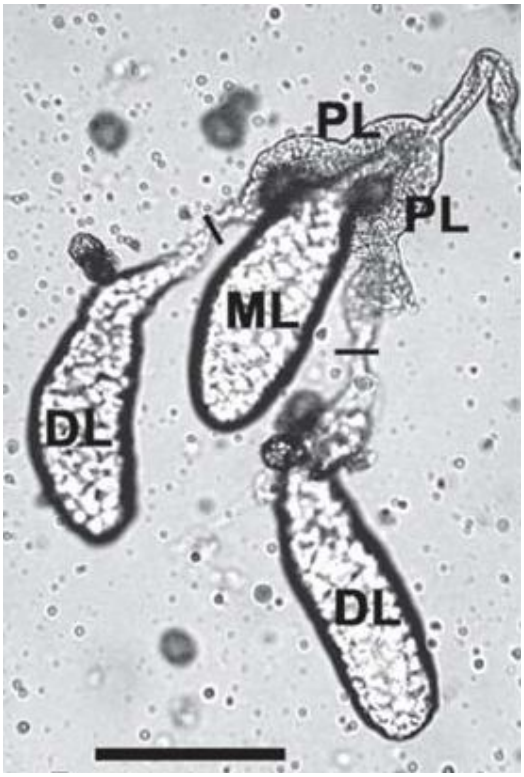

Fig 2.2: Representative salivary glands of anopheline female adult mosquito. (Adapted from Jariyapan et al., 2007).
Key-PL: proximal region of the lateral lobe; ML: median lobe; DL: distal region of the lateral lobe. Bar represents 500µm

The proximal regions of the lateral lobes in females express and secrete salivary gland products such as amylases and α1-4 glucosidases that are involved in sugar feeding, with these lobes appearing to overlap with the functions of male salivary glands (James, 1994; Arcà *et al.,* 1999; Lehane, 1991). Conversely, gene expression of the medial lobe and distal-lateral lobes is associated with products involved in hematophagy such as apyrases, anticoagulants and vasodilating agents (Beerntsen *et al.,* 1999; Arcà *et al.,* 1999; Smartt *et al.,* 1995; Champagne *et al.,* 1995; Stark and James, 1998).

Moreover, physiological variation of the surface properties of the different salivary gland lobes has also been demonstrated by differential binding of lectins and monoclonal antibodies raised to whole salivary glands in the female mosquito (Barreau *et al.,* 1999; Barreau *et al.,* 1995; Perrone *et al.,* 1986). These differences are especially important because sporozoites have previously been shown to preferentially invade the distal-lateral and medial lobes of the female glands, demonstrating the potential of transmission blocking vaccines (Beerntsen *et al.,* 2000; Golenda *et al.,* 1990; Sterling *et al.,* 1973; Rossignol *et al.,* 1984; Pimenta *et al.,*1994).

2.3.2 Saliva Components (Sialotranscriptomes) of Anopheline Mosquitoes

Mosquito saliva and salivary glands are central to the interaction between parasite, vector and mammalian host. Recent advances in proteomics and bioinformatics have enabled the creation and reporting of large databanks of proteosome (termed the 'sialome' from the Greek word *sialo,* saliva) and salivary gland gene expression (transcriptosome) that provides a useful platform of knowledge on the salivary gland components of anopheline mosquitoes and their putative roles (Arcà *et al.,* 2005; Calvo *et al.,* 2004; Calvo *et al.,* 2007; Calvo *et al.,* 2006; Calvo *et al.,* 2009; Francischetti *et al.,* 2002; Valenzuela *et al.,* 2003; Valenzuela *et al.,* 2002). The entire body of transcripts or expressed genes is assembled into a clusterized database that can be broadly be classified into four functional categories consisting of transposable elements (TE), housekeeping (H), secreted (S) and those genes of unknown function (U). Transposable elements are

now widely acknowledged as an important feature in the genomes of eukaryotes and their transcripts are a regular finding in most sialotranscriptomes.

The H category consists of several gene clusters further characterized into subgroups according to function including genes associated with protein synthesis machinery and energy metabolism being the most prevalent. Other abundant subgroups include genes encoding for conserved proteins including of unknown function presumably associated with cellular metabolism, proteins associated with signal transduction, protein modification, and protein export machineries. Remaining aspects of housekeeping with less frequent gene expression include those encoding for cytoskeletal proteins, proteasome machinery, transcription factors, carbohydrate metabolism, nuclear regulation, transcription machinery, oxidant metabolism and amino acid metabolism.

The (S) category of secreted proteins can be systematically reviewed in groupings based on their exclusivity and/or functions. By comparing elucidated gene and protein sequences with other known sialotranscriptomes and through the blasting other databases such as the United States of America's National Centre for Biotechnology Information (NCBI), it is possible to organize the (S) category using criteria such as all secreted proteins that are found ubiquitously, or exclusively in Diptera, exclusively in mosquitoes and exclusively in the Anophelines. The functions of a few of these secreted proteins are known but many remain unknown perhaps owing to the fact that previous studies have primarily focused on the antihemostatic role of saliva in the host during blood feeding and antimicrobial/defense properties, overshadowing other possible roles in the vector physiology.

Ubiquitously distributed proteins consist of the AG5 protein family and enzymes that are found in the salivary glands of all mosquitoes. The AG5 family is found in the salivary glands of many hematophagous insects and ticks (Francischetti *et al.,* 2002; Valenzuela *et al.,* 2002; Li *et al.,* 2001). This family belongs to the cysteine-rich secretory proteins (CAP family; AG5 proteins of insects; pathogenesis-related protein 1 of plants) (Megraw *et al.,* 1998). Four (4) proteins of this family have been identified in the *An. gambiae* sialotranscriptome, of which one is richly expressed in the adult female salivary glands (Arcà *et al.,* 2005). The function of this protein family nevertheless remains unknown.

Ubiquitous enzymes include maltase, amylase and α1-4 glucosidases that are involved in carbohydrate catabolism, predicted serine proteases thought to be involved in host specific proteolytic events that prevent clotting or the complement cascade, lysozyme which has antimicrobial activity and prevents microbial growth in the insect crop, and the 5′ nucleotidase that enable the degradation of purinergic mediators of platelet aggregation and inflammation respectively (Arcà *et al.,* 1999; Calvo *et al.,* 2007; Rodriguez and Hernandez-Hernandez, 2004; Marinotti *et al.,* 1990). The 5′ nucleotidase family is composed of apyrase and 5′ nucleotidase, both of which prevent the aggregation of platelets during blood feeding (Champagne *et al.,* 1995; Valenzuela *et al.,* 2003; Valenzuela *et al.,* 2002; Ribeiro and Valenzuela, 2003). Apyrase can hydrolyse adenosine diphosphate (ADP) and adenosine triphosphate (ATP) that are important for the platelet-mediated clotting of ruptured blood vessels (Ribeiro *et al.,* 1984). Additionally, apyrase has been shown to be of particular importance in the probing and location of blood vessels behavior of *Ae.*

Aegypti and *An. gambiae* (Boisson *et al.,* 2006; Champagne *et al.,* 1995; Ribeiro, 2000).

In *Aedes aegypti*, the main antiplatelet activity is because of apyrase (Champagne *et al.,* 1995; Ribeiro, 2000), whereas *Anopheles* mosquitoes primarily depend on the use of a potent vasodilatory salivary peroxidase and anticlotting molecule, antithrombin (Champagne and Valenzuela, 1996; Valenzuela *et al.,* 1999; Ribeiro, 2000; Waidhet-Kouadio *et al.,* 1998). Other identified antihaemostatic salivary proteins include an antifactor Xa-directed protein from the salivary glands of *Ae. Aegypti* (Stark and James, 1998), platelet-activating factor that hydrolyses phospholipase C required for platelet aggregation from the salivary glands of *C. quinquefasciatus* (Ribeiro and Francischetti, 2001) and adenosine deaminase and nucleosidase that have vasodilatory and antiplatelet abilities from the salivary glands of *Ae. Aegypti* and *C. quinquefasciatus* (Ribeiro and Valenzuela, 2003; Ribeiro *et al.,* 2001).

Among the (S) proteins exclusively found in Diptera is the D7 protein family which is the most abundant secreted protein in the salivary glands of mosquitoes (Valenzuela *et al.,* 2002). It belongs to the superfamily of the odorant-binding proteins (Hekmat-scafe *et al.,* 2000) and is found in the salivary glands of blood-feeding Nematocera such as mosquitoes, sand flies and the Culicoides (Arca *et al.,* 2002; Campbell *et al.,* 2005; Valenzuela *et al.,* 2002). Two forms of D7, short (approx. 17kDa) and long (approx. 30kDa) are recognized, with the short forms only occurring in mosquitoes (Arca *et al.,* 2002; Calvo *et al.,* 2002; Valenzuela *et al.,* 2003; Malafronte *et al.,* 2003). Both forms consist of highly divergent polypeptides and they are therefore thought to have evolved diverse biochemical properties and

binding affinities (Arca *et al.,* 2002; Valenzuela *et al.,* 2002). Some of them have been associated with binding of biogenic amines such as serotonin, histamine and norepinephrine that may contribute to vasodilation, inhibition of platelet aggregation and suppressing the inflammatory effects of histamine (Arca *et al.,* 2002). Additionally, hamadarin (a short D7 protein from *An. stephensi*) has been shown to prevent kallikrein activation by coagulation Factor XIIa (Isawa *et al.,* 2002). Five short and three long D7 proteins are secreted in *An. gambiae* salivary glands (Lanfrancotti *et al.,* 2002; Arca *et al.,* 2002; Calvo *et al.,* 2007). The function of these proteins however remains unknown or merely speculative including an anticoagulant, a proteolytic enzyme, and the others probably involved in blood feeding (Lanfrancotti *et al.,* 2002).

Another abundant secretion is the 30kDa glycoprotein family that is found exclusively in the transcriptomes of mosquitoes (Calvo *et al.,* 2007; Simons and Peng, 2001; Valenzuela *et al.,* 2003). Only one gene is known in *An. gambiae* and it has enriched expression in the salivary glands of adult females. The function of this family has not been elucidated, but the location of its secretion, abundance and sex specificity suggest that it could be involved in blood feeding. Another group of abundant proteins that are found exclusively in mosquitoes is the mucins and their function is primarily lubrication (Calvo *et al.,* 2007).

Lastly several secreted proteins are thought to be unique to anopheline mosquitoes including the gSG1 family, gSG2 family, gSG6 peptide, gSG7 family, cE5/Anophelin family, 8.2kDa family and the 6.2kDa family. In the *An. gambiae*, six genes of the gSG1 family are known whose mature molecular weight is approx. 41kDa. Their transcripts are found uniquely or enriched in the salivary glands of

adult females, suggestive of a function in blood feeding (Arcà *et al.,* 2005). The gSG2 family in *An. gambiae* consists of the glycine- and proline-rich SG2 and SG2a proteins that are enriched in female salivary glands and are thought to assist in sugar feeding possibly as an antimicrobial (Otvos, 2000). The gSG6 peptide (approx. 10kDa) is richly expressed in the female salivary glands of *An. gambiae* and is therefore speculated to have a blood-feeding function; likewise with the gSG7 family, 8.2kDa family and the 6.2kDa family (Arcà *et al.,* 2005). cE5/Anophelin family plays a key role in *Anopheles* mosquitoes primarily as a potent vasodilatory salivary peroxidase and anticlotting molecule with antithrombin activity (Champagne and Valenzuela, 1996; Valenzuela *et al.,* 1999; Ribeiro, 2000; Waidhet-Kouadio *et al.,* 1998). Anophelin is also an inhibitor of serine proteases (Valenzuela *et al.,* 2003).

Additionally, several postulated families of peptides speculated to have antimicrobial and/or blood-feeding function exist. These include hypothetical family 13, 15/17, and 10/12 whose functions are thought to be housekeeping or antimicrobial, blood-feeding and antimicrobial respectively (Arcà *et al.,* 2005; Calvo *et al.,* 2007; Francischetti *et al.,* 2002; Valenzuela *et al.,* 2003).

2.3.3 Saliva - Plasmodium Interactions in the Mosquito Midgut

During feeding, it has been demonstrated that mosquitoes continuously secrete saliva in pulses (Clements, 1992) and some of this saliva is ingested with the blood into the midgut (Luo *et al.,* 2000). The effect of this saliva in the mosquito midgut remains poorly understood.

Mosquito salivary gland excretions contain many proteins of diverse functions including antihemostasis effect during blood-feeding, catabolic enzymes, protease inhibitors and antimicrobial peptides that may interact with the midgut and its processes (Arcà *et al.,* 2005; Calvo *et al.,* 2004; Calvo *et al.,* 2007; Calvo *et al.,* 2006; Francischetti *et al.,* 2002; Valenzuela *et al.,* 2003). Whether all these factors affect the development of the malaria parasite is not yet fully understood. The salivary glands of female *An. stephensi* have been shown to express xanthurenic acid that has exflagellation inducing activity on the male gametocyte (Hirai *et al.,* 2001; Billker *et al.,* 1998). Additionally, mosquito salivary glands have been demonstrated to contain molecules that are potent inhibitors of bacteria and fungi (Francischetti *et al.,* 2002; Rossignol and Lueders, 1986). The presence of bacteria has been shown to up-regulate the mosquito's innate immune system resulting in increased refractoriness to *Plasmodium* development in the gut (Dong *et al.,* 2009). However it remains to be demonstrated whether the antimicrobial properties reported in saliva are active in the midgut, thus aiding parasite development in the midgut. Similarly, the cE5/Anophelin protein family's protease inhibitor properties (Valenzuela *et al.,* 2003) may also have an effect on the blood-meal digestion in the midgut, possibly affecting *Plasmodium* development. Moreover sialotranscriptomes of female mosquitoes reveal the presence of other molecular weight molecules that are secreted from the salivary glands and whose role in the mosquito or on parasite development remains unknown.

2.4 The Mosquito Midgut

The midgut is a remarkable interface where the interaction between the mosquito, malaria parasite, bacteria and salivary gland excretions takes place. With the recent sequencing of the *An. gambiae* genome, several studies identifying differential gene expression between blood fed versus sugar fed mosquitoes have been undertaken (Dana *et al.,* 2005; Ribeiro, 2003; Holt *et al.,* 2002). These studies have also been expanded to include the differential gene expression of mosquitoes infected with *Plasmodium* and the gene expression of the parasite development in the mosquito (Dana *et al.,* 2005; Bonnet *et al.,* 2001; Blandin *et al.,* 2009). These studies have shed new light on the digestion of the proteinaceous blood meal and coordinated processes associated with *Plasmodium* development, the mosquito's immune responses, oocyte development and vitellogenesis.

The midgut is therefore a central part of the alimental canal playing a key role in the digestion and absorption of nutrients. Nectar, the main food source of males and females, is stored in the crop and digested and absorbed at the anterior midgut (AMG), whereas blood which is imbibed by anautogenous females passes to the posterior midgut (PMG) for digestion and absorption (Billingsley, 1990; Terra and Ferreira, 1994). The midgut structure consists of a single-cell layer epithelium with a basal laminae on the outside and microvilli on the inside. The dramatic distension of the midgut by blood feeding induces the epithelium cells to secrete an anatomical structure, the peritrophic membrane or peritrophic matrix (PM) that is continous along the length of the midgut (Lemos *et al.,* 1996; Tellam *et al.,* 1999; Dinglasan *et al.,* 2009; Freyvogel and Jaquet, 1965).

The PM surrounds the food bolus and is made up of a matrix of proteins (peritrophins) and chitin to which other components such as food molecules and enzymes associate. It is thought the PM functions as a restrictive layer protecting the midgut epithelium from proteolytic digestive enzymes and hematin crystals that form following the breakdown of hemoglobin. Additionally, the PM may also protect the mosquito from blood-borne pathogens such as bacteria and malaria parasites (Peters, 1992). In *An. gambiae* the PM can be visualized as early as 12 hours post blood feeding (PBF) by electron microscopy and is fully formed by 48 hours PBF (Berner *et al.*, 1983; Freyvogel and Jaquet, 1965). In between the PM and midgut epithelium is the ectoperitrophic space where enzymes involved in intermediate digestion are found free in the ectoperitrophic fluid, whereas enzymes of terminal digestion are membrane bound at the midgut cell microvilli (Fig 2.2).

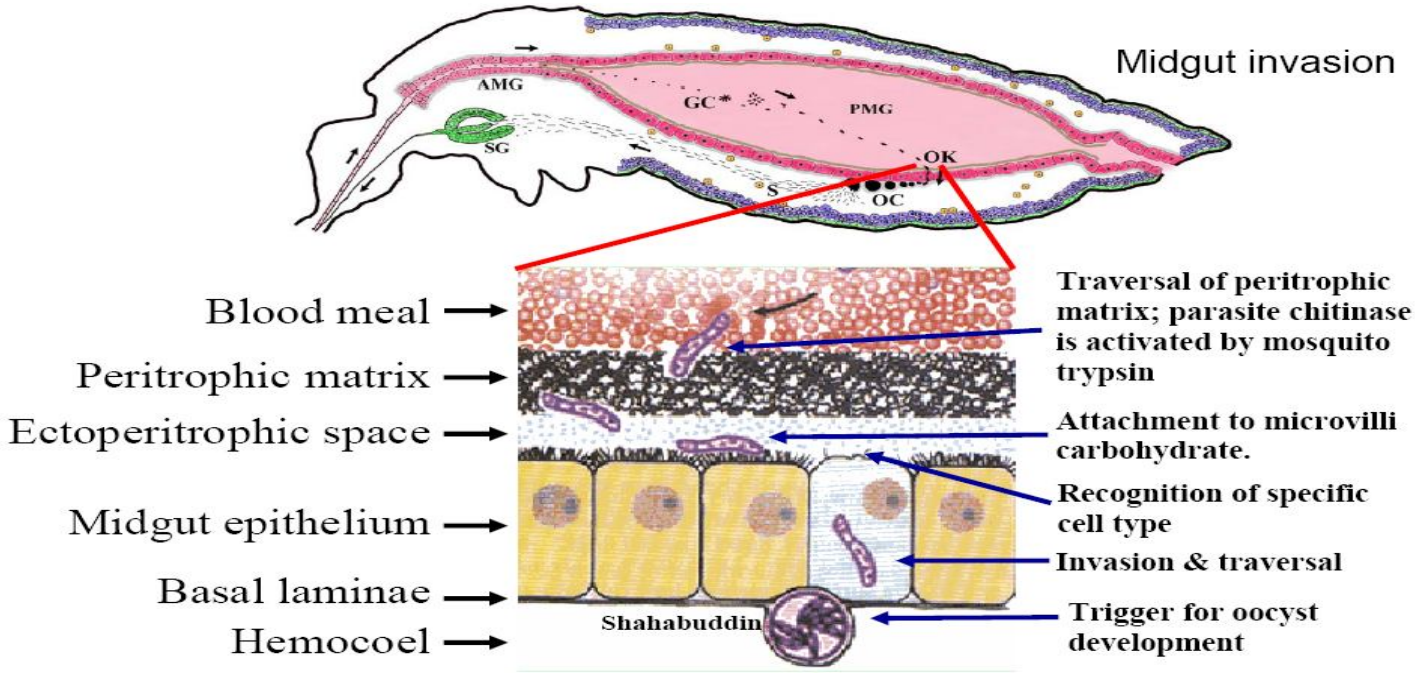

Fig 2.3: Diagrammatic representation of mosquito midgut and its interaction with Plasmodium (Adapted from Dimopolous group: www.dimopoulosgroup.org/research.html)

Key: AMG – Anterior midgut; SG – Salivary gland; GC – Gametocytes; PMG – Posterior midgut; OK – Ookinete; OC – Oocyst; S – Sporozoite.

