UNIVERSITY OF GHANA

COLLEGE OF BASIC AND APPLIED SCIENCES

IN VITRO STUDIES OF THE EFFECT OF *ANOPHELES GAMBIAE* MIDGUT BACTERIA ON THE DEVELOPMENT OF *PLASMODIUM FALCIPARUM*

BY

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DECLARATION

I, Godwin Williams Ametsi, do certify that this project aside other cited works was carried out by me under the supervision of Dr. Jewelna Efua Birago Akorli and Dr. Yaw Aniweh, and that reference made to the works of others have been duly acknowledged. I certify that no part of this thesis has been previously submitted for a degree or any other qualification

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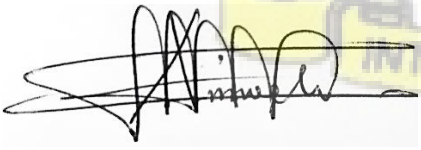

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ABSTRACT

During blood feeding, female *Anopheles* mosquitoes may ingest *Plasmodium* gametocytes which undergo transformation in the gut and develop into sporozoites that are infectious to humans. Bacteria inhabit the mosquito gut, and the number and diversity of these bacteria change following blood feeding. The presence of some bacteria species results in the reduced intensity of developing *Plasmodium* parasites. Little attention has been given to understanding this direct mechanism of bacteria on *Plasmodium* parasites, and the effects of bacteria on malaria parasite developmental genes are not completely understood. This limits the scope of how gut bacteria, for example *Enterobacter* and *Serratia*, which have been found with anti-*Plasmodium* effects can be further explored for alternative disease control strategies. Therefore, this study investigated the lethal effect of cell-free secreted bio-products of *E. cloacae* and *S. marcescens* on a key *Plasmodium* parasite developmental gene (Gamete release gene, *GAMER*) for its potential as a target for malaria transmission-blocking.

Plasmodium falciparum 3D7 and Dd2 cultures at 1% parasitaemia were independently exposed to spent Luria-Bertani (LB) medium from varying concentrations of *Enterobacter cloacae* and *Serratia marcescens*. The parasite killing effect of the bacteria were assessed with SYBR green fluorescent assay after 48 hours of co-culture. Spent media with final bacteria concentration between $10e^{+10}$-$10e^{+20}$ reduced parasitaemia ($P<0.001$) compared to parasite culture without bacteria treatment. Using real-time (quantitative) PCR, it was found that the expression of *GAMER* was down regulated by 2 folds after 1 hour of screening *P. falciparum* 3D7 with cell-free spent medium of *E. cloacae* cultured for 8 hours in LB broth (*Ec*-8). However, the expression of *GAMER* was unaffected after 6 and 12 hours of screening *P. falciparum* 3D7 with *Ec*-8. These data provide information for further studies on gene and protein targets for transmission blocking interventions.

DEDICATION

This work is dedicated to my mother, Mrs. Beatrice Setor for her continuous encouragement over the years and her selfless contribution to my education. God bless you; mum and I love you so dearly.