2.4.1 Blood-Meal Digestion in the Mosquito Midgut

The acquisition of a blood-meal stimulates midgut proteolytic activity whereby 80% of the protein content is digested within 24 hours (Lemos *et al.*, 1996; Jahan *et al.*, 1999; Billingsley and Hecker, 1991). The blood-meal is sequentially digested by hydrolytic enzymes in three phases. Initial digestion involves dispersion of the food bolus and reduction in molecular size of the substrate polymers into oligomers. This occurs inside the peritrophic membrane. Secondary or intermediary digestion then ensues in the ectoperitrophic space where the oligomers are further reduced in molecular size to dimers. Lastly, the dimers are finally broken down to monomers at the surface of the midgut cells by integral microvillar enzymes or enzymes trapped in the glycocalyx (Terra and Ferreira, 2005).

In addition, microorganisms residing in the gut such as bacteria, fungi or protozoa that may be symbiotic, fortuitous contaminants from the external environment, or otherwise, are also thought to account for some of the hydrolase activity. However, few studies have clearly demonstrated their role in nutrition and digestion (Douglas and Beard, 1996; Campbell, 1990; Tanada and Kaya, 1993). For example, some wood and humus feeding insects have been shown to depend on fungi and certain filamentous bacteria for the digestion of lignin, a phenolic polymer associated with plant cell wall (Dillon and Dillon, 2004). *Rhodnius prolixus*, the Chagas disease vector, harbors a mutualistic actinomycete *Rhodococcus rhodnii* in its hindgut, where the bacterium is thought to be involved in the sequestration of B complex vitamins utilized by the host in blood-meal digestion (Beard *et al.*, 2002). In the case of a *Plasmodium* infective blood-meal, parasite derived chitinase is

activated by mosquito secreted trypsin, facilitating ookinete parasite traversal of the PM and commencement of secondary digestion (Shahabuddin *et al.,* 1993).

2.4.1.1 Nomenclature and Classification of Digestive Enzymes

Digestive enzymes are called hydrolases. Blood-meal digestion is carried out by peptidases, the enzymes that act on peptide bonds. The peptidases are classified into two main groups: the proteinases and the exopeptidases. The Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (Enzyme Commission) has classified and numbered the peptidases thus (peptide hydrolases, EC 3.4), proteinases (endopeptidases, EC 3.4.21-24) and exopeptidases (EC 3.2.4.11-19).

Endopeptides are further divided into subclasses based on their catalytic mechanism as shown with specific reagents or effect of pH, with specifity being used only to identify individual enzymes within subclasses. The subclasses consist of Serine proteinases (EC 3.4.21) that possess a serine and a histidine in the active site; Cysteine proteinases (EC 3.4.22) that both possess a cysteine in the active site and are inhibited by mercurial compounds; Aspartic proteinases (EC 3.4.23) whose optimum pH is below 5, owing to involvement of a carboxyl residue in catalysis; and lastly the Metalloproteinases (EC 2.3.24) that require a metal ion in the catalytic process.

The group exopeptidase includes enzymes that hydrolyze single amino acids from the N-terminus (aminopeptidases, EC 3.4.11) or from the C-terminus

(carboxypeptidases, EC 3.4.16-18) of the peptide chain and also enzymes specific for dipeptides (dipeptide hydrolases, EC 3.4.13).

2.4.1.2 Trypsin, Chymotrypsin and Aminopeptidase in An. gambiae Blood-Meal Digestion and Plasmodium Development

Trypsin, chymotrypsin and aminopeptidase are important proteases, with trypsin and chymotrypsin both classified as endopeptidase, subclass serine proteases and aminopeptidase in class exopeptidase, subclass metalloproteinase. Trypsin and chymotrypsin are the main digestive proteases in Diptera midguts and are involved in many aspects of the vector-parasite relationship (Horler and Briegel, 1997; Briegel, 1975; Ramalho-Ortigão *et al.,* 2003; Billingsley and Hecker, 1991).

Eight genes of the *An. gambiae* trypsin family located on chromosome 3R and encoding six functional proteins have so far been characterized (Dana *et al.,* 2005). These consist of Trypsins 1, 2, 3, 4 and 7 of which 1 and 2 are induced by a blood meal peaking at 24 hr post blood feeding (PBF) albeit with Trypsin 1 expressed at higher levels (Muller *et al.,* 1995). This coincides with the height of midgut invasion by *P. falciparum* ookinetes in *An. gambiae*. Conversely, Trypsins 3, 4 and 7 are constitutively expressed prior to blood meal (Chege *et al.,* 1996; Muller *et al.*, 1995; Billingsley and Hecker, 1991). Trypsin 4 is down-regulated following a blood meal to undetectable levels by 4 hr PBF using Northern and RT-PCR techniques (Muller *et al.,* 1995). It then remains undetected until 20 hr PBF reaching its peak at 48 hr PBF towards the end of the gonotrophic cycle. Similarly, Trypsins 3 and 7 are down-regulated to undetectable until 28 hr PBF (Muller *et al.,* 1995).

Lastly, Dana *et al.,* (2005) reported a trypsin-like serine protease featuring high amino acid similarity with Trypsin 4 but whose location on chromosome 3R did not fall within division 30A. Up-regulation of this protease was induced to greater than twofold within 6 hr PBF but repressed during the peak of digestion. However, unlike the constitutive Trypsins 3, 4 and 7, the expression profile differs in that it was not highly expressed at 48 hrs PBF (Dana *et al.,* 2005). An elaborate bi-phasic expression patterns of serine proteases therefore emerges whereby the constitutive Trypsins 3-7 are active prior to and during blood feeding but are down-regulated by the blood meal which induces expression of the late Trypsins 1 and 2.

The *An. gambiae* chymotrypsin family made up of three genes located on chromosome 2L and encoding three functional proteins AnChym 1, AnChym 2, AgChyL has previously been characterized (Vizioli *et al.,* 2001; Shen *et al.,* 2000). AnChym 1 and 2 are blood meal induced by 12 hr PBF but unlike Trypsins 1 and 2 that decline sharply from 24 hr remain abundant until 48 hr PBF (Vizioli *et al.,* 2001). Conversely, AgChyL is constitutive with the expression profile corresponding to that of Trypsins 3-7 (Shen *et al.,* 2000). A fourth *An. gambiae* chymotrypsin reported as "AS 2243" also located on chromosome 2L was characterized by Dana *et al.,* (2005). The expression profile of the gene product was shown to be similar to that of Trypsins 1 and 2, peaking between 12 – 24 hr PBF and declining sharply by 48 hr PBF.

In addition Dana *et al.,* (2005) identified two *An. gambiae* genes of aminopeptidase proteins labeled "AS 340" and "AS 430" respectively. Gene expression of the former peaked at 24 hr PBF, concurring with previous reports of

aminopeptidase activity in *An. gambiae* (Lemos *et al.*, 1996; Billingsley and Hecker, 1991), whereas that of the later peaked at 48 hr PBF. Interestingly, similar aminopeptidase expression profiles were reported in *An. stephensi* whereby two divergent peaks were observed based on whether the enzyme was soluble or membrane-associated with the soluble aminopeptidase's profile concurring with that of AS 340 (Jahan *et al.*, 1999).

Early trypsin activity has been found to be essential as signal transducers for the transcription and subsequent expression of late trypsins in *Ae. aegypti* (Barillas-Mury *et al.*, 1995). Additionally, *Ae. aegypti* produces a proline-rich decapeptide known as the trypsin-modulating oostatic factor (TMOF) that inhibits egg development as well as biosynthesis of trypsin and chymotrypsin-like enzymes by binding to a specific gut epithelial cell receptor and stopping biosynthesis (Borovsky, 2003). TMOF is therefore likely involved in the regulation of serine proteases and gonotrophic cycle as indicated by its expression profile whereby it's induced 18 hr PBF in the *Ae. aegypti*, peaking at 33 hr and rapidly declining to a minimum at 48 h after the blood meal (Borovsky *et al.*, 1994).

Bonnet *et al.*, (2001) reported that trypsin expression was down-regulated at 18 hr PBF by the presence of gametocytes in the blood. *Plasmodium gallinaceum* ookinetes have previously been shown to be sensitive to trypsin *in vitro* (Gass and Yeates, 1979); suggesting down-regulation of trypsin may be a parasite defense mechanism to evade degradation by trypsin during ookinete invasion of the midgut which for *P. falciparum* peaks at 24 hr PBF in *An. gambiae* (Bonnet *et al.*, 2001). Conversely, trypsins have also been shown to be necessary for the activation of

ookinete-secreted chitinase that enables the parasite's penetration of PM, the midgut's defensive barrier (Huber *et al.,* 1991; Shahabuddin and Kaslow, 1994).

2.5 Bacteria in the Anopheles Midgut

The complex nature of gut microbe interactions has in the past led to the overlooking of the impact of gut microbiota on the biology of the host (Dillon and Dillon, 2004). For example, the consortium of microbes inhabiting the human gut (10^{14}) has been estimated to outnumber the somatic and germ cells of the body (10^{13}) (Savage, 1977). The metabolic activity of this consortium has been equated to that of the human liver (Berg, 1996). The development of molecular techniques such as microarrays technology is now equipping scientists with the tools to shed new light on these complex systems that range from pathogenic to obligate mutualism. This new perspectives stand to revolutionize how we perceive and understand the biology of any higher order species with a new argument advanced that expands the comprehensive genetic view of an organism beyond its genome to include the microbiome, a term coined to represent the genomes of indigenous microbiota (Lederberg and McCray, 2001). It has been estimated that the combined genomes of human microbiota may contain 50-100 times more genes than the human genome (Hooper and Gordon, 2001).

Similarly, insect species are inhabited by large and diverse communities of organisms residing primarily in the gut that likely outnumber their own cells (Dillon and Dillon, 2004), the study of which can provide a simpler model for understanding the human microbiome and integrated physiology. Additionally, the study of mosquito microbiota is experiencing a revival in the wake of the development of

paratransgenic mosquitoes for the mitigation of malaria transmission that seeks to employ naturally occuring bacterial symbionts of the midgut to block *Plasmodium* development. A symbiotic relationship has been defined to mean the acquisition and maintenance of the microorganism by the insect, resulting in novel structures or metabolism (Zook, 1998). Other terms like mutualism, commensalisms or pathogen, are applied to further define the nature of the relationship in terms of beneficial or harmful relationships.

Bacteria in the midgut can be found in the lumen, adhering to the peritrophic membrane, attached to the midgut surface, or within cells. In insects, intracellular bacteria are usually found in special cells known as mycetocytes that may be organized in groups referred to as mycetomes (Terra and Ferreira, 2005).

2.5.1 Bacteria in Larvae Midguts

Bacteria are today acknowledged to be an important if not essential part of the mosquito larvae's diet (Lindh, 2007). Some of the earliest studies on the role of bacteria demonstrated that it is not possible to rear *Aedes aegypti* Linnaeus larvae in sterile media (Rozeboom, 1935). However, what larvae eat remained a fundamental question in the study of larval mosquito ecology due to the lack of adequate methods for examining gut contents. Walker *et al.,* (1988) were the first to definitely analyze larval gut content and to demonstrate bacteria, algae, protozoans and organic detritus in fourth instar *Aedes triseriatus* (Say), *An. quadrimaculatus* (Theobald) and *Coquillettidia pertubans* (Walker). More importantly, bacteria were found to make up the bulk of the living particles in each of the three species (Walker *et al.,* 1988). In addition the treatment of rearing water with antibiotics has been reported to result

in significantly smaller *An. gambiae* larvae than in untreated water (Wotton *et al.*, 1997); and high mortality of vast majority of the larvae before pupation (Touré *et al.*, 2000).

2.5.2 Bacteria in Adult Midguts

The presence of bacteria in the adult midgut has been well documented in field caught and laboratory reared mosquitoes, where the γ-proteobacteria have been the most frequently identified, the majority of these belonging to the family Enterobacteriacea (summarized in Table 2.1). Significantly, bacteria species of the γ-proteobacteria and Enterobacteriacea have frequently been found and classified as symbionts in insects (Zientz *et al.*, 2001; Wernegreen, 2002; Pontes and Dale, 2006). Initially, the presence of bacteria in the midgut was understood to be unstable residents due to the low prevalence of bacteria (Prevalence = percentage of mosquitoes with bacteria in the midgut out of the total number of mosquitoes investigated). However, the majority of bacteria in nature do not grow in standard culturing media (Rappe and Giovannoni, 2003; Amann *et al.*, 1995). This has been thought to account for the wide ranging variation of bacteria prevalence reported in the literature that have depended mainly on traditional culture-based techniques (Pumpuni *et al.*, 1996; Pumpuni *et al.*, 1993; Lindh *et al.*, 2005; Gonzalez-Ceron *et al.*, 2003; Straif *et al.*, 1998).

The pupa stage, during which the mosquito undergoes complete metamorphosis, is a non-feeding stage with a notable lack of mouthparts. Two previous studies have suggested transstadial transfer of bacteria from larvae to adults in *Anopheles* mosquitoes: early studies whereby *Pseudomonas sp.* were fed to *An.*

quadrimaculatus larvae and the same species later isolated from adult guts (Jadin *et al.,* 1966), and later studies by Pumpuni *et al.,* (1996) who fed *Escherichia coli* HS5 to *An. gambiae* larvae and similarly recovered the same from one adult. However, these results were challenged by an effective gut sterilization mechanism described recently that occurs during the pupa stage and adult emergence, suggesting the improbability of such a transstadial transfer of bacteria from larvae to adult mosquito (Moll *et al.,* 2001).

The employment of modern molecular techniques has since established that the gut sterilization is not complete during the larva-adult transition as evidenced by a growing body of work, and that certain bacteria are retained in the gut of *Anopheles* mosquitoes (Rani *et al.,* 2009; Briones *et al.,* 2008; Favia *et al.,* 2007). Favia *et al.,* (2007) reported the presence of the α-proteobacteria genus *Asaia* to be stably associated with *An. stephensi* and demonstrated the presence of *Asaia* DNA in egg, larvae, pupae and adult stages in laboratory reared mosquitoes. Briones *et al.,* (2008) documented the γ-proteobacteria *Thorsellia anophelis* as the dominant bacterium in the midgut of adult *An. gambiae sensu lato* occurring in the Central Province, Mwea irrigation scheme. This species was also detected in the surface microlayer of the rice paddies indicating that aquatically derived bacteria such as *T. anophelis* can be transstadially transmitted, becoming established in the adult mosquito midgut.

Table 2.1: Bacteria species isolated and/or identified in studies of Anopheline midguts (Adapted from Lindh 2007)

Mosquito species	**Bacteria species**			**Field/Lab** [a]	**Prev** [b]	**Ref**
	Other than γ-Proteobacteria	-Belonging to phylum γ-Proteobacteria	-And family Enterobacteriaceae			
Anopheles stephensi (Liston)	*Staphylococcus spp.*		*Ewingella americana, Serratia marcescens*	Lab	Pools	Pumpuni *et al*., 1993
Anopheles albimanus (Wiedemann)	*Flavobacterium spp*	*Acinetobacter spp., Pseudomonas cepacia*	*Pantoea agglomerans*[c], *Serratia spp.*	Lab	17%	Pumpuni *et al*., 1996
Anopheles gambiae (Giles)	*Flavobacterium*	*Ps. cepacia, Pseudomonas gladioli*	*Aeromonas hydrohila, Cedecea lapaget, Klyvera cryocrescens, P. agglomerans, Serratia spp.*	Lab	73%	Pumpuni *et al*., 1996
An. stephansi	*Flavobacterium*	*Ps. Cepacia*	*A. hydrophila, C. lapagei, P. agglomerans*	Lab	90%	Pumpuni *et al*., 1996
An. gambiae	*Achromobacter xylosoiydans*[e], *Bacillus cereus, Bacillus coagulans, Bacillus mucoides, Bacillus thuringiensis, Hydrogenophaga pseudoflava*	*Psedomonas putida, Pseudomonas stutzeri*	*Cedecea davisae, Escherichia coli, Klebsiella pneumoniae, Morganella morgani, Pantoea ananas, P. agglomerans, Salmonella cholerasuis, Salmonella enteritidis*	Field	14.4/17.9% [g]	Straif *et al*., 1998
Anopheles funestus	*Bacillus megaterium, Brevundiumonas diminuta, Comamonas testeroni*[h],	*Pseudomonas mendocina, Ps. stutzeri, Stenotrophomonas*	*C. davisae, E. coli, Erwinia chrysanthenum,K. pneumoniae, Klyvera cryosceens, Pantoea agglomerans, P. ananas, S. cholerasuis*	Field	28.5/21.2% [g]	Straif *et al*., 1998

	Flavobacterium resinovorum, Gluconobacter cerinus	*maltophilia*[f]				
An. albimanus			*Enterobacter amnigenus, Enterobacter cloacae*[d]*, S. marcescens, Enterobacter spp., Serratia spp.*	Field	60%	Gonzalez-Ceron *et al.*, 2003
An. gambiae	*Anaplasma ovis, Rhodococcus corynebacteriodes Acidovorax spp, Anaplasma spp, Bacillus spp., Bacillales spp., Mycoplasma spp., Paenibacillus spp.,*	*Thorsellia anophelis,* *St. maltophilia, Vibrio metschnikovii Pseudomonas spp.,*	*Aeromonas spp., Enterobacteriacea spp.*	Field	16%	Lindh *et al.*, 2005
An. funestus	*Janibacter anophelis, Spiroplasma spp.*			Field	8%	Lindh *et al.*, 2005
An. stephensi	*Asaia bogorensis, Asaia siamensis, Gluconobacter asaii, Acetobacter aceti, Sphingomonas rhizogenes*			Lab	100%	Favia *et al.*, 2007
Anopheles maculipennis	*Asaia spp., Staphylococcus spp.*		*Serratia spp.*	Field	100%	Favia *et al.*, 2007
An. gambiae	*Asaia spp., Sphingomonas spp., Phenilobacterium spp., Burkolderia spp., Aquabacterium*	*Acinetobacter spp.,* *Pseudomonas spp.*		Field	100%	Favia *et al.*, 2007

	spp.,					
An. gambiae		*T. anopheles*		Field	Pools	Briones *et al.*, 2008
An. stephensi	*Agrobacterium tumefaciens, Chryseobacterium meningosepticum, Elizabethkingia meningosepticum, Comamonas spp.*	*Pseudomonas mendocina*	*Serratia marcescens, Klebsiella spp.,*	Lab	Pools	Rani *et al.*, 2009
An. stephensi	*Staphylococcus hominis, Staphylococcus saprophyticus, Paenibacillus alginolyticus, Paenibacillus chondroitinus, Achromobacter xylosoxidans, Chrsyseobacterium indologenes, Micrococcus spp., Herbaspirillum spp., Bacillus spp.,* Flexibacteriaceae	*Acinetobacter lwofii, Acinetobacter hemolyticus, Acinetobacter radioresistens, Acinetobacter johnsonii, Citrobacter freundii, Pseudomonas putida, Pseudomonas synxantha, Xenorhabdus nematodiphila, Leminorella grimontii*	*Enterobacter cloacae, Enterobacter sakazaki, Escherichia hermani, Serratia marcescens, Serratia nematodiphila, Serratia proteamaculans,*	Field	Pools	Rani et al 2009

[a]Field = field caught mosquitoes, Lab = insectary reared mosquitoes. [b]Prevalence = The percentage of mosquitoes with bacteria in the midgut out of the total number investigated. [c]Synonym: (among others) *Enterobacter agglomerans* (Gavini *et al.,* 1989). [d]Synonym: (among others) *Aerobacter cloacae*. [e]Synonym: (among others) *Alcaligenes xylosoxydans* (Yabuuchi *et al.,* 1998). [f]Synonym: (among others) *Xanthomonas maltophila* (Palleroni and Bradbury 1993), *Stenotrophomonas africana* (Coenye *et al.,* 2004). [g]For Gram-negative/Gram-positive bacteria respectively. [h]Synonym: (among others) *Pseudomonas testosterone*.

2.5.3 Physiology of Bacteria – Midgut Interactions

Monitoring individual 5 day old *Anopheles gambiae* for five consecutive generations, Dong *et al.,* (2009) determined the average bacteria load of sugar fed mosquitoes to be 10^4 CFU per midgut, which increased to 10^6 CFU per midgut in blood fed mosquitoes. Their results also demonstrated a great variation in both the bacterial loads and species composition, confirming earlier reports (Pumpuni *et al.,* 1996; Favia *et al.,* 2007; Demaio *et al.,* 1996; Lindh *et al.,* 2005; Straif *et al.,* 1998). This fluidity should therefore be carefully considered in defining the biological relationship with the mosquito of an individual bacterial species, together with the resource conditions in which the bacteria is examined (Klepzig *et al.,* 2001).

Whereas the relationship between an individual bacteria species and the anopheline mosquito may be dependent on time frame and resource conditions, the biological relationship of the collective microbiota is much clearer. Bacteria have been demonstrated to contribute to larval nutrition and development in anopheline mosquitoes, and increased parasite attrition and mosquito survival rates in the *Plasmodium* infected adults (Okech *et al.,* 2007; Walker *et al.,* 1988; Wotton *et al.,* 1997; Dong *et al.,* 2009). Thus the mosquito benefits immensely even as it hosts a fluctuating albeit significant microbiome and the biological relationship can be deemed to be predominantly mutualistic.

2.5.4 Bacteria – Plasmodium Interactions in the Midgut

Midgut microbiota interactions with the malaria parasite encompass various scenarios affecting the different parasite stages within the bloodmeal. Bacteria may interact directly by the production of various enzymes and toxins or as a physical

barrier that hinders *Plasmodium* ookinetes-midgut epithelium interaction (Azambuja *et al.,* 2005). Alternately, indirect effect on parasite development may be achieved through altering the physiology of the mosquito itself such as changes of host metabolism that would affect the composition of essential mosquito derived molecules, and/or induction of immune responses that are cross-reactive to both bacteria and *Plasmodium* (Dong *et al.,* 2009). *Wolbachia pipientis* strain *w*MelPop has recently been shown to reduce mosquito longevity and inhibit the development of filarial nematodes in *Ae. aegypti* by up-regulation of the innate immune system, giving proof of concept that suitable bacteria – mosquito interactions can be developed as a tool for the control of mosquito-borne parasitic diseases (McMeniman *et al.,* 2009; Kambris *et al.,* 2009).

Comparisons of parasite loads in septic and aseptic mosquito midguts for the *An. gambiae* - *P. falciparum* combination have indicated that bacteria have no effect on pre-invasive ookinete stages, but rather at the point of invasion where a 2.5 fold increase in the aseptic mosquitoes was noted (Dong *et al.,* 2009). Microarray-based genome wide gene expression of the mosquito was used to determine gene up-regulation and down-regulation by the presence of microbial flora during ookinete invasion of the midgut epithelium. 121 genes were found to be up-regulated and 64 down-regulated compared to antibiotic treated aseptic mosquitoes.

CHAPTER THREE

3.0 MATERIALS AND METHODOLOGY

3.1 Mosquito Colony

Anopheles gambiae mosquitoes (MBITA strain) originally collected from water bodies at Mbita Point, Suba District, western Kenya (Seynoum *et al.*, 2002) were obtained from the International Centre for Insect Physiology and Ecology (*icipe*), Duduville campus in Nairobi. These were maintained in the insectary at CBRD-KEMRI, Nairobi. Mosquito rearing procedures followed standard protocols in use at CBRD-KEMRI. Briefly, adult mosquito colony were maintained at 27±2ºC and 70±10% RH at a 12:12 L:D photoperiod. Adults were kept in cages made of plastic with netting on top or metal frames covered with netting. They were then maintained on 10% glucose solution *ad libitum.* Additionally, adult mosquitoes were blood fed on a hamster once a week. Eggs were laid on wet filter paper and transferred to water trays for hatching. Larvae were maintained separately at 32±2ºC, 90±10% RH, 12:12 L:D photoperiod in rectangular plastic pans of 30 x 40 cm that were flooded with dechlorinated tap water and fed on Tetramin® fish food.

3.2 Experimental Animals

BALB/c mice and Syrian golden hamsters used for blood feeding mosquitoes were bred and maintained at KEMRI's animal house facility under standard hygienic

conditions. This was done in compliance with Animal Care and Use Committee (ACUC) guidelines of KEMRI.

3.3 Salivary Glands and Midgut Dissections

Mosquito midguts and salivary glands were dissected as described and demonstrated elsewhere (Coleman *et al.,* 2007; Xi *et al.,* 2007), with few modifications whereby only dissecting pins were used in lieu of fine tipped forceps. Simple dissecting pins were fashioned from 1 ml, 29 G insulin needles manufactured by Becton, Dickinson and Company (BD Micro-Fine™ *Plus*, 1 Becton Drive, Franklin Lakes, NJ, USA). Where experiments called for sterile technique, fresh needles (sterile) were used for each dissection to prevent cross contamination of bacteria from one midgut to another.

3.4 To Determine the Effect of Salivary Gland Homogenate on the Mosquito Midgut Proteases and on Bacterial Population Dynamics

3.4.1 To Establish Midgut Microbiota Presence

Initially, three female mosquitoes of ages 20-30 days obtained from established *An. gambiae* colony were dissected for midguts under sterile conditions using aseptic technique. Each midgut was ruptured and cultured overnight in brain heart infusion (BHI) broth media at 37ºC. A blank test tube of BHI not inoculated with midgut was also cultured overnight as a quality control measure. Turbidity was observed for the 3 midgut cultures following incubation, a sign of successful culture.

The midgut cultures were streaked on 3 selective media, McConkey, XLD and blood-free selective media for Campylobacter (as a further QC). The plates were then incubated overnight at 37ºC and observed the following morning for the colony characteristics and subsequent subculture for identification using microscopy and biochemical tests.

3.4.2 Determining the Effect of Salivary Gland Homogenate on Midgut Bacteria

5-7 day old female *An. gambiae* mosquitoes were dissected for salivary glands, with 100 (50 pairs) salivary glands being homogenized in 100µl phosphate buffered saline (PBS) and stored at -20ºC. Whole salivary gland homogenates and <30kDa, 30≥x<100kDa and ≥100kDa fractions obtained by centrifugal concentrators using molecular weights were assayed for antibacterial activity. The classic disk diffusion susceptibility test was used whereby bacteria isolates were cultured in the presence of small filter paper disks impregnated with 2 µl of salivary gland homogenate and the measurements of the zone of inhibition recorded. Antibacterial activity was assessed against 8 bacteria colonies: 3 Gram (+) rods; 2 Gram (+) cocci; and 3 Gram (-) cocci, previously isolated from mosquito. This were cultured at 30°C in the presence of SGH as described above and the plates monitored for inhibition zones at 24 and 48 hours post plating.

3.4.3 Determining the Effect of Salivary Gland Homogenate on Midgut Proteases

Five (5) day old *An. gambiae s.s.* (Mbita strain) mosquitoes reared as described above were used. Chloroquine sensitive *Plasmodium falciparum* strain D6 parasites were cultured and gametocytogenesis induced by artemesinin drug pressure using a method devised at KEMRI (Kangethe *et al.,* personal communication). Successful *Plasmodium* infection in *An. gambiae* was demonstrated using gametocytes derived from this method prior to this study to ensure viability. Salivary gland homogenates (SGH) were prepared from salivary glands dissected from 5 day old *An. gambiae s.s.* Mbita strain female mosquitoes in cold phosphate buffered saline (PBS) at 4ºC and under sterile conditions. Salivary glands were pooled in concentrations of 25 pairs (50 glands) in 50µl of PBS and immediately stored at -70ºC until required.

Three groups (A, B and C) consisting of 70, 100 and 125 female mosquitoes were starved for 7 hours prior to artificial membrane feeding on blood, blood + gametocytes, and blood + gametocytes + SGH respectively. 0.5 ml gametocyte culture at 1.62% parasitaemia, mixed thoroughly (50:50) with fresh human blood was used for the infective blood-meals in groups B & C, whilst 50µl of salivary gland homogenate was added into and mixed thoroughly in group C to give an equivalent of 1/10th salivary gland per fully engorged blood meal estimated volume of 2µl. Mosquitoes were maintained at normal insectary conditions for the feed and at 4 hours post feeding, all mosquitoes that were not fully engorged were removed from the three cages. From each group 2 midguts were dissected in cold PBS at 4ºC,

under sterile conditions at 6 hr, 12 hr, 18 hr, 24 hr and 48 hrs post feeding. Each midgut was homogenized in 200µl PBS and immediately stored at -70ºC for subsequent enzyme assays. Enzyme assays were conducted using modifications of the methods described by Billingsley and Hecker (1991). Briefly, synthetic substrate of trypsin, BAPNA, was dissolved in 2%*N*-dimethylformamide (DMF) and synthetic substrates of chymotrypsin and aminopeptidase, N-Succinyl-Ala-Ala-Pro-Phe-pNA and L-leucine-p-nitraonilide respectively, were dissolved in dimethyl sulfoxide (DMSO). The midgut homogenate supernatants were pooled and added in triplicates to synthetic substrates in microwell plates containing buffer (Tris-HCl at a pH 8.0), and the reaction left to run. The change in absorbance at 405nm was measured by a spectrophotometer and mean optical densities derived for analysis using ANOVA in SPSS.

3.5 To Determine the Effect of Bacteria Resident in the Mosquito Midgut on Vector Survival

3.5.1 To Determine the Effect of Bacteria Resident in Midgut on Mosquito Larvae Survival by Simulating Field Conditions

One day old *An. gambiae* hatchlings were introduced in 24-well plates at a density of 1 larvae per well filled with approx. 10 ml of water. The experiment was set in duplicate as follows:

a. Plate 1. Larvae reared in distilled water alone. (Water = dH_20)

b. Plate 2 Larvae reared in autoclaved distilled water

c. Plate 3 Larvae reared in distilled water + soil*

d. Plate 4 Larvae reared in autoclaved distilled water + autoclaved soil

Soil used in this experiment was collected from active *An. gambiae* breeding sites in the Mwea rice irrigation scheme (Kirinyaga District, Central Kenya) which is endemic for *An. gambiae s.l.* Soil treatments were prepared by suspending 5gms of soil in 15 ml of water and the larvae reared in the supernatant. The water was replenished and larvae fed daily on slurry of parts 3:1 of yeast to Tetramin fish food. To control for differences in nutrition, all larvae were maintained on 0.2 mg/larva upon hatching, 0.3 mg/larva at 1 day post hatching, 0.4 mg/larva on 2-4 days post hatching, and 0.6 mg/hatching for ≥5 days post hatching to pupation. Larval development time (from hatchlings to pupae), and rate of pupae formation (pupae forming per day) were monitored and recorded. To test for presence of bacteria in the larvae rearing media, a drop of water from each treatment was streaked on MacConkey agar plates and incubated at 37°C for 24 hours and colony morphology characteristics and gram stain determined. The emerging adult mosquitoes were maintained on 10% glucose and observed for mortality in the adult survival study. Two (2) female adult mosquitoes from each group were culled; midguts were then dissected under sterile condition, homogenized and plated on enriched media (blood agar) to establish bacteria counts (colony forming units). Individual bacteria colonies were isolated and further subcultured on nutrient and McConkey agars and gram stain, morphology and biochemical tests determined for identification purposes. These tests included the Indole, Methyl Red, Voges-Proskauer, and Citrate tests (IMVC), plus Hydrogen sulphide (H_2S) and motility tests.

In addition, *An. gambiae* larvae were also reared in conventional breeding pans as described above (see 3.1) that were similarly treated as follows: Treatment 1- distilled water (dH_2O); or Treatment 2 - autoclaved dH_2O; or Treatment 3 - dH_2O + 60g soil (60 g soil in 400ml dH_2O); or Treatment 4 - autoclaved dH_2O + 60g autoclaved soil (60 g autoclaved soil in 400ml autoclaved dH_2O). For each treatment n=40, with the experiment carried out in duplicate so that total "n" for each treatment N=80. The emerging adult mosquitoes were maintained on 10% glucose and observed for mortality in the adult survival study.

3.5.2 To Determine the Effect of Bacteria Residence in Midgut on Mosquito Survival by Rearing Bacteria-free Mosquitoes

Groups of 20 *An. gambiae* hatchlings were reared in water treated with 10µg/ml and 15µg/ml of broad spectrum antibiotics solution (streptomycin, penicillin and amphotericin B, Sigma®). The experiment was carried out in triplicate and included a control group reared under normal insectary conditions. Larval mortality rates were recorded and cumulative mortalities and survival estimates between groups analyzed using one way ANOVA and Kaplan Meier analysis.

Additionally, approximately 1,000 *An. gambiae* eggs were floated to hatch in water treated with 10µg/ml antibiotics. Two hundred (200) larvae were then picked for inclusion in the experiment, 3 days post hatching in the treated water, to further hedge against mortality. A control group was picked from the same batch of eggs that were reared in normal water. The experiment included 10 replicates for treatments and 3 replicates for control where n=20 per replicate. Upon pupating, the pupae were collected and enclosed in empty cages for the adults to emerge. These

were then separated by gender, and only female mosquitoes (malaria vectors) included in the adult survival study. The female mosquitoes were provided with 10% glucose solution *ad libitum* and maintained at normal insectary conditions; 27±2°C and 70±10% RH, 12-12 L:D photoperiod.

3.5.3 To Determine the Effect of Bacteria Residence in Midgut on Mosquito Survival by Converting from Septic to Aseptic Mosquitoes

Larvae were reared under normal conditions using dechlorinated tap water and emergent adult mosquitoes maintained on 10% glucose solution treated with antibiotics (streptomycin, penicillin, amphotericin B) at a concentration of 15µg/ml and a control group fed 10% glucose solution alone. The control group consisted of mosquitoes maintained on 10% glucose alone. The experiment was carried out in triplicate where n = 25 pupae per treatment with emerging adults maintained as described above and monitored daily for mortality.

3.5.4 To Determine the Effect of Bacteria Residence in Midgut on Mosquito Survival by Comparing Aseptic vs. Bacteria-fed Mosquitoes

Bacteria species was isolated from the midgut of female *An. gambiae s.s.* (Mbita strain) colony mosquitoes using sterile technique as described in section 3.3. The species was successfully cultured and positively identified as *Enterobacter cloacae* using API 20 E strip developed by Biomérieux®.

Three groups (A, B, C) consisting of 20 female mosquitoes aged 4-5 days were sequestered in separate cages and starved for 7 hours at 32±2°C and 40±10% RH during the light period. Thereafter, the mosquitoes were maintained at normal

colony conditions as described above and group A maintained on 10% autoclaved glucose solution which was supplemented with 20µl broad spectrum antibiotics antimycotic (Sigma: 10,000 units penicillin, 10mg streptomycin and 25µg Amphotericin B per ml). Group B was fed on 10% glucose mixed with $1.5x10^3$ cfu/ml *Enterobacter cloacae* according to the method described by Lindh *et al.*, (2006). Briefly, bacteria previously isolated from the *Anopheles gambiae* colony was cultured overnight at 28°C in LB broth. The culture was centrifuged and washed once with 10% autoclaved glucose solution at x6000 rpm and CFU determined. Bacteria-laced glucose was then delivered using sterile filter paper capillary action and the mosquitoes allowed feed for a period of 2-3 hours, following which the mosquitoes were maintained on 10% autoclaved glucose solution *ad libitum*. Group C was maintained on 10% glucose solution as the control group. No blood-meal was provided to any of the groups with mortality monitored daily.

3.6 To Determine the Effect of Salivary Gland Homogenate on P. falciparum Development in Experimentally Infected An. gambiae

3.6.1 To Determine the Effect of Salivary Gland Homogenate on P. falciparum Oocyst Development

Five (5) day old *An. gambiae s.s.* (Mbita strain) mosquitoes reared as described above were used. Chloroquine sensitive *P. falciparum* strain D6 parasites were cultured and gametocytogenesis induced as described in 3.4.3. Successful *Plasmodium* infection in *An. gambiae* was demonstrated using gametocytes derived from this method prior to this study to ensure viability. Salivary gland homogenates

were dissected from 5 day old *An. gambiae s.s* Mbita strain female mosquitoes in cold phosphate buffered saline (PBS) at 4ºC and under sterile conditions. Salivary glands were pooled in concentrations of 25 pairs (50 glands) in 50µl of PBS and immediately stored at -70ºC until required.

The 5 day old female mosquitoes were split into two groups of 25 mosquitoes each and starved for 7 hours prior to artificial membrane feeding. One group served as control group and was fed on blood + gametocytes, whilst the experimental group was served on blood + gametocytes + SGH respectively. Briefly, the infective feeds were prepared as follows: 0.5 ml of gametocyte culture at 1.62% parasitaemia, mixed thoroughly (50:50) with fresh human blood was used for the infective blood-meals in both groups, whilst 50µl of salivary gland homogenate was added and mixed thoroughly in experimental saliva group. Mosquitoes were maintained at normal insectary conditions for the feed and at 4 hours post feeding, all mosquitoes that were not fully engorged were removed from the cages. All mosquitoes were dissected at 7 days post feed, midguts observed under light microscopy and oocysts enumerated.

3.7 Data Management and Analysis

Data analysis was carried out using Excel, Stata and SPSS. One way analysis of variance (ANOVA) was used to analyze the experimental midgut protease assays. Log-rank test of Kaplan-Meier survival curves and Cox regression were used to analyze differences in survival rates between the groups of mosquitoes. Student's t-test, ANOVA and suitable non-parametric tests were utilized for comparison of means between control versus experimental groups in the other experiments, as appropriate.

CHAPTER FOUR

4.0 RESULTS

4.1 The Effect of Salivary Gland Homogenate on the Mosquito Midgut Proteases and on Bacterial Population Dynamics

4.1.1 Establishing the Presence of Microbiota in Mosquito Midgut

Broth cultures for the 3 midguts were turbid whilst the blank culture remained clear. Growth for all 3 midguts was observed on McConkey and XLD media but not on selective Campylobacter media. Colony observation, gram stain microscopy and biochemical tests as basis for qualitative analysis indicated presence of *Klebsiella* spp. in all 3 midguts (Table 4.1).