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LIST OF ABBREVIATIONS

ACTs - Artemisinin based combination therapies

AQ - Amodiaquine

AS - Artesunate

Bti - *Bacillus thuringiensis subspecies Israelensis*

CQ - Chloroquine

DHFR - Dihydro folate reductase

DHPS - Dihydropterate synthase

DNA - Deoxyribonucleic acid

GAMER - Gamete release gene

HADO - Haloacid dehalogenase domain ookinete protein gene

HRP2 - Histidine-rich protein 2

IPT - Intermittent preventive treatment

IPTc - Intermittent preventive treatment in children younger than 5 years

IPTi - Intermittent preventive treatment in infants

IPTp - Intermittent preventive treatment in pregnant women

IRS - Indoor residual spraying

ITNs - Insecticide-treated nets

IVM - Integrated vector management

LAMP - Loop-mediated isothermal amplification

LLINs - Long-lasting insecticide-treated bed nets

PCR - Polymerase chain reaction

PE - Protective efficacy

Pf - *Plasmodium falciparum*

PfCRT - *Plasmodium falciparum* chloroquine resistant transporter,

PfEMP1 - *Plasmodium falciparum* erythrocyte membrane protein 1

PfHRP2 - *Plasmodium falciparum* histidine-rich protein 2

PfHRP3 - *Plasmodium falciparum* histidine-rich protein 3

pLDH - *Plasmodium* lactate dehydrogenase

PVM - Parasitophorous vacuolar membrane

qPCR - Quantitative polymerase chain reaction

qPCR - Quantitative real time polymerase chain reaction

RDT - Rapid diagnostic test

RNA - Ribonucleic acid

RT-qPCR - Reverse transcriptase real time polymerase chain reaction

s.l - *Anopheles gambiae senso lacto*

s.s - *Anopheles gambiae senso stricto*

SIT - Sterile insect technique

SP - Sulfadoxine-Pyrimethamine

TBV - Transmission blocking vaccine

TTM - Transfusion-transmitted malaria

WHO - World Health Organization

CHAPTER ONE

1.0 GENERAL INTRODUCTION

1.1 Introduction

Malaria is an infectious disease caused by an obligate intracellular protozoan parasite of the genus *Plasmodium* and transmitted through the bites from infected female *Anopheles* mosquitoes. Although there are several species responsible for malaria, the six (6) species known to infect humans are *Plasmodium falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, *P. knowlesi* and *P. cynomolgi* (World Health Organization, 2021). In 2020, the World Health Organization (WHO) reported 241 million malaria cases worldwide with 627, 000 deaths; 95% of these cases were recorded in Africa. Children (≤ 5 years) and pregnant women are the most susceptible (World Health Organization, 2021). The disease is mainly controlled by activities that effectively kill mosquitoes and the use of drugs that target the parasites within the human host.

Vector control interventions remain important in fighting malaria (Benelli and Beier, 2017). These typically exploit the unique breeding, feeding and resting dynamics of the *Anopheles* vector. The consistent use of pyrethroids for indoor residual spraying (IRS) and in insecticide-treated nets (ITNs) represent key means to eliminate the vectors. Larvivorous fishes are also employed as biological agents for reducing mosquito population (Howard, Zhou and Omlin, 2007; Kondrashin *et al.*, 2017). Other biological strategies include the use of niche competitors, entomopathogens, (Ramirez *et al.*, 2018) and plasmodial symbiotic organisms (Geissbühler *et al.*, 2009; Abdul-Ghani, Al-Mekhlafi and Alabsi, 2012). Personal protective interventions for the at-risk population and roll-out of small-scale environmental management strategies are also helpful in vector control (Kondrashin *et al.*, 2017). The WHO initiated an Integrated Vector Management approach to achieve significant results in the control of mosquito-borne diseases

as most strategies have not been successful when used as stand-alone projects (Ministry of Health-Republic of Ghana, 2009; WHO, 2015; Herdiana, Sari and Whittaker, 2018).

Malaria treatment (Chemotherapy) involves the use of anti-malarial medicines to target different pathways of the parasite. Currently, Artemisinin-based combination therapy (ACT) has been recommended by WHO as frontline antimalarial drugs for the treatment of malaria (World Health Organization, 2019). However, increased reports of parasite resistance to antimalarial drugs (World Health Organization, 2010) especially ACTs (Dondorp *et al.*, 2009; Ariey *et al.*, 2014) threaten the global efforts to fight the disease. It is in this regard that vaccines are being considered as alternatives for use as drugs in malaria treatment. Vaccines have shown sufficient efficacy in the control and prevention of diseases like smallpox (Rieckmann *et al.*, 2017), poliomyelitis and measles. However, there is currently no effective anti-malaria vaccine as the most promising anti-malaria vaccine RTS,S/AS01 undergoing WHO-recommended pilot implementation in some parts of Africa, has been reported to have low prevention and efficacy indices (Moorthy and Okwo-Bele, 2015) and its efficacy declines over time (RTS, 2014; Olotu *et al.*, 2016). The periodic emergence of anti-malarial drug resistance, lack of effective anti-malarial vaccines and failing vector-control methods may have contributed to the estimated 627, 000 malaria-related deaths reported globally in 2020, compared with the 416, 000 estimated deaths in 2019 as recorded in the WHO 2021 World Malaria Report. This alarming statistic calls for the need for a paradigm shift to identify novel approaches for the control of malaria.