Table 4.1: Biochemical test results showing identification of Klebsiella sp. isolated from colony maintained An. gambiae midguts

ID #	Gram stain	Motility	INDO	ORN	SC	TSI			OXD
						H_2S	A/K/A/A	G	
Midgut1	-	+	-	+	+	-	A/A	+	-
Midgut2	-	+	-	+	+	-	K/A	+	-
Midgut3	-	+	-	-	+	-	K/A	+	-

Key: ID # - identification no. ; INDO – indole, ORN – ornithone, SC – simon citrate, TSI – triple sugar iron, H_2S – Sulfur reduction, A – acid production, K – alkaline reaction, G – gas production, OXD – oxidase test

4.1.2 The Effect of Salivary Gland Homogenate on Bacteria Isolated from An. gambiae Midguts

Neither the whole salivary gland homogenate nor its fractions, exhibited antibacterial properties (zone of inhibition = 0 mm diameter) against the 8 colonies following incubation of bacteria in their presence, 48 hours post inoculation.

4.1.3 Establishing the Effect of Salivary Gland Homogenate on Midgut Proteases

Overall enzyme trends demonstrated inhibition of enzyme activity in treatment B (Blood + *Plasmodium* fed) and further suppression in treatment C (Blood + *Plasmodium* + SGH), compared to treatment A (Blood fed only) (Table 4.2).

Table 4.2: Overall trends in enzyme levels across treatments (mean optical densities)

Treatment	Mean	SD	N	95% CI for Mean	
				Lower	Upper
A (Blood)	.1797	.05714	150	0.1776	0.1818
B (Blood +*Plasmodium*)	.1362	.04031	150	0.1341	0.1383
C (Blood+*Plasmodium* + SGH)	.1061	.02611	150	0.1040	0.1081

Analysis of variance (ANOVA) was used to model variability in enzyme levels (mean optical densities) using three factors namely; type of blood meal coded "Treatment", duration post blood feeding coded "hr PBF", and the different proteases assayed (trypsin, chymotrypsin or aminopeptidase) coded "Enzyme". A saturated model was fitted using these factors with the outcome summarized below (Table 4.3).

Table 4.3 Analysis of variability in levels of Trypsin, Chymotrypsin and Aminopeptidase (mean optical densities) in mosquito midgut at 6, 12, 18, 24 and 48 hr PBF

Source of variability	Sum of Squares	df	Mean Square	F	P value
Treatment	0.41110	2	0.205550420	1211.80	<0.001*
hr PBF	0.38201	4	0.095501610	563.02	<0.001*
Enzyme	0.01269	2	0.006346455	37.41	<0.001*
Treatment * hr PBF * Enzyme	0.36677	36	0.010188108	60.06	<0.001*
Residual (Error)	0.06870	405	0.000169624		
Total	1.24127	449			

R Squared =0.945 (Adjusted R Squared = 0.939)
* Variability is significant at P<0.05.

Adjusting for hr PBF, Enzyme and the interaction term (Treatment * hr PBF * Enzyme), the ANOVA model revealed a significant Treatment effect on enzyme levels (mean optical densities) (P<0.001). Similarly, adjusting for Treatment, Enzyme and the interaction term (Treatment * hr PBF * Enzyme), hr PBF had a significant effect on variability in enzyme levels (mean optical densities) (P<0.001). The effect of Enzyme on enzyme levels (mean optical densities) was equally significant upon adjusting for Treatment, hr PBF and the interaction term (Treatment * hr PBF * Enzyme). Total variability in enzyme levels (mean optical densities) explained by the model was 94.5%. Variability due to the treatment effect accounted

for the highest variation with 33.1%, followed by duration PBF effect accounting for 30.8%, the interaction term (Treatment * hr PBF * Enzyme) accounting for 29.6% and lastly the effect due to Enzyme accounting for 1.0% of the total variability.

Subsequent ANOVA univariate modeling and Tukey HSD post hoc analysis was carried out to investigate the effects of each treatment (A, B and C), on each enzyme (trypsin, chymotrypsin and aminopeptidase) at 6, 12, 18, 24 and 48 hr PBF respectively (Annex 1).

4.1.3.1 Enzyme Activity in Blood Fed Mosquitoes

The chymotrypsin activity levels in the mosquitoes declined between 6 and 18 hr after a blood meal, indicative of constitutive enzyme down regulation but increased to peak at 18hr PBF after which it steadily declined indicative of the enzyme activity induced by the blood meal (Fig 4.1). Trypsin activity followed a similar pattern to chymotrypsin but at lower levels (Fig 4.2). Whereas the serine proteases (chymotrypsin and trypsin) reached peak activity at 18hr PBF, aminopeptidase levels peaked at the initial measurement time point (6hr PBF) after which the activity levels declined over the entire experimental period albeit with a small “peak” detected at 18hr PBF (Fig 4.3).

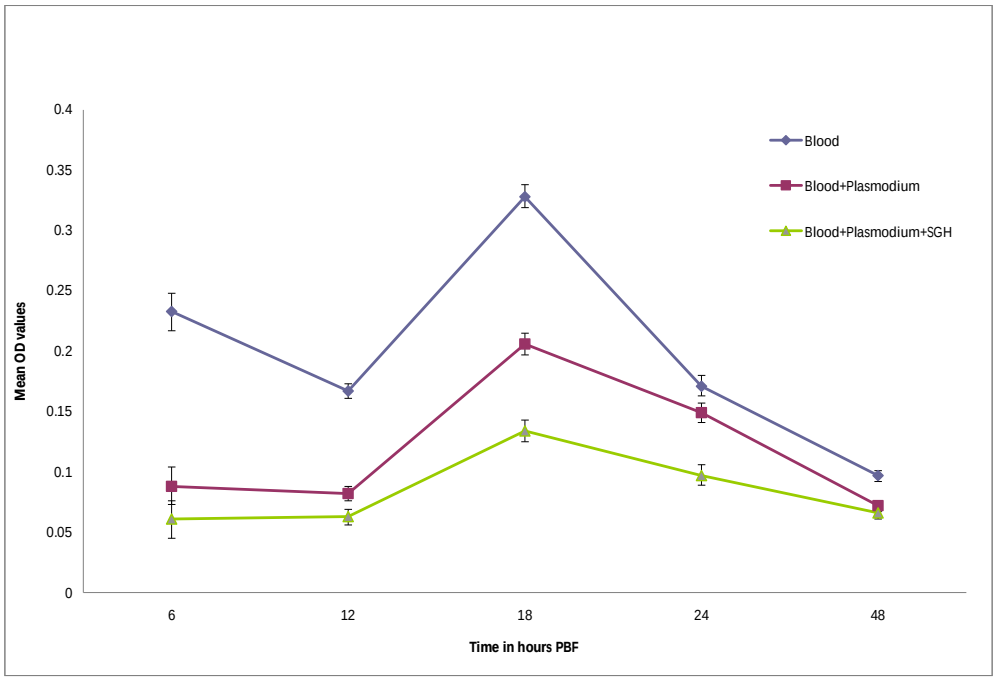

Fig 4.1 Chymotrypsin activity in treatments A (Blood), B (Blood + Plasmodium) and C (Blood+Plasmodium+SGH) showing 95% confidence intervals

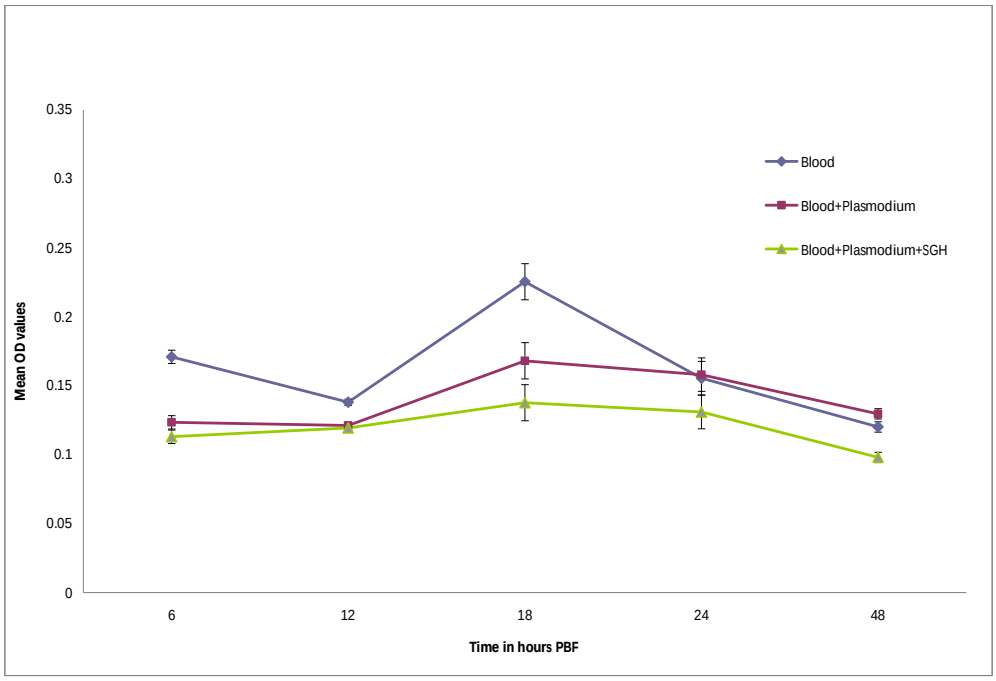

Fig 4.2 Trypsin activity in treatments A (Blood), B (Blood + Plasmodium) and C (Blood+Plasmodium+SGH) showing 95% confidence intervals

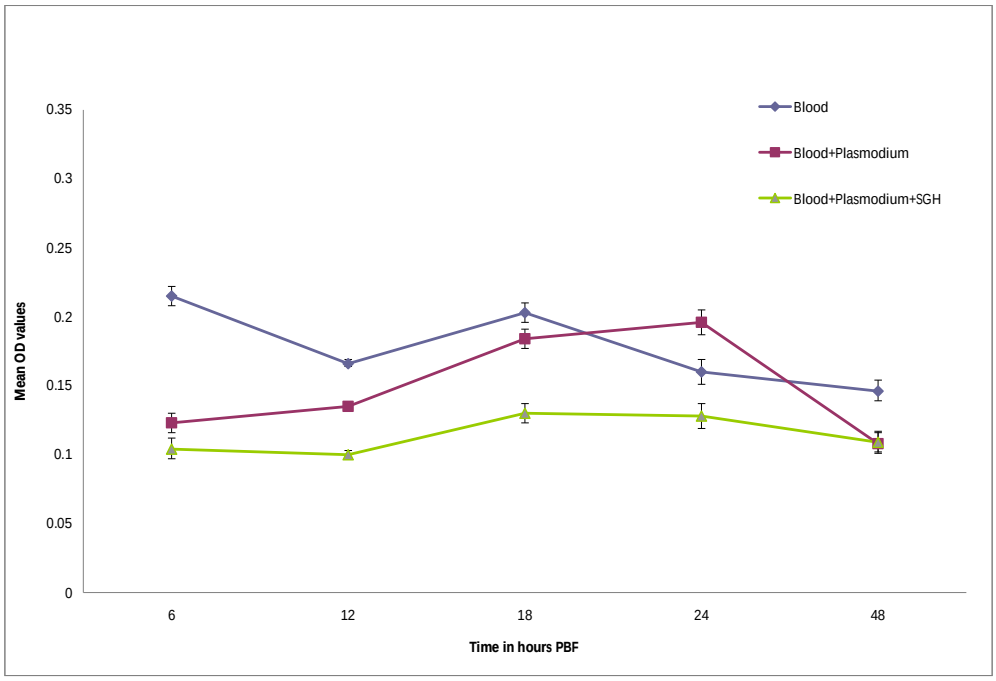

Fig 4.3 Aminopeptidase activity in treatments A (Blood), B (Blood + Plasmodium) and C (Blood+Plasmodium+SGH) showing 95% confidence intervals

4.1.3.2 Effect of Malaria Parasites on Enzyme Activity

The presence of *P. falciparum* parasites in the blood meal depressed the activity levels of chymotrypsin, trypsin and aminopeptidase by 62%, 29% and 26% at the 6 hour time point, respectively (Figs 4.1-4.3). This reduced level of protease activity was statistically significant (Tukey HSD post hoc, $P<0.001$) for each enzyme indicating the down regulation of constitutively expressed enzymes by the presence of *Plasmodium* in the blood meal, with the most reduction observed for chymotrypsin, trypsin and aminopeptidase in that order. Similarly, at 18 hours PBF the suppression of enzyme activity by *P. falciparum* was evident by 37% (Tukey HSD post hoc, $P<0.001$), 26% (Tukey HSD post hoc, $P<0.001$) and 9% (Tukey HSD post hoc, $P<0.001$) for chymotrypsin, trypsin and aminopeptidase respectively indicating significant down regulation of blood meal induced enzyme activity.

However, whereas the trends for serine proteases in the infective blood meal were indicative of enzyme down regulation by the malaria parasite (Figs 4.1-4.2), a steady increase in aminopeptidase levels was evidenced from 6 hr PBF, peaking at 24 hr PBF with activity enhanced by 22.5% compared to treatment A (blood only) (Tukey HSD post hoc, $P<0.001$), but sharply declining thereafter with enzyme activity suppressed by 26% (Tukey HSD post hoc, $P<0.001$) at 48hr PBF (Fig 4.3). Serine protease suppression may therefore suggest a negative impact on parasite development with the upregulation of aminopeptidase peaking at 24 hr PBF indicative of a positive interaction, coinciding with *P. falciparum* ookinetes' peak traversal of midgut.

4.1.3.3 Effect of Malaria Parasites plus Salivary Glands Homogenate on Enzyme Activity

A significant effect of treatment (blood meal alone or blood meal plus malaria parasites or blood meal plus malaria parasites plus saliva) on enzyme activity in the midgut of mosquitoes ($F = 1211.80$, $P < 0.001$) was observed (Fig 4.4). The addition of salivary gland homogenate to blood meals with malaria parasites further reduced the enzyme activities in the mosquito midgut. Enzyme activities in mosquito midguts were significantly inhibited ($P<0.001$) by the presence of malaria parasites and saliva in the blood meal (Tukey HSD post hoc, $P<0.001$) when compared to treatment A (blood only) for chymotrypsin, trypsin and aminopeptidase at 6, 12, 18, 24 and 48 hr PBF (Figs 4.1-4.4).

Six hours after the infective blood meal with saliva, chymotrypsin activity further reduced by 31%, trypsin by 8% and aminopeptidase by 15% compared to treatment B (Blood + *Plasmodium* only) indicative of constitutively expressed enzymes down regulation. Additionally, suppression of both the serine proteases and aminopeptidase blood meal induced enzymes was evidenced with chymotrypsin activity further reduced by 35%, trypsin by 18% and aminopeptidase by 29% at 18 hr PBF when compared to treatment B indicative of blood meal induced enzyme activity down regulation. Also worthy of note is the effective suppression of aminopeptidase's 24 hour peak in treatment B (Blood + *Plasmodium* only) by the addition of saliva in treatment C (Figs 4.3 and 4.4).

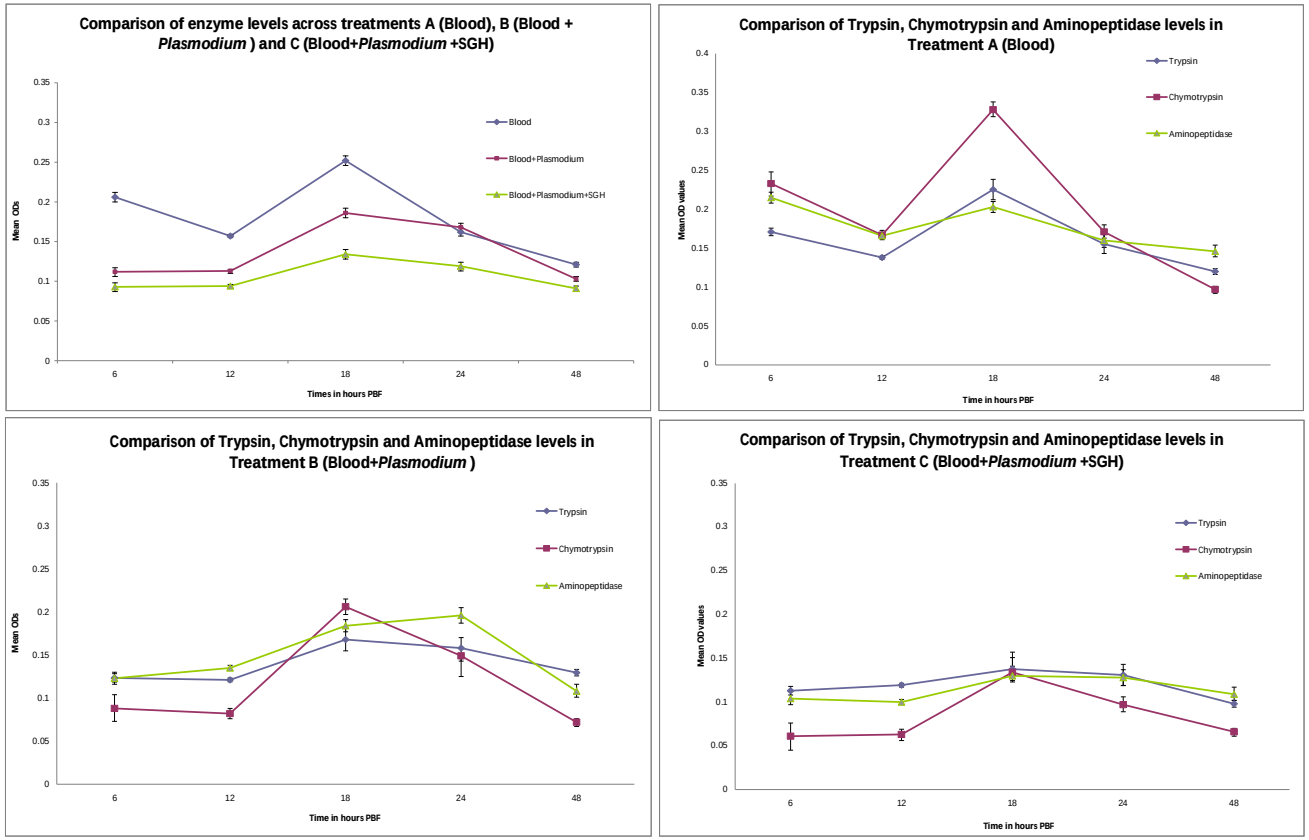

Fig 4.4 Comparison of overall enzyme trends across treatments vis a vis individual enzymes trend in each treatment

4.2 The Effect of Resident Midgut Bacteria on Vector Survival

4.2.1 The Effect of Resident Midgut Bacteria on Mosquito Larvae Survival in Simulated Field Conditions

The culturing of bacteria from a drop of rearing milieu collected from each of the treatments established that efforts to eliminate bacteria in treatments 1 (distilled water), 2 (distilled autoclaved water) and 4 (autoclaved distilled water and autoclaved soil) were unsuccessful and could only be described as "bacteria poor" *vis a vis* the "bacteria rich" treatment 3 (distilled water + soil) (Table 4.4).

Table 4.4: Bacteria presence in milieu of bacteria rich and poor water

Inoculum	**Gram stain**	**Morphology**
Distilled water	Negative	Round, flat
Autoclaved distilled water	Negative	Round, flat
Distilled water + soil	Negative	Round, flat
Autoclaved distilled water + soil	Negative	Round, flat

Treatments three and four (distilled water + soil and autoclaved water + autoclaved soil respectively) had the greatest variation of distinct colonies/unique species with 7 each, followed by treatment 1 (distilled water) with 6 colonies and lastly treatment 2 (autoclaved water) with 4 colonies (Table 4.5). Interestingly, treatment 4 also had the highest bacteria CFU counts (> 300 for colony I). However, gram stain, morphology and the IMVC biochemical tests proved insufficient for species identification.

Table 4.5: Midgut cultures and CFU counts on blood agar plates and the characteristics of bacteria based on morphology

Treat-ment	Unique Colony id #	CFU count	Colony Morphology (Blood agar)		
			Colour	Size	Characteristics
1	I	1	Cream	Medium	Round, raised, smooth edge
	II	12	Yellow/ Brown	Large	Round, raised, mucoidal, smooth edge
	III	2	Pale	Medium	Rough surface, raised edge, sunken centre, smooth edge
	IV	1	White	Medium	Round, mucoidal centre, well defined surface, smooth edge
	V	3	Colourless	Small	Concave "dots" in media with "halo"
	VI	1	White	Large	Flat, serrated edge
2	I	1	White	Small	Raised, smooth
	II	15	Pale	Small	Raised, smooth, mucoid
	III	1	Pale	Small	Round, sunken centre (concave), rough surface
	IV	3	Colourless	Small	Concave "dots" in media without "halo"
3	I	2	Pink	Large	Round-oval, raised, striated
	II	9	Pink, beige	Small	Round, raised, mucoid
	III	1	White	Small	Concave "dots" in media without "halo"
	IV	1	Cream	Medium	Round (perfect circle), raised, well defined smooth edges, mucoid
	V	1	Cream	Tiny	Raised, smooth edges
	VI	1	Cream	Large	Round, flat with sunken centre, serrated edges
	VII	1	Pale	Medium	Round, raised, rough surface and edges
4	I	>300	Pale	Medium	Round, raised (convex), smooth surface and edge, well defined edges, mucoid
	II	3	White	Small	Round, flat, rough surface, edge not well defined
	III	26	Light yellow	Tiny	Round, raised, smooth, mucoid
	IV	1	Pale/ yellow	Medium	Round, raised (convex), smooth edge, mucoid
	V	8	White	Tiny	Round, raised, smooth, mucoid
	VI	17	White	Small	Round, raised (convex), well defined edge, mucoid
	VII	10	Colourless	Small	Concave "dots" in media with "halo"

Key: Treatment 1 = Distilled water, Treatment 2 = Autoclaved distilled water, Treatment 3 = Distilled water + Soil, Treatment 4 = Autoclaved distilled water + Autoclaved soil

Larval development to pupa took an average of 6.45 days (CI: 6 – 7 days) in all the four treatments (mosquito larvae reared in 1. distilled water, 2. autoclaved distilled water, 3. distilled water + soil and 4. autoclaved distilled water + autoclaved soil) with the soil treatments (3 and 4) taking the shortest time on average to pupate indicating derived advantage from the presence of bacteria and soil (Table 4.6). Comparison of the average time that the larvae in each treatment took to pupate by linear regression showed that the differences were significant: F-ratio = 13.67, P = 0.0013, R-squared = 0.3832.

Table 4.6: The duration of larvae-pupa development in bacteria rich and bacteria poor water

Treatment	Mean days(CI)	Median days
1 - Distilled water	6.6 (5.4 – 7.8)	6.5
2 - Autoclaved distilled water	7.5 (5.4 – 9.6)	7.5
3 - Distilled water + Soil	6 (5.1 – 6.9)	6
4 - Autoclaved distilled water + autoclaved soil	6 (5.1 – 6.9)	6
Collective	6.45 (6-7)	6

Larvae reared under the bacteria poor (distilled water, autoclaved distilled water, and autoclaved distilled water + autoclaved soil) and bacteria rich (distilled water + soil) had statistically significant survival times (Fig. 4.5) when their Kaplan Meier survival charts were analysed using the Mantel-Cox Chi2 test or Log Rank test: χ^2 = 14.35, df = 3, P = 0.0025). However, further comparisons between each treatment using Cox regression analysis against the distilled water group treatment as the standard was not significant for any group (P>0.05): Autoclaved water HR = 2.51

(CI: 0.87-7.23), Distilled water + soil HR = 0.15 (0.02-1.25), Autoclaved water + autoclaved soil HR = 0.65 (0.16-2.60).

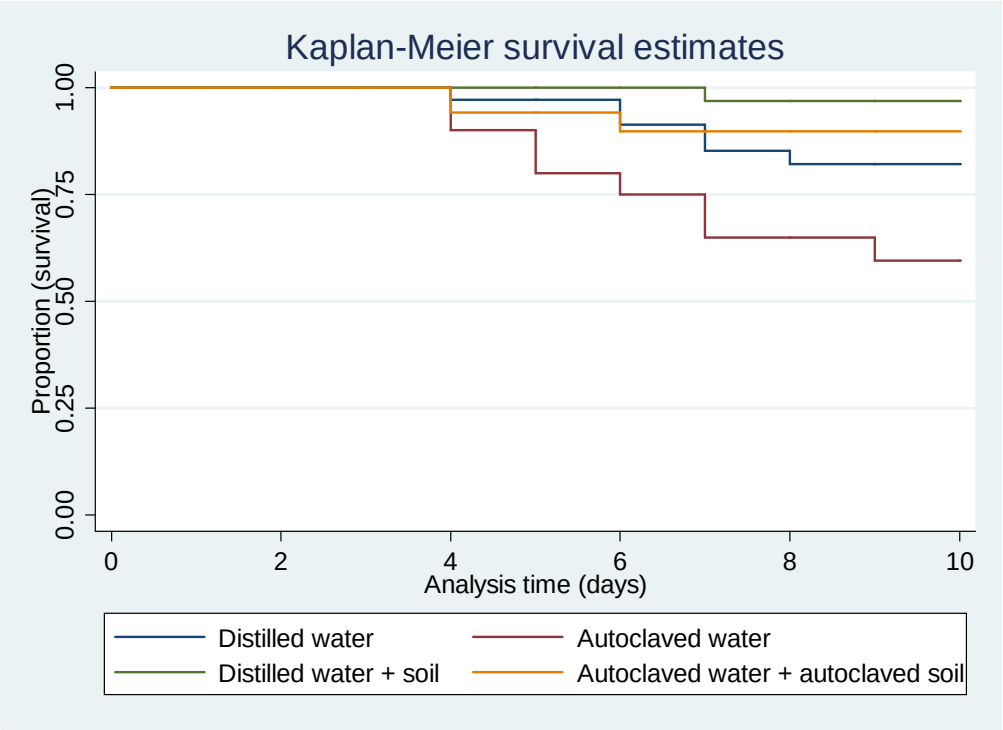

Fig 4.5: Kaplan-Meier survival functions of mosquito larvae reared in bacteria rich and bacteria poor water

The differences in survival rates of adult mosquitoes bred in bacteria rich and bacteria poor environments were significant (Fig 4.6: KM Log-rank test χ^2 = 16.26, df = 3, P = 0.001). Cox regression analysis against the distilled water group treatment was significant for each group, autoclaved water (HR = 0.47, CI: 0.29-0.75, P = 0.02), distilled water + soil (HR = 0.56, CI: 0.35-0.88, P = 0.013), autoclaved water + autoclaved soil (HR = 0.45, CI: 0.29-0.71, P = 0.001).

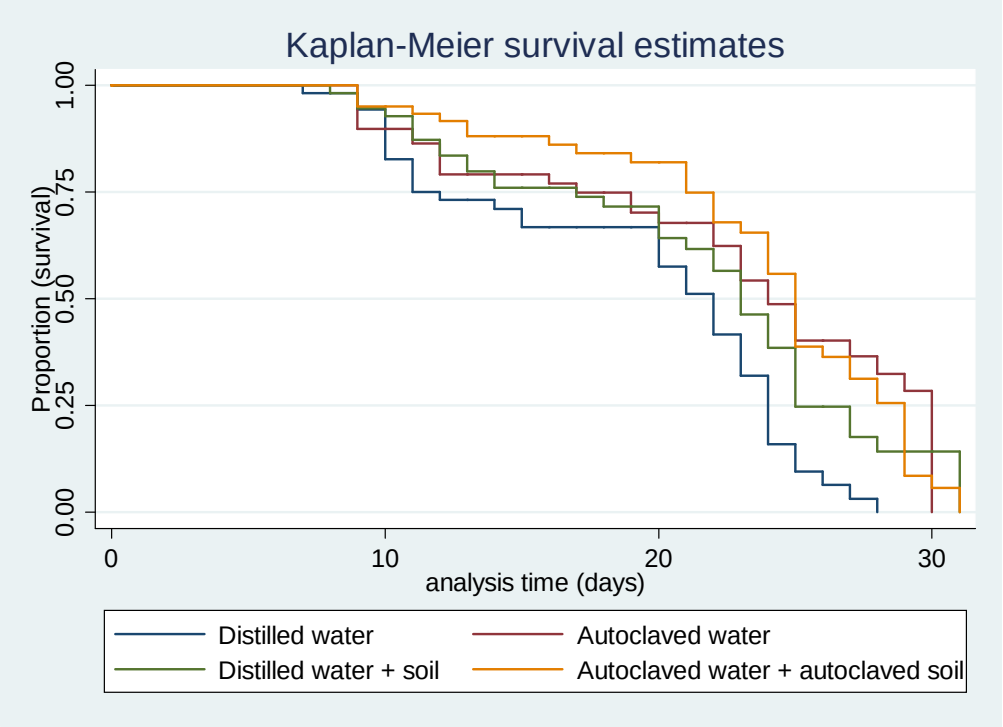

Fig 4.6: Kaplan-Meier survival functions of adult mosquito reared in bacteria rich and bacteria poor water

4.2.2 The Effect of Resident Midgut Bacteria on Mosquito Survival:

Rearing of Bacteria-free Mosquitoes

Mortalities of 90% and 95% (Table 4.7) and prolonged larval stage duration of up to 17 days and arrested development (Fig 4.7) compared to larvae reared in bacteria inclusive milieu (Table 4.6; Fig 4.5) was observed in larvae reared in water treated with 10µl/ml and 15 µl/ml of antibiotic solution (Sigma: 10,000 units penicillin, 10mg streptomycin and 25µg/ml amphotericin B). The mean cumulative mortality across the treatments differed significantly between the treatments (ANOVA, F = 13.98, df = 2, P = 0.0055).

Table 4.7: Mosquito survival rate of larvae bred in antibiotic treated, and untreated milieu

Treatment	%Mortality (n=20)	Mean mortality (n=20)	Median mortality
10µl/ml Antibiotic	90	17.7 (CI: 9.7 – 25.7)	19
15µl/ml Antibiotic	95	19 (CI: 16.5 – 21.5)	19
Control	50	9.3 (CI: 3 – 15.6)	9

Log-rank test analysis of the Kaplan Meier survival charts (Fig 4.7) also demonstrated significant differences (χ^2 = 31.11, df = 2, P < 0.0000). Further analysis using Cox regression indicated no significant difference between the two groups treated with 10µl/ml and 15 µl/ml antibiotic solution (HR = 0.77, CI: 0.53-1.12, P = 0.174) but significant difference between either antibiotic concentration group and the control group (HR = 0.32, CI: 0.20-0.50, P < 0.000).

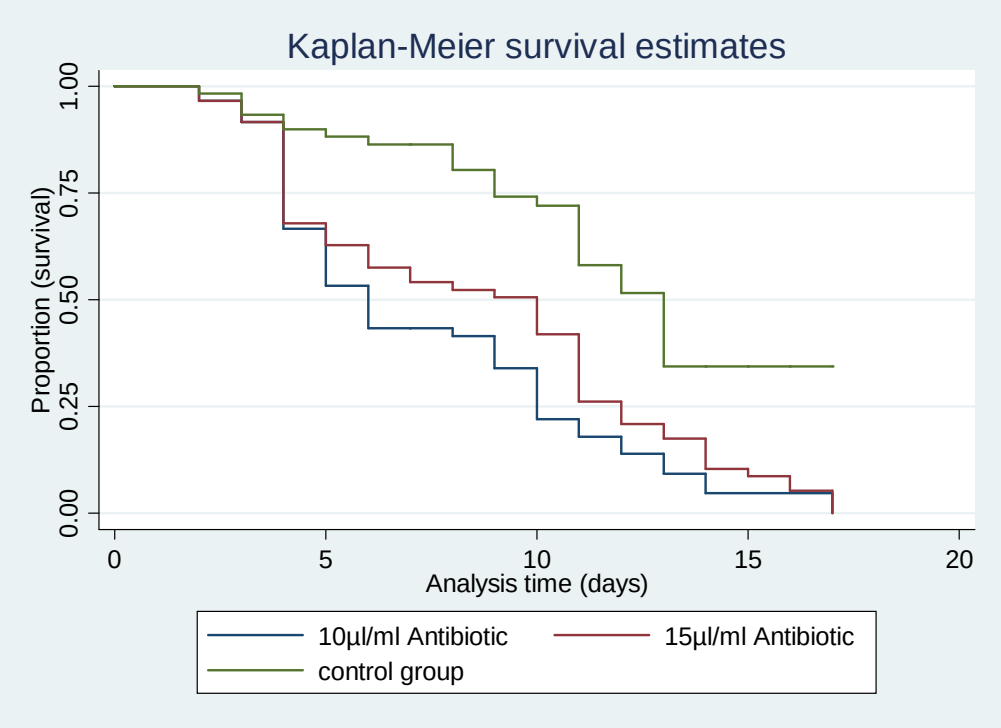

Fig 4.7: Kaplan-Meier survival functions of larvae bred in antibiotic treated water vs. control group

Improved survival rates of 68% (136 out of 200) compared to 10% in similar treatment (Table 4.7) was achieved when the assay was repeated with survival for 3 days post hatching in water treated with 10µl/ml of the antibiotic solution (Sigma: 10,000 units penicillin, 10mg streptomycin and 25µg/ml amphotericin B) as the inclusion criteria for larvae in the experiment group (Fig 4.8). In the control group 54 out of 60 (90%) survived to pupa stage. The differences in larvae survival rates (Fig 4.8) between the two groups were significant (Log-rank test: χ^2 = 11.99, df = 1, P = 0.0005). Cox regression analysis of the larval survival rates between the two antibiotic treatment and control group was also significant (HR = 0.27, CI: 0.11-0.61, P = 0.002).

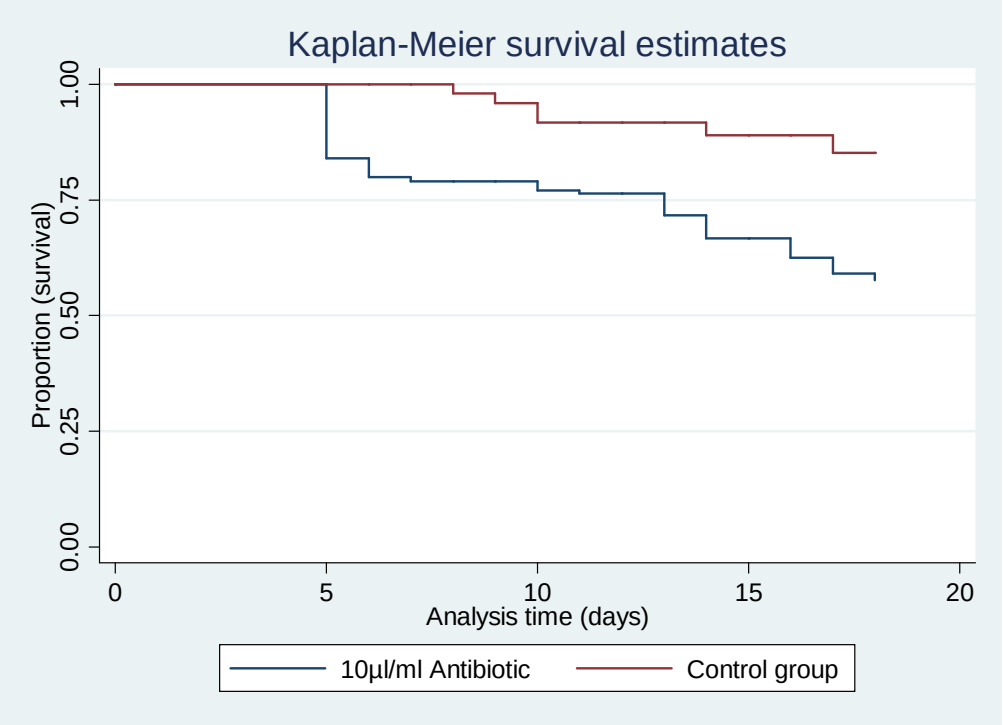

Fig 4.8: Kaplan-Meier survival functions of larvae reared in 10µl/ml antibiotic treated water vs. control group

Adult mosquitoes emerging from the repeated assay with enhanced survival rates were segregated by sex and twenty seven (27) female mosquitoes collected from the antibiotics treatment for inclusion in the adult assay as the experimental group and another twenty three (23) female mosquitoes from the controls as the control group. These were maintained at normal insectary conditions and mortalities recorded daily. Their survival rates (Fig 4.9) differed significantly (Log-rank test: χ^2 = 4.37, df = 1, P = 0.0365). However, Cox regression analysis did not find significant difference between the antibiotic treatment and control group in the emergent female adults (HR = 0.58, CI: 0.32-1.04, P = 0.07).

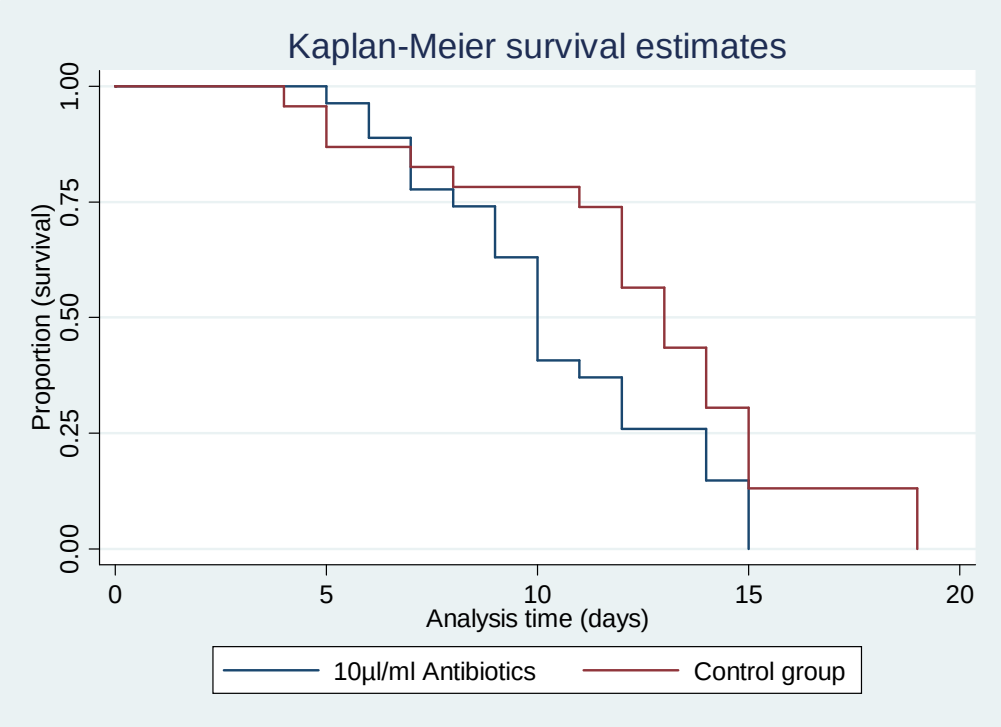

Fig 4.9: Kaplan-Meier survival functions of female mosquitoes reared in 10µl/ml antibiotic vs control group

4.2.3 The Effect of Resident Midgut Bacteria on Mosquito Survival: Converting From Septic to Aseptic Mosquitoes

The survival rate of mosquitoes reared under normal insectary conditions but converted from septic to aseptic by maintenance on 10% glucose laced with 15µl/ml antibiotic solution (Sigma: 10,000 units penicillin, 10mg streptomycin and 25µg/ml amphotericin B) differed significantly from the control group maintained on glucose alone (Fig 4.10; Log-rank test: χ^2 = 7.09, df = 1, P = 0.0077) indicating advantage accrued from the elimination of midgut bacteria in the adult mosquito. Cox regression analysis of the survival rates between antibiotic treated group and control group was also significant (HR = 1.56, CI: 1.09-2.24, P = 0.016).