Mosquito midgut microbiota is found to be major players in the ecology and physiology of mosquitoes including impact on resistance to mosquito control agents (Berticat *et al.*, 2002; Duron *et al.*, 2006), fitness (Strand, 2018) and vector competence (Dennison, Jupatanakul and Dimopoulos, 2014). In recent times, there has been heightened interest in research to identify suitable commensal microbes (bacteria, fungi and/or viruses) in disease vectors such as blood-

sucking bugs, tsetse flies and mosquitoes that can be explored for control of diseases like leishmaniasis (Karimian *et al.*, 2019), trypanosomiasis and malaria (Azambuja, Garcia and Ratcliffe, 2005; Geiger *et al.*, 2009). This knowledge has been important for the development of novel microbial-mediated (e.g *Wolbachia*) mosquito control strategies (Moreira *et al.*, 2009; Iturbe-Ormaetxe, Walker and O' Neill, 2011; Kamtchum-Tatuene *et al.*, 2017; Niang *et al.*, 2018; O'Neill, 2018).

The presence of some microbes, mainly bacteria species in the mosquito midgut, has significant effects on vector competence. Several bacteria families including Enterobacteriaceae, Acetobacteriaceae and Neisseriaceae have been associated with the regulation of Anopheline vector competence and anti-Plasmodial activities (Cirimotich *et al.*, 2011; Boissière *et al.*, 2012; Gendrin and Christophides, 2013). For example, the colonization of the midgut with *Chromobacterium* (*Csp_P*) reduces malaria and dengue infection in vector mosquitoes (Ramirez *et al.*, 2014). *Asaia sp.* also activate the immune system of *An. stephensi* to produce anti-microbial peptides that reduce *P. berghei* infection in the vector (Capone *et al.*, 2013). *Enterobacter* and *Serratia* are genera of bacteria belonging to the *Enterobacteriaceae* family and play key roles in parasite development via direct interactions with bacteria-produced anti-*Plasmodium* factors (Cirimotich *et al.*, 2011), secretion of various biomolecules such as enzymes and toxins (Azambuja, Garcia and Ratcliffe, 2005), and by the formation of a physical barrier that obstructs the contact between *Plasmodium* ookinetes and the midgut epithelium (Bando *et al.*, 2013; Bahia *et al.*, 2014; Song *et al.*, 2018). *Enterobacter* sp. and *Serratia* sp. also inhibit the sporogonic development of *P. vivax* in *Anopheles albimanus* (Gonzalez-Ceron *et al.*, 2003). *Serratia* and *Enterobacter*, therefore, have the potential for use in the control of malaria parasites in various *Anopheles* vectors and can be further explored for a novel intervention to control the disease.