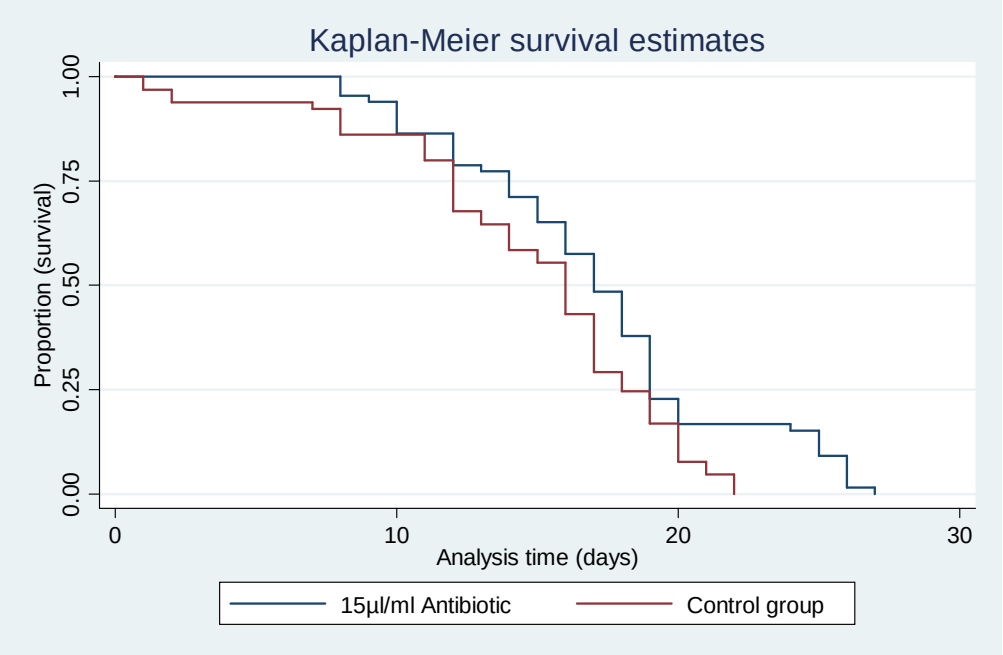

Fig 4.10 Kaplan-Meier survival functions of aseptic vs septic mosquitoes

4.2.4 The Effect of Resident Midgut Bacteria on Mosquito Survival: Comparing Aseptic vs. Bacteria-fed Mosquitoes

The comparison of survival rates between female mosquitoes similarly converted from septic to aseptic by maintenance on 10% glucose laced with 20µl/ml antibiotic solution (Sigma: 10,000 units penicillin, 10mg streptomycin and 25µg/ml amphotericin B) and septic bacteria fed on 10% glucose laced with $1.5x10^3$ cfu/ml of *E. cloacae* indicated accrued advantage from the presence of midgut bacteria in the adult female mosquito which was contrary to the results in 4.2.3 (Fig 4.11). Log-rank test of the survival table indicated the difference was statistically significant (χ^2 = 12.69, df = 2, P = 0.0018). Cox regression analysis comparing the survival rates of bacteria fed and control group (maintained on glucose alone) vs the antibiotics fed group as the standard showed significant difference for bacteria (HR = 0.33, CI: 0.16-0.69, P = 0.003) and control (HR = 0.35, CI: 0.17-0.73, P = 0.005) respectively.

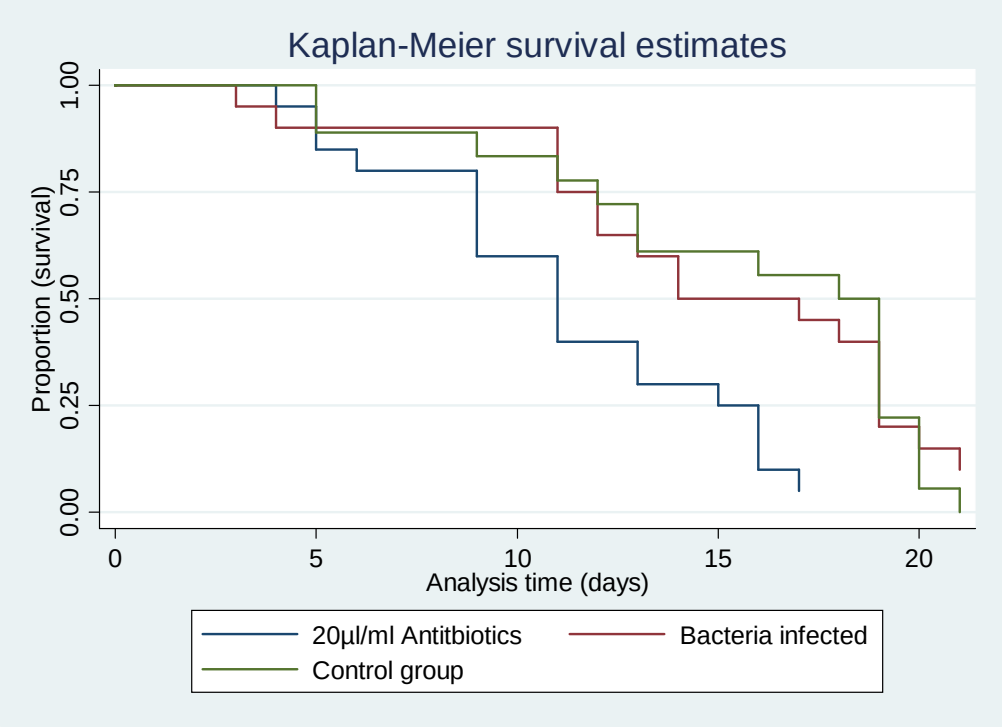

Fig 4.11: Kaplan-Meier survival functions of aseptic vs septic and E. cloacae sugar fed female mosquitoes

4.3 The Effect of Salivary Gland Homogenate on P. falciparum Development in Experimentally Infected An. gambiae Mosquito

Twenty four (24) *P. falciparum* infections (Plate 4.1) were recorded in the saliva group compared to only 10 in the control group. Oocyte densities ranged from 4 to 113 and 16 to 107 in the saliva and control groups respectively, with the former following normal distribution. Spearman rank correlation indicated no statistical significance for oocyst densities between the two groups (P = 0.9601).

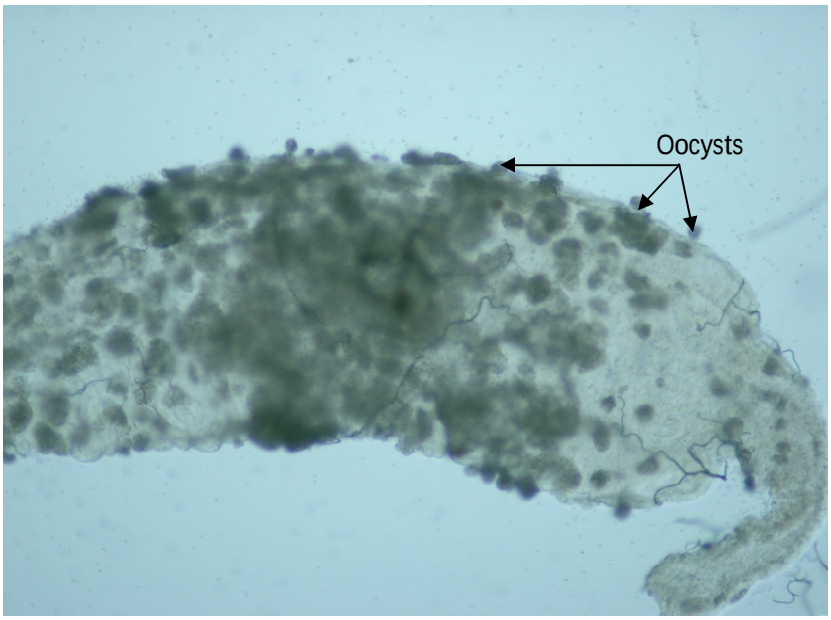

Plate 4.1 Mosquito midgut inundated by oocyst as observed under light microscope (x10)

Plate 4.2 Mosquito midgut without P. falciparum infection as observed under light microscope (x10)

CHAPTER FIVE

5.0 DISCUSSION

5.1 The Effect of Salivary Gland Homogenate on the Mosquito Midgut Proteases and on Bacterial Population Dynamics

5.1.1 Establishing the Presence of Microbiota in Mosquito Midgut

Klebsiella species complex was isolated and positively identified from each of the midguts demonstrating bacteria-mosquito coexistence in the midgut. Although the sampling number was very small, the uniformity of bacteria flora in all three mosquitoes may be an indicator of the common insectary rearing environment. Anopheline mosquito larvae have previously been shown to ingest bacteria from the surface micro layer (Walker *et al.,* 1988). Moreover, using culture-independent techniques, Briones *et al.,* (2008) demonstrated that bacteria derived from the larval breeding habitat such as *Thorselia anophelis* can be able to persist in the midgut through the larval-adult metamorphosis (transtadial transmission) to become stably established in the adult mosquito. *Klebsiella pneumoniae/ozonae/rhinoscleromatis* has a ubiquitous distribution and can be found in soil, water, wood and animals and as such could come into contact with the colony from common source such as larval breeding water.

5.1.2 The Effect of Salivary Gland Homogenate on Midgut Bacteria

Neither whole salivary gland homogenate nor fractions thereof showed any antimicrobial activity against 8 midgut derived bacteria colonies. Unfortunately, efforts to identify the bacteria species in this evaluation through biochemical tests proved ill suited for the purposes of this study. This is because the culture media used and biochemical tests undertaken are based on the premise of medical bacteriology, whereas the symbiotic bacteria targeted in this study inhabit invertebrate, poikilotherm organisms of divergent physiological parameters. The difficulties associated with the detection, culturing and identification of bacteria from insects and/or the environment are well documented (Amann *et al.*, 1995; Rappe and Giovannoni, 2003).

Whereas the results indicating zero antibacterial properties contrast with saliva transcriptome study predictions (Francischetti *et al.*, 2002), they are not altogether surprising, keeping in mind that none of the 8 colonies tested were Gram (-) negative bacilli. Moreover, studies by (Favia *et al.*, 2007) demonstrated that alpha-Proteobacteria of the genus *Asaia* colonizes all mosquito body organs necessary for malaria parasite development and transmission, including female gut and salivary glands. In contrast, *An. gambiae* salivary glands have been shown to primarily respond to sporozoites invasion in the *Plasmodium* lifecycle by an increased expression of a small number of genes involved in the innate immune response. Among them, upregulated genes associated with defensive antimicrobial peptides (AMPs) *Defensin1*, *GNBP*, *Serpin6* and *Cecropin2* have been identified,

indicating that mosquito saliva possesses antimicrobial qualities (Rosinski-Chupin *et al.,* 2007).

Indeed, earlier antimicrobial assays using purified AMPs demonstrated that *Cecropin 2* has broad spectrum activity against both Gram-positive and Gram-negative bacteria (Vizioli *et al.,* 2001); whilst *Defensin 1* is mainly active against Gram-positive bacteria (Vizioli *et al.,* 2001), thus suggesting that negative results in the present study can be attributed to low AMP concentrations in the saliva homogenate. However, this is unlikely owing to the large number of salivary gland pooled in the homogenate (50 pairs) and further concentration of the same through the molecular weight cut-off filters.

An alternate hypothesis is that AMPs being immune-associated defensive agents are specifically induced by invasion events as has has been reported for *Serpin 6* whose expression is spatially localized at the point of sporozoite invasion of the salivary gland epithelial membrane (Pinto *et al.,* 2008). The divergent paths followed by *Plasmodium* (alimental canal - traversal of midgut – heamolymph - traversal of salivary gland epithelial) versus the non-invasive path of bacteria (intra-alimental canal) may therefore account for the limited role of saliva in bacteria inhibition in light of the innate immunity's activation mechanism. This view is supported by Favia *et al.,* (2007) who demonstrated the colonization of *An. stephensi* salivary glands by green fluorescent protein (GFP) tagged-bacteria of the *Asaia* spp that is a midgut endosymbiont.

5.1.3 The Effect of Salivary Gland Homogenate on Midgut Proteases

The results in treatment A (mosquitoes fed on blood only) whereby chymotrypsin has the highest enzyme activity indicates the validity of the study as serine proteases are known to be the main digestive proteases in midguts of the Diptera (Ramalho-Ortigão *et al.,* 2003). This is further supported by the biphasic pattern characterized by a "dip" at 12 hr PBF and "peak" at 18 hr PBF (Fig 4.4) indicating the constitutive (pre-blood meal) proteases that are down-regulated by the blood meal which induces the secretion of post-blood meal proteases as previously reported for chymotrypsin, trypsin and aminopeptidase (Dana *et al*., 2005).

The addition of *P. falciparum* gametocytes to the blood meal (treatment B) resulted in the suppression of serine protease activity (Fig 4.1-4.2) that has previously been reported as a likely defense mechanism to evade catabolic degradation during ookinete invasion of the midgut (Bonnet *et al.,* 2001). In addition, significant upregulation of aminopeptidase to peak at 24 hr PBF was also noted (Fig 4.3). Aminopeptidase is expressed as an integral part of the mosquito midgut membrane and has recently been discovered as transmission blocking candidate that acts as a receptor for ookinetes in the midgut invasion process (Dinglasan *et al.,* 2007). This may therefore be advantageous to the parasite as it coincides with the height of midgut invasion by *P. falciparum* ookinetes in *An. gambiae* at 24 hr PBF.

This study is also the first to demonstrate that saliva may play an important physiological role in the vector *in vivo* by suppressing midgut protease activity (Fig 4.4). *An. gambiae* saliva contains six proteins of serine proteases predicted in the sialotranscriptome (Arca *et al.,* 2005) that were initially expected to augment protease activity in treatment C (Blood + *Plasmodium* + SGH). Four of these serine

proteases are thought to facilitate blood feeding through antihemostatic activity by preventing clotting and the complement cascade, with the remaining two proteins being similar to prophenoloxidase-activating enzymes and therefore likely to be involved in the mosquito immune system (Arca *et al.*, 2005). However, the opposite was evident with significant suppression of chymotrypsin (Fig 4.1), trypsin (Fig 4.2) and aminopeptidase (Fig 4.3).

Saliva's protease suppression properties may however be attributed to the cE5/Anophelin family of proteins which is known to have serine protease inhibition properties (Valenzuela *et al.*, 2003). Anopheline mosquitoes depend on their unique cE5/Anophelin family for vasodilatory, anticlotting and antithrombin activity (Champagne and Valenzuela, 1996; Valenzuela *et al.*, 1999; Ribeiro, 2000; Waidhet-Kouadio *et al.*, 1998). This protein is therefore highly up-regulated to facilitate blood feeding in the *An. gambiae* salivary gland excretions. Additionally, the up-regulation of serine protease inhibitors has previously been reported in response to sporozoite invasion of salivary glands (Rosinski-Chupin *et al.*, 2007), in particular the Serpin defensive proteins may therefore hypothetically contribute towards suppression of enzyme activity in older sporozoite bearing mosquitoes *in vivo*.

5.2 The Effect of Resident Midgut Bacteria on Mosquito Survival

5.2.1 The Effect of Resident Midgut Bacteria Residence on Mosquito Larvae Survival in Simulated Field Rearing Conditions

The larval breeding conditions in all four treatments were found to harbor gram negative bacteria (Table 4.4) and as such the treatments can only be described

as either “bacteria poor” rearing milieu where elimination of bacteria by autoclaving was attempted in treatments 2 and 4 (autoclaved water and autoclaved water + autoclaved soil, respectively), or “bacteria rich” by the addition of soil in treatment 3 (distilled water + soil), with treatment 1 (distilled water only) serving as control. Likewise, the dissected midguts from adult mosquitoes in each treatment harboured bacteria. Surprisingly, whereas the midguts from the autoclaved treatments were expected to have the least bacteria loads, treatment 4 (autoclaved water + autoclaved soil) had the highest cfu counts and highest species diversity, whereas treatment 2 (autoclaved distilled water only) had few cfu counts and the lowest species diversity.

In this study the simulation of field conditions by adding soils to the rearing water significantly shortened the duration of larvae development to pupae in both the autoclaved and unautoclaved soil treatments (treatments 3 and 4). Linear regression showed the difference in mean pupation rates to be significant ($P = 0.0013$), with type of treatment substantially accounting for 38% of the variation in means. Moreover, the survival rates of larvae in the different treatments differed significantly (Log rank test, $P = 0.0025$) indicating that bacteria play a crucial role in the survival and development of larval stage mosquito (Table 4.6; Fig 4.5). This is underscored by the hazard ratios (HRs) that indicated a 2.5 fold increase in hazard exposure in bacteria impoverished treatment 2 (autoclaved water only) as opposed to a 6.6 fold reduction in hazard exposure in the bacteria-enriched distilled water + soil (treatment 3) when compared to treatment 1 (distilled water only) as a control. This concurs with previous findings of larval nutrition studies (Atkin and Bacot, 1917; Hinman, 1933; Trager, 1935).

Okech *et al.,* (2007) using a similar experimental setup evaluated the impact of larval breeding habitats on vector competence by rearing *An. gambiae* larvae in clay soil, sandy soil or water drawn from Lake Victoria respectively. Their study demonstrated that rearing larvae on soil substrates significantly shortened the larval development time, and that autoclaving of the soil substrates resulted in significantly smaller adult mosquitoes of significantly reduced vectorial capacity. This suggested an important nutritional role for organic matter and microbial fauna occurring in the soil on mosquito fitness and vector competence, corroborating the findings of this study whereby significantly shorter duration of larval development and higher pupation rates are demonstrated.

Differences in the survival rates of the adult mosquitoes reared in the different treatments were statistically significant (Log-rank test, P = 0.001) indicating that larval breeding conditions affect adult mosquito survival (Fig. 4.6). However, these differences could not be correlated to the bacteria poor or bacteria rich nature of the treatment as evidenced by a consistent 2.3, 1.7 and 2.2-fold decrease in hazard exposure for treatments 2 (autoclaved water), 3 (distilled water + soil) and 4 (autoclaved water + autoclaved soil) respectively, when compared to treatment 1 (distilled water only) as control. Adult mosquitoes are known to possess a robust innate immune system that regulates bacterial infection and proliferation, hence presenting a confounding factor in the evaluation of bacteria's impact on adult survival due as a limitation in this experimental design (Meister *et al.*, 2009).

Nevertheless, it is important to note that the presence of bacteria in the midgut of mosquitoes in each treatment was established (Table 4.5). This could have

been derived from the bacteria contamination evidenced in the rearing milieu (Table 4.4) and/or from subsequent cage confinement in the insectary. This is characteristic of mosquito interaction with endosymbionts, which is established horizontally from the environment as opposed to vertically through trans-ovarial transmission as is often the case for other vectors of public health concern. Interestingly, the highest cfu counts came from group 4 treatment (autoclaved distilled water + autoclaved soil). The top 3 most common colonies all occurred in this group with the highest scoring a cfu of more than 300 representing an outlier compared to the rest of the cfu counts (range: 1-26).

A repeat of this experimental setup but employing a second soil sample from Mwea also gave similar results corroborating this outlier cfu count (unpublished). Endosymbiotic bacteria derived from the soil such as *Thorselia anophelis* (Briones *et al.*, 2008) were eliminated from the soil substrate by autoclaving. Such bacteria with stable association with the mosquito midgut may serve to protect against gut colonization by pathogens and other microbes (Douglas, 2009; Douglas and Beard, 1996; Dillon and Dillon, 2004). It is therefore possible that autoclaving of the soil substrate nullified similar benefits that could have accrued from the presence of soil-derived bacteria, resulting in the "outlier" high cfu counts observed in treatment 4.

5.2.2 The Effect of Resident Midgut Bacteria on Mosquito Survival: Rearing of Bacteria-free Mosquitoes

Efforts to rear bacteria-free mosquitoes by the use of broad spectrum antibiotics solution in the rearing milieu were frustrated by arrested development and high mortalities (Fig 4.7). The vast majority of larvae reared in water treated with

10µl/ml and 15µl/ml antibiotics solution died, with significant difference in cumulative mortality ($P = 0.0055$) and survival functions ($P < 0.0000$) compared to control. Cox regression hazard ratios indicated a 3 fold reduction in the survival odds when compared to the control group. This supports the findings of the field simulation study by demonstrating that bacteria are crucial if not necessary for the survival and development of mosquito larvae. The high mortalities (90-95%) observed in the antibiotic groups (Table 4.7) are also corroborated by Touré *et al.,* (2000) who reported that most 4th instar larvae of *An. gambiae* died before pupation when introduced to rearing water treated with 10 and 15µl/ml of a combination of penicillin, gentamycin, and streptomycin antibiotics. Similarly, Lindh (2007) reported that addition of antibiotics to the rearing water of *Ae. aegypti* stopped larval development indicating that bacteria may be a necessary component of the larval diet. Wotton *et al.,* (1997) also reported that *An. gambiae* and *An. quadrimaculatus* (Theobald) larvae reared in the presence of gentamycin were significantly smaller than larvae reared without the antibiotic, although no report on pupation rate and adult survival is given.

However, in the present study, a few larvae survived to pupa and adult stages indicating that a subpopulation of the colony stock can be able to survive and develop without bacteria. Efforts to boost the numbers by screening for larvae that were more likely survive using the inclusion criteria of 3 day old larvae hatched in antibiotics treated rearing milieu were successful with improved rates of 68% of larvae surviving to pupa stage. It is possible that this subpopulation may be representative of a genetic trait that allows them to develop despite the sterile diet, or

they may harbour small antibiotic resistant populations of bacteria that enable them to survive through symbiosis. It is also possible that this subgroup may be able to survive by cannibalizing of weaker larvae, a phenomenon that is not altogether uncommon, and thus the few stronger ones may access essential nutrients that they cannot synthesize or otherwise obtain from their diet for development.

Larval survival rates for the experimental group selected using the above inclusion criteria further demonstrated the crucial role of bacteria in mosquito larvae survival with significant difference in survival rates (Log rank test, P = 0.0005) and a 3.7 fold reduction in the survival odds compared to a control group of larvae from the same batch of eggs but not reared in antibiotic treated water (Fig 4.8). Moreover, comparison of survival functions between the adult female mosquitoes from the two groups were border line significant (Log rank test, P = 0.0365), indicating that rearing of bacteria free mosquitoes using antibiotics may adversely impact survival in the adult female mosquito that is of medical importance (Fig 4.10). However, Cox regression analysis was not significant (P = 0.07) with HR (1.04) indicating similar survival odds for the bacteria-free reared mosquito and control group reared under normal insectary conditions. Again, this may be attributed to the horizontal mosquito-bacteria association whereby endosymbionts are acquired from the environment. The bacteria-free reared mosquito though initially disadvantaged can boost their survival odds by acquiring bacteria from the environment and through sugar meals. Conversely, if disadvantaged the control group by harbouring bacteria can utilize the immune system to regulate bacteria population hence achieving similar survival odds with the experimental group.

Key to the interpretation of the results of the present study is the need to understand nutritional needs of the mosquito and the properties of antibiotics; either antibiotics are toxic to the larvae or that bacteria plays an essential role in the development of larvae. Hinman (1933) reported the development of a small number of bacteria-free *Ae. aegypti* larvae that were reared in filter sterilized water that was rich in organic matter. However, attempts to rear *Ae. aegypti* on various autoclaved media, bacteria killed at 60°C or by means of formaldehyde, and on filtrates from bacteria cultures in his subsequent studies were unsuccessful, indicative of a symbiotic relationship (Hinman, 1933). These early nutrition studies suggest that it is the absence of living bacteria in the larvae rearing milieu that is responsible for the mortality evidenced in the present study rather than the toxicity of antibiotics, which are routinely used for rearing of larvae in completely defined media that is able to support their development without any adverse effects (Akov, 1964).

5.2.3 The Effect of Resident Midgut Bacteria on Mosquito Survival: Converting From Septic to Aseptic Mosquitoes

In order to further elucidate the impact of bacteria association in the adult stage of mosquito lifecycle, conventionally reared mosquitoes were maintained on sugar meal laced with antibiotics to eliminate midgut microbiota and associated survival functions compared against conventionally maintained control group. The log rank test indicated significant difference ($P = 0.0077$) indicating accrued benefits associated with elimination of bacteria. This was further demonstrated by HR with a 1.56 fold increase in survival odds of the aseptic mosquitoes compared to the septic control group, which was significant (Cox regression, $P = 0.016$).

Interestingly, this is in contrast with the beneficial bacteria's symbiotic relationship demonstrated in the immature larval stages relationship in the preceding experiments. Whereas the nutritional requirements of all insects tend to be uniform (Dadd, 1985); their diets are remarkably diverse with microorganisms having long been implicated in the provision of B vitamins, amino acids and/or other essential nutrients to insects lacking both the capacity to synthesize them and unable to access them from their dietary sources (Douglas, 2009). Mosquitoes undergo complete metamorphosis in their transition from larva to adult that reflect different nutritional needs and dietary sources. The anopheline mosquito's larvae diet consists primarily of organic detritus, followed by bacteria, algae and protozoa, respectively that occur in the micro surface layer of the breeding habitat to meet their growth and developmental nutritional needs (Walker *et al.*, 1988); whereas adult mosquitoes utilize natural sugar sources such as nectar and plant sap to meet their equally divergent nutritional needs (Theobald, 1901; Knab, 1907). It is therefore conceivable for bacteria to have such a role reversal from beneficial symbiont in larval development to parasite/pathogen in the adult mosquito.

Additionally, symbiotic bacteria population sizes are known to increase dramatically following a blood-meal, with Dong *et al.*, (2009) reporting an increased average bacteria load of 10^6 cfu per midgut in blood fed *An. gambiae*, up from 10^4 cfu/midgut in sugar fed mosquitoes. This dramatic increase in bacteria population is regulated by *An. gambiae*'s innate immune system that recognizes peptidoglycan on bacteria cell wall and activates pathways that confer resistance to the infections

(Meister *et al.,* 2009), indicating that bacteria-mosquito interactions are dynamic and can shift from a mutually beneficial symbiosis to a harmful/parasitic relationship.

5.2.4 The Effect of Resident Midgut Bacteria on Mosquito Survival: Comparing Aseptic vs. Bacteria-fed Mosquitoes

Building on the previous study, the experimental in the present study was improved by introducing a bacteria-fed group for comparison with the antibiotic group. However, this experiment differs from the previous study (Experiment 3.5.3) in two key aspects; first, it evaluates longevity of female mosquito adult population only, and second it uses a higher concentration of antibiotics (20µl/ml). Surprisingly, the elimination of bacteria in the present study resulted in reduced longevity in contrast to the previous experiment where it was beneficial to the mosquitoes. Whereas the higher concentration of antibiotic may contribute to toxicity, these results are supported by the findings in Experiment 3.5.2 in which only half the dose (10µl/ml) is used for rearing bacteria-free mosqutoes, with significant (Log rank test, P = 0.0365) accrued benefits in the survival of female adult mosquitoes compared to the control group (Fig 4.6). This suggests that the segregation of mosquito population by gender may be a key factor in the interpretation of these results.

Nevertheless the role of bacteria in the adult mosquito is ambivalent at best owing to the constant flux in species constitution and their numbers as observed throughout this series of studies and also reported by Dong *et al.,* (2009). Adult mosquitoes possess robust innate immune systems that regulate symbiont bacteria

populations (Dong *et al.,* 2009; Meister *et al.,* 2009). It is therefore possible to conclude that the interaction of anopheline larvae with symbiotic midgut microbiota ranges from commensual to mutualistic (i.e. beneficial if not necessary), whereas it is even broader in the adult mosquito population where it changes from harmful to beneficial from time to time.

5.3 The Effect of Salivary Gland Homogenate on P. falciparum Development in Experimentally Infected An. gambiae

P. falciparum oocyst development in experimentally infected mosquitoes that were provided with an infective blood meal spiked with salivary gland homogenate did not differ significantly (P = 0.9601) when compared with a control group that was fed an infective blood meal alone indicating saliva did not enhance nor impede parasite development in the midgut.

The salivary glands of *An. stephensi* contain xanthurenic acid, which has been shown to induce exflagellation activity of the male gametocyte *in vitro* (Bilker *et al.,* 1998; Hirai *et al.,* 2001). The present study also anticipated an indirect effect on *Plasmodium* development through saliva's suppression of protease activity. This was deemed to be advantageous on the one hand by enhancing *Plasmodium* defensive mechanism in the suppression of serine proteases, but also disadvantageous by abrogating *Plasmodium* upregulation of aminopeptidase, a primary parasite receptor for midgut invasion (Fig 4.4).

Exflagellation of the male gametocyte in the midgut is also induced by other factors such as temperature, pH and levels of CO_2 (Billker *et al.,* 1997); thus

presenting confounding factors in the interpretation of the results in this experiment. Similarly, the added effect of SGH to the infective blood meal is both for and against the parasite. It is therefore instructive that no benefit accrued to either the mosquito or the parasite in the early *Plasmodium* development stages, was derived from the experimental addition of salivary gland homogenate in the blood meal. However, this demonstrates the need for further studies that will determine and isolate the SGH constituents with suppression activity of serine proteases and aminopeptidase in order to evaluate their potential for transmission blocking separately.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

Salivary glands and their secretions are central to the interaction between parasite, vector and mammalian host. Saliva that is secreted into the host during blood feeding plays a critical role in overcoming the challenges posed to the mosquito by the host: pain and itch responses, immune defenses and hemostasis thus facilitating blood feeding and contributing to pathogen transmission (Ribeiro 1995; Ribeiro and Francischetti 2003). However, genomic and proteomic analysis of mosquito salivary glands documented the presence of numerous proteins of diverse molecular weights that are secreted from the salivary glands, the majority of which the role remains unknown (Arca et al., 2005). Whereas previous studies have dealt with the properties of saliva and its role in the facilitation of blood feeding and transmission blocking studies, this study is the first that investigates the role of saliva in the physiology of the vector as pertains to *Plasmodium* development in the midgut.

The present study has demonstrated that although saliva is associated with several antimicrobial proteins, salivary gland homogenate and fractions thereof failed to inhibit endosymbiont bacteria *in vitro,* speculating that this may be due to the non-invasive nature of bacteria acquisition as a key component of the larval diet. Additionally, the present study also evaluated the effect of midgut endosymbiont bacteria on mosquito survival demonstrating key differences in the immature larval-

stage whereby bacteria plays a crucial role in development and survival, as opposed to in the adult mosquito where the role of bacteria becomes ambivalent.

This is also the first study to demonstrate that saliva is capable of suppressing midgut protease activity *in vivo*. Interestingly, the presence of *Plasmodium* alone (without augmented saliva) was observed to have both a defensive mechanism in the downregulation of serine proteases and an advantageous upregulation of aminopeptidase that serves as a ligand receptor to ookinetes during midgut invasion in the *Plasmodium* life cycle. However, oocyst counts in the saliva spiked treatment did not differ significantly when compared with *Plasmodium* alone indicating that saliva's impediment of the aminopeptidase upregulation did not affect parasite development in the mosquito. It is therefore possible to conclude that saliva may play an important role in vector physiology by suppressing protease activity but does not affect *Plasmodium* development in the midgut.

The findings of this study are both timely and significant in that the new national malaria strategy calls for the elimination of malaria by the year 2017 (GoK, 2009). This bold and ambitious goal though envisaged to be achieved using the current interventions, will undoubtedly encounter new challenges in the maintenance of zero transmission that IRS, ITNs and ACT chemotherapy are ill equipped to handle. The adoption of new innovative tools that exploit emerging knowledge of the malaria vector and its transmission of *Plasmodium* is therefore recommended. The findings of this study contribute to the body of knowledge that can be exploited for the control and eradication of malaria through future studies.

This includes the promotion of *Bacillus thuringiensis israelensis* (Bti) and *B. sphaericus* as a biological control strategy that exploits the crucial role of bacteria in the mosquito larval diet as a larvicidal. In addition, further studies employing culture-independent techniques should be carried out to bioprospect for stably associating endosymbiont bacteria that persist in the midgut of *An. gambiae* through trans-stadial transmission. Such bacteria should be evaluated for their potential in paratransgenics whereby they can be exploited in transmission blocking mechanisms and/or sterile insect technique amenable to the area-wide vector control paradigm (Chen *et al.*, 2008; McMeniman *et al.*, 2009; Kambris *et al.*, 2009; Catteruccia *et al.*, 2009; Robinson *et al.*, 2009).

Similarly, protease suppression properties can and should be harnessed for malaria control/elimination. The characterization of compounds/proteins in saliva that possess the protease suppression properties and the mode of action is therefore recommended. The trypsin modulating oostatic factor (TMOF) is one such molecule with protease suppression properties that was initially discovered in the *Ae. aegypti* female mosquito. By suppressing trypsin, TMOF would impede digestion in larvae therefore leading to death from starvation, with this protease suppression property having since been exploited as a larvicidal (Borovosky, 2003).

Alternatively, the protease suppression properties may be harnessed using paratransgenics and exploited to reverse insecticide resistance which has recently been linked to the over expression of serine proteases in *Culex pipiens* (Gong *et al.*, 2005). Lastly, the mode of aminopeptidase suppression should be investigated further and carefully examined to determine how it can be exploited in transmission

blocking mechanisms. Moreso, in light of aminopeptidase's upregulation by *Plasmodium* and its relatively new found status as a TBV candidate target for its role in ookinete invasion of the midgut (Dinglasan *et al.*, 2007).

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APPENDIX I

ANALYSIS AND COMPARISON OF ENZYMES ASSAY DATA

A. Enzyme levels (mean optical densities)

Table A.1: Overall trends for combined (trypsin, chymotrypsin and aminopeptidase) enzyme levels (mean optical densities) across treatments

Treatment	Mean	s.e	95% CI for Mean Lower	Upper
Blood	0.1797	0.0011	0.1776	0.1818
Blood +Plasmodium	0.1362	0.0011	0.1341	0.1383
Blood +Plasmodium +SGH	0.1061	0.0011	0.1040	0.1081

A.1. ANOVA modeling of enzyme levels (mean optical densities)

Analysis of variance (ANOVA) was used to model variability in enzyme levels (mean optical densities) using three factors namely; type of blood meal coded "Treatment", duration post blood feeding coded "hr PBF", and the different proteases assayed (trypsin, chymotrypsin or aminopeptidase) coded "Enzyme". A saturated model was fitted using these factors. The outcome of the model is shown in Table A.2.

Table A.2: Analysis of variability in enzyme levels (mean optical densities)

Source of variability	Sum of Squares	df	Mean Square	F	P value
Treatment	0.41110	2	0.205550420	1211.80	<0.001*
Hr PBF	0.38201	4	0.095501610	563.02	<0.001*
Enzyme	0.01269	2	0.006346455	37.41	<0.001*
Treatment * hr PBF * Enzyme	0.36677	36	0.010188108	60.06	<0.001*
Residual (Error)	0.06870	405	0.000169624		
Total	1.24127	449			

R Squared =0.945 (Adjusted R Squared = 0.939)
* Variability is significant at P<0.05.

Adjusting for hr PBF, Enzyme and the interaction term (Treatment * hr PBF * Enzyme), the ANOVA model revealed a significant Treatment effect on enzyme levels (mean optical densities) (P<0.001). Similarly, adjusting for Treatment, Enzyme and the interaction term (Treatment * hr PBF * Enzyme), hr PBF had a significant effect on variability in enzyme levels (mean optical densities) (P<0.001). The effect of Enzyme on enzyme levels (mean optical densities) was

equally significant upon adjusting for Treatment, hr PBF and the interaction term (Treatment * hr PBF * Enzyme).
Total variability in enzyme levels (mean optical densities) explained by the model was 94.5%. Variability due to the treatment effect accounted for 33.1% while the hr PBF effect accounted for 30.8%. The effect due to Enzyme was small compared to the rest accounting for 1.0% of the total variability. The interaction term significantly accounted for 29.6% of the total variability in enzyme levels (mean optical densities).