The vector-control potential of an *Enterobacter* species in *Aedes* and *Culex* mosquitoes was investigated by incorporating mosquito-larvicidal genes, *cry4B* from *Bacillus thuringiensis subsp. israelensis* (*Bti*) and binary toxin genes from *Bacillus sphaericus* into the genome of *E. amnigenus* strain isolated from *Anopheles* (Tanapongpipat *et al.*, 2003). It was established that the engineered *E. amnigenus* strain expressed the mosquito lethal genes which led to the death of the *Aedes* and *Culex* mosquito larvae (Tanapongpipat *et al.*, 2003). The parasite inhibiting potential of *E. agglomerans* was demonstrated by engineering the *E. agglomerans* to secrete anti-Plasmodium molecules and this significantly suppressed the development of *P. falciparum* and *P. berghei* oocysts (Wang *et al.*, 2011). Several studies have demonstrated that mosquito vector defence against malaria parasites is regulated by the activation of the vector's immune system (Meister *et al.*, 2009; Bahia *et al.*, 2014). RNA transcription profiles of aseptic and septic mosquitoes identified several genes that are highly expressed by enteric bacteria, including several anti-*Plasmodium* factors (Dong, Manfredini and Dimopoulos, 2009). A comprehensive transcriptional profile of the genes involved in the development of *Plasmodium berghei* in *Anopheles gambiae* midgut revealed two genes, Gamete release gene *(GAMER)* and Haloacid dehalogenase (HAD) domain ookinete protein gene *(HADO)*, that play critical roles in the development of parasites in the vector (Akinosoglou *et al.*, 2015). Gamete release (*GAMER*) gene encodes a 10.7kD protein that is associated with male gamete release. Inhibition of this gene results in significantly low ookinete numbers. Haloacid dehalogenase (HAD) domain ookinete protein (HADO) gene encodes a 44.7 kD amino acid protein that has a putative magnesium phosphatase role. The protein is found on the concave periphery of ookinetes. Disruption of HADO compromises ookinete development leading to a significant decline in oocyst numbers (Akinosoglou *et al.*, 2015).

1.2 Rationale

In a recent study, an *Enterobacter* sp. isolated from wild Anopheline populations in Zambia rendered mosquitoes resistant to infection with *Plasmodium falciparum* by blocking the development of ookinete, oocysts and sporozoites, and this has been shown to occur via a mosquito-independent mechanism with the parasite that involves the secretion of reactive oxygen species (Cirimotich *et al.*, 2011). This suggests that the effects of bacteria on parasites in mosquito vectors is not exclusively through an immune-dependent mechanism and could be a direct interaction between bacteria and parasite. Little attention has been given to understanding this direct mechanism of bacteria on *Plasmodium* parasites, and the effects of bacteria on malaria parasite developmental genes are not completely understood. This limits the scope of how gut bacteria, for example *Enterobacter* and *Serratia*, which have been found with anti-*Plasmodium* effects can be further explored for alternative disease control strategies. Therefore, this study investigated the lethal effect of cell-free secreted bio-products of *E. cloacae* and *S. marcescens* on a key *Plasmodium* parasite developmental gene (*GAMER*) for its potential as a target for malaria transmission-blocking.

1.3 Hypothesis

Anti-Plasmodial activities of *E. cloacae* and *S. marcescens* isolated from *Anopheles* mosquito midgut are achieved through the secretion of bio-products that affect the expression of *Plasmodium* developmental genes.

1.4 Objectives

1.4.1 Main objective

The main aim of this study is to determine the direct effects of *E. cloacae* and *S. marcescens* secreted bio-products (cell-free spent media) on *P. falciparum in vitro*.

1.4.2 Specific objectives

The specific objectives of the study are:

1. Compare the concentration-dependent anti-plasmodial effect of cell-free spent media of *Enterobacter cloacae* and *Serratia marcescens in vitro*
2. Investigate the regulation of *GAMER* gene following exposure to *Enterobacter cloacae cell*-free spent media *in vitro*

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Epidemiology of malaria

Malaria is an infectious parasitic disease caused by *Plasmodium* species and transmitted by female *Anopheles* mosquitoes. Malaria is generally associated with non-specific symptoms such as fever, chills and fatigue. However, anaemia, lactic acidosis, multiple-organ failure and coma have also been observed in severe cases of the disease (Evans *et al.*, 2006). The clinical manifestations of malaria are caused by the asexual blood stages of the parasites (Maitland and Marsh, 2004; Arévalo-Herrera *et al.*, 2015, 2016). The disease is characterized by high mortality and morbidity