Table A.3: Comparisons of overall enzyme level changes across treatments (type of feed) using Tukey HSD post hoc analysis

(I) Treatment	(J) Treatment	Mean Difference (I-J)	s.e.	95% CI for Mean Difference (I-J) Lower	Upper	P value
Blood	Blood +Plasmodium	0.043	0.002	0.041	0.046	<0.001
Blood	Blood +Plasmodium +SGH	0.074	0.002	0.071	0.077	<0.001
Blood +Plasmodium	Blood +Plasmodium +SGH	0.030	0.002	0.027	0.033	<0.001

B. Overall enzyme levels (mean optical densities) at 6, 12, 18, 24 and 48 hr post feed end points

Table B.1: Overall trends in enzyme levels (mean optical densities) across treatments at 6, 12, 18, 24 and 48 hr post feed end points

End Point	Treatment	Mean	s.e	95% CI for Mean Lower	Upper
6 hours	Blood	0.206	0.003	0.200	0.212
	Blood +Plasmodium	0.112	0.003	0.106	0.117
	Blood +Plasmodium +SGH	0.093	0.003	0.087	0.098
12 hours	Blood	0.157	0.001	0.155	0.159
	Blood +Plasmodium	0.113	0.001	0.110	0.115
	Blood +Plasmodium +SGH	0.094	0.001	0.092	0.096
18 hours	Blood	0.252	0.003	0.246	0.258
	Blood +Plasmodium	0.186	0.003	0.180	0.192
	Blood +Plasmodium +SGH	0.134	0.003	0.128	0.140
24 hours	Blood	0.162	0.003	0.157	0.168
	Blood +Plasmodium	0.168	0.003	0.162	0.173
	Blood +Plasmodium +SGH	0.119	0.003	0.113	0.124
48 hours	Blood	0.121	0.002	0.118	0.124
	Blood +Plasmodium	0.103	0.002	0.100	0.106
	Blood +Plasmodium +SGH	0.091	0.002	0.088	0.094

Table B.2: Analysis of Variability in overall enzyme levels (mean optical densities) due to treatment (type of feed) and Enzyme at 6, 12, 18, 24 and 48 hr post feed end points

End Point	Source of variability	Sum of Squares	df	Mean Square	F	P value
6 hours [a]	Treatment	0.22184	2	0.110917878	439.17	<0.001
	Enzyme	0.00617	2	0.003082800	12.21	<0.001
	Treatment * Enzyme	0.03781	4	0.009453022	37.43	<0.001
	Residual (Error)	0.02046	81	0.000252562		
	Total	0.28627	89			
12 hours [b]	Treatment	0.06303	2	0.031514359	781.61	<0.001
	Enzyme	0.01460	2	0.007297600	180.99	<0.001
	Treatment * Enzyme	0.02265	4	0.005662437	140.44	<0.001
	Residual (Error)	0.00327	81	0.000040320		
	Total	0.10354	89			
18 hours [c]	Treatment	0.21099	2	0.105493872	429.30	<0.001
	Enzyme	0.04653	2	0.023262979	94.67	<0.001
	Treatment * Enzyme	0.05030	4	0.012575642	51.18	<0.001
	Residual (Error)	0.01990	81	0.000245737		
	Total	0.32772	89			
24 hours [d]	Treatment	0.04263	2	0.021317411	90.50	<0.001
	Enzyme	0.00730	2	0.003647781	15.49	<0.001
	Treatment * Enzyme	0.01328	4	0.003320565	14.10	<0.001
	Residual (Error)	0.01908	81	0.000235543		
	Total	0.08229	89			
48 hours [e]	Treatment	0.01369	2	0.006842915	92.53	<0.001
	Enzyme	0.03349	2	0.016743826	226.40	<0.001
	Treatment * Enzyme	0.00628	4	0.001569030	21.22	<0.001
	Residual (Error)	0.00599	81	0.000073957		
	Total	0.05944	89			

[a] – R Squared = 0.968 (Adjusted R Squared = 0.965)
[b] – R Squared = 0.968 (Adjusted R Squared = 0.965)
[c] – R Squared = 0.939 (Adjusted R Squared = 0.933)
[d] – R Squared = 0.768 (Adjusted R Squared = 0.745)
[e] – R Squared = 0.899 (Adjusted R Squared = 0.889)

Table B.3: Comparisons of enzyme level changes across treatments (type of feed) at 6, 12, 18, 24 and 48 hr post feed end points (Tukey HSD)

End Point	(I) Treatment	(J) Treatment	Mean Difference (I-J)	s.e.	95% CI for Mean Difference (I-J) Lower	Upper	P value
6 hours	Blood	Blood +Plasmodium	0.095	0.004	0.086	0.103	<0.001
	Blood	Blood +Plasmodium +SGH	0.114	0.004	0.105	0.122	<0.001
	Blood +Plasmodium	Blood +Plasmodium +SGH	0.019	0.004	0.011	0.027	<0.001
12 hours	Blood	Blood +Plasmodium	0.045	0.002	0.041	0.048	<0.001
	Blood	Blood +Plasmodium +SGH	0.063	0.002	0.060	0.066	<0.001
	Blood +Plasmodium	Blood +Plasmodium +SGH	0.019	0.002	0.015	0.022	<0.001
18 hours	Blood	Blood +Plasmodium	0.066	0.004	0.058	0.074	<0.001
	Blood	Blood +Plasmodium +SGH	0.118	0.004	0.110	0.126	<0.001
	Blood +Plasmodium	Blood +Plasmodium +SGH	0.052	0.004	0.044	0.060	<0.001
24 hours	Blood	Blood +Plasmodium +SGH	0.043	0.004	0.035	0.051	<0.001
	Blood +Plasmodium	Blood	0.005	0.004	-0.002	0.013	0.177
	Blood +Plasmodium	Blood +Plasmodium +SGH	0.049	0.004	0.041	0.057	<0.001
48 hours	Blood	Blood +Plasmodium	0.018	0.002	0.013	0.022	<0.001
	Blood	Blood +Plasmodium +SGH	0.030	0.002	0.026	0.034	<0.001
	Blood +Plasmodium	Blood +Plasmodium +SGH	0.012	0.002	0.008	0.017	<0.001

C. Trypsin levels (mean optical densities) at 6, 12, 18, 24 and 48 hr post feed end points for Trypsin

Table C.1: Overall trends in trypsin levels (mean optical densities) across treatments at 6, 12, 18, 24 and 48 hr post feed end points

End Point	Treatment	Mean	s.e	95% CI for Mean Lower	Upper
6 hours	Blood	0.1709	0.0024	0.1661	0.1758
	Blood +Plasmodium	0.1234	0.0024	0.1186	0.1283
	Blood +Plasmodium +SGH	0.1130	0.0024	0.1081	0.1179
12 hours	Blood	0.1380	0.0011	0.1358	0.1402
	Blood +Plasmodium	0.1211	0.0011	0.1189	0.1233
	Blood +Plasmodium +SGH	0.1194	0.0011	0.1172	0.1215
18 hours	Blood	0.2254	0.0064	0.2123	0.2385
	Blood +Plasmodium	0.1680	0.0064	0.1549	0.1812
	Blood +Plasmodium +SGH	0.1377	0.0064	0.1246	0.1508
24 hours	Blood	0.1554	0.0059	0.1432	0.1676
	Blood +Plasmodium	0.1580	0.0059	0.1459	0.1702
	Blood +Plasmodium +SGH	0.1309	0.0059	0.1188	0.1431
48 hours	Blood	0.1201	0.0018	0.1163	0.1238
	Blood +Plasmodium	0.1295	0.0018	0.1258	0.1333
	Blood +Plasmodium +SGH	0.0980	0.0018	0.0942	0.1017

Table C.2: Analysis of Variability in trypsin levels (mean optical densities) due to treatment (type of feed) at 6, 12, 18, 24 and 48 hr post feed end points

End Point	Source of variability	Sum of Squares	Df	Mean Square	F	P value
6 hours [a]	Treatment	0.01907	2	0.009535626	170.33	<0.001
	Residual (Error)	0.00151	27	0.000055982		
	Total	0.02058	29			
12 hours [b]	Treatment	0.00212	2	0.001059693	94.36	<0.001
	Residual (Error)	0.00030	27	0.000011230		
	Total	0.00242	29			
18 hours [c]	Treatment	0.03967	2	0.019837226	48.39	<0.001
	Residual (Error)	0.01107	27	0.000409923		
	Total	0.05074	29			
24 hours [d]	Treatment	0.00447	2	0.002233270	6.36	0.005
	Residual (Error)	0.00948	27	0.000351210		
	Total	0.01395	29			
48 hours [e]	Treatment	0.00525	2	0.002624137	79.76	<0.001
	Residual (Error)	0.00089	27	0.000032901		
	Total	0.00614	29			

[a] – R Squared = 0.927 (Adjusted R Squared = 0.921)
[b] – R Squared = 0.875 (Adjusted R Squared = 0.866)
[c] – R Squared = 0.782 (Adjusted R Squared = 0.766)
[d] – R Squared = 0.320 (Adjusted R Squared = 0.270)
[e] – R Squared = 0.855 (Adjusted R Squared = 0.845)

Table C.3: Comparisons of trypsin level changes across treatments (type of feed) at 6, 12, 18, 24 and 48 hr post feed end points (Tukey HSD)

End Point	(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	95% CI for Mean Difference (I-J) Lower	Upper	P value
6 hours	Blood	Blood +Plasmodium	0.048	0.003	0.041	0.054	<0.001
	Blood	Blood +Plasmodium +SGH	0.058	0.003	0.051	0.065	<0.001
	Blood +Plasmodium	Blood +Plasmodium +SGH	0.010	0.003	0.004	0.017	0.004
12 hours	Blood	Blood +Plasmodium	0.017	0.001	0.014	0.020	<0.001
	Blood	Blood +Plasmodium +SGH	0.019	0.001	0.016	0.022	<0.001
	Blood +Plasmodium	Blood +Plasmodium +SGH	0.002	0.001	-0.001	0.005	0.258
18 hours	Blood	Blood +Plasmodium	0.057	0.009	0.039	0.076	<0.001
	Blood	Blood +Plasmodium +SGH	0.088	0.009	0.069	0.106	<0.001
	Blood +Plasmodium	Blood +Plasmodium +SGH	0.030	0.009	0.012	0.049	0.002
24 hours	Blood	Blood +Plasmodium +SGH	0.024	0.008	0.007	0.042	0.007
	Blood +Plasmodium	Blood	0.003	0.008	-0.015	0.020	0.756
	Blood +Plasmodium	Blood +Plasmodium +SGH	0.027	0.008	0.010	0.044	0.003
48 hours	Blood	Blood +Plasmodium +SGH	0.022	0.003	0.017	0.027	<0.001
	Blood +Plasmodium	Blood	0.009	0.003	0.004	0.015	0.001
	Blood +Plasmodium	Blood +Plasmodium +SGH	0.032	0.003	0.026	0.037	<0.001

D. Chymotrypsin levels (mean optical densities) at 6, 12, 18, 24 and 48 hr post feed end points

Table D.1: Overall trends in chymotrypsin levels (mean optical densities) across treatments at 6, 12, 18, 24 and 48 hr post feed end points

End Point	Treatment	Mean	s.e	95% CI for Mean Lower	Upper
6 hours	Blood	0.233	0.008	0.217	0.248
	Blood +Plasmodium	0.088	0.008	0.073	0.104
	Blood +Plasmodium +SGH	0.061	0.008	0.045	0.076
12 hours	Blood	0.167	0.003	0.161	0.173
	Blood +Plasmodium	0.082	0.003	0.076	0.088
	Blood +Plasmodium +SGH	0.063	0.003	0.056	0.069
18 hours	Blood	0.328	0.005	0.319	0.338
	Blood +Plasmodium	0.206	0.005	0.197	0.215
	Blood +Plasmodium +SGH	0.134	0.005	0.125	0.143
24 hours	Blood	0.171	0.004	0.163	0.180
	Blood +Plasmodium	0.149	0.004	0.141	0.157
	Blood +Plasmodium +SGH	0.097	0.004	0.089	0.106
48 hours	Blood	0.097	0.002	0.092	0.101
	Blood +Plasmodium	0.072	0.002	0.067	0.076
	Blood +Plasmodium +SGH	0.066	0.002	0.061	0.070

Table D.2: Analysis of Variability in chymotrypsin levels (mean optical densities) due to treatment (type of feed) at 6, 12, 18, 24 and 48 hr post feed end points

End Point	Source of variability	Sum of Squares	df	Mean Square	F	P value
6 hours [a]	Treatment	0.17039	2	0.085197181	148.27	<0.001
	Residual (Error)	0.01551	27	0.000574609		
	Total	0.18591	29			
12 hours [b]	Treatment	0.06159	2	0.030794193	341.18	<0.001
	Residual (Error)	0.00244	27	0.000090257		
	Total	0.06403	29			
18 hours [c]	Treatment	0.19279	2	0.096397026	464.68	<0.001
	Residual (Error)	0.00560	27	0.000207447		
	Total	0.19840	29			
24 hours [d]	Treatment	0.02879	2	0.014394059	87.49	<0.001
	Residual (Error)	0.00444	27	0.000164522		
	Total	0.03323	29			
48 hours [e]	Treatment	0.00541	2	0.002705293	54.74	<0.001
	Residual (Error)	0.00133	27	0.000049418		
	Total	0.00674	29			

[a] – R Squared = 0.917 (Adjusted R Squared = 0.910)
[b] – R Squared = 0.962 (Adjusted R Squared = 0.959)
[c] – R Squared = 0.972 (Adjusted R Squared = 0.970)
[d] – R Squared = 0.866 (Adjusted R Squared = 0.856)
[e] – R Squared = 0.802 (Adjusted R Squared = 0.788)

Table D.3: Comparisons of chymotrypsin level changes across treatments (type of feed) at 6, 12, 18, 24 and 48 hr post feed end points (Tukey HSD)

End Point	(I) Treatment	(J) Treatment	Mean Difference (I-J)	s.e.	95% CI for Mean Difference (I-J) Lower	Upper	P value
6 hours	Blood	Blood +Plasmodium	0.144	0.011	0.122	0.166	<0.001
	Blood	Blood +Plasmodium +SGH	0.172	0.011	0.150	0.194	<0.001
	Blood +Plasmodium	Blood +Plasmodium +SGH	0.028	0.011	0.006	0.050	0.016
12 hours	Blood	Blood +Plasmodium	0.085	0.004	0.076	0.094	<0.001
	Blood	Blood +Plasmodium +SGH	0.104	0.004	0.096	0.113	<0.001
	Blood +Plasmodium	Blood +Plasmodium +SGH	0.019	0.004	0.010	0.028	<0.001
18 hours	Blood	Blood +Plasmodium	0.122	0.006	0.109	0.135	<0.001
	Blood	Blood +Plasmodium +SGH	0.194	0.006	0.181	0.207	<0.001
	Blood +Plasmodium	Blood +Plasmodium +SGH	0.072	0.006	0.059	0.085	<0.001
24 hours	Blood	Blood +Plasmodium	0.022	0.006	0.011	0.034	0.001
	Blood	Blood +Plasmodium +SGH	0.074	0.006	0.062	0.086	<0.001
	Blood +Plasmodium	Blood +Plasmodium +SGH	0.052	0.006	0.040	0.063	<0.001
48 hours	Blood	Blood +Plasmodium	0.025	0.003	0.018	0.031	<0.001
	Blood	Blood +Plasmodium +SGH	0.031	0.003	0.025	0.038	<0.001
	Blood +Plasmodium	Blood +Plasmodium +SGH	0.006	0.003	0.000	0.013	0.060

E. Aminopeptidase levels (mean optical densities) at 6, 12, 18, 24 and 48 hr post feed end points

Table E.1: Overall trends in aminopeptidase levels (mean optical densities) across treatments at 6, 12, 18, 24 and 48 hr post feed end points

End Point	Treatment	Mean	s.e	95% CI for Mean Lower	Upper
6 hours	Blood	0.215	0.004	0.208	0.222
	Blood +Plasmodium	0.123	0.004	0.116	0.130
	Blood +Plasmodium +SGH	0.104	0.004	0.097	0.112
12 hours	Blood	0.166	0.001	0.164	0.169
	Blood +Plasmodium	0.135	0.001	0.132	0.138
	Blood +Plasmodium +SGH	0.100	0.001	0.097	0.103
18 hours	Blood	0.203	0.003	0.196	0.210
	Blood +Plasmodium	0.184	0.003	0.177	0.191
	Blood +Plasmodium +SGH	0.130	0.003	0.123	0.137
24 hours	Blood	0.160	0.004	0.151	0.169
	Blood +Plasmodium	0.196	0.004	0.187	0.205
	Blood +Plasmodium +SGH	0.128	0.004	0.119	0.137
48 hours	Blood	0.146	0.004	0.139	0.154
	Blood +Plasmodium	0.108	0.004	0.101	0.116
	Blood +Plasmodium +SGH	0.109	0.004	0.102	0.117

Table E.2: Analysis of Variability in aminopeptidase levels (mean optical densities) due to treatment (type of feed) at 6, 12, 18, 24 and 48 hr post feed end points

End Point	Source of variability	Sum of Squares	df	Mean Square	F	P value
6 hours [a]	Treatment	0.07018	2	0.035091115	276.10	<0.001
	Residual (Error)	0.00343	27	0.000127095		
	Total	0.07361	29			
12 hours [b]	Treatment	0.02197	2	0.010985348	564.17	<0.001
	Residual (Error)	0.00053	27	0.000019472		
	Total	0.02250	29			
18 hours [c]	Treatment	0.02882	2	0.014410904	120.25	<0.001
	Residual (Error)	0.00324	27	0.000119840		
	Total	0.03206	29			
24 hours [d]	Treatment	0.02266	2	0.011331211	59.36	<0.001
	Residual (Error)	0.00515	27	0.000190896		
	Total	0.02782	29			
48 hours [e]	Treatment	0.00930	2	0.004651544	33.33	<0.001
	Residual (Error)	0.00377	27	0.000139551		
	Total	0.01307	29			

[a] – R Squared = 0.953 (Adjusted R Squared = 0.950)
[b] – R Squared = 0.977 (Adjusted R Squared = 0.975)
[c] – R Squared = 0.899 (Adjusted R Squared = 0.892)
[d] – R Squared = 0.815 (Adjusted R Squared = 0.801)
[e] – R Squared = 0.712 (Adjusted R Squared = 0.690)

Table E.3: Comparisons of aminopeptidase level changes across treatments (type of feed) at 6, 12, 18, 24 and 48 hr post feed end points (Tukey HSD)

End Point	(I) Treatment	(J) Treatment	Mean Difference (I-J)	s.e.	95% CI for Mean Difference (I-J) Lower	Upper	P value
6 hours	Blood	Blood +Plasmodium	0.092	0.005	0.082	0.102	0.000
	Blood	Blood +Plasmodium +SGH	0.111	0.005	0.100	0.121	0.000
	Blood +Plasmodium	Blood +Plasmodium +SGH	0.019	0.005	0.008	0.029	0.001
12 hours	Blood	Blood +Plasmodium	0.032	0.002	0.028	0.036	0.000
	Blood	Blood +Plasmodium +SGH	0.066	0.002	0.062	0.070	0.000
	Blood +Plasmodium	Blood +Plasmodium +SGH	0.035	0.002	0.031	0.039	0.000
18 hours	Blood	Blood +Plasmodium	0.019	0.005	0.009	0.029	0.001
	Blood	Blood +Plasmodium +SGH	0.073	0.005	0.063	0.083	0.000
	Blood +Plasmodium	Blood +Plasmodium +SGH	0.054	0.005	0.044	0.064	0.000
24 hours	Blood	Blood +Plasmodium +SGH	0.031	0.006	0.019	0.044	0.000
	Blood +Plasmodium	Blood	0.036	0.006	0.023	0.049	0.000
	Blood +Plasmodium	Blood +Plasmodium +SGH	0.067	0.006	0.055	0.080	0.000
48 hours	Blood	Blood +Plasmodium	0.038	0.005	0.027	0.049	0.000
	Blood	Blood +Plasmodium +SGH	0.037	0.005	0.026	0.048	0.000
	Blood +Plasmodium +SGH	Blood +Plasmodium	0.001	0.005	-0.010	0.012	0.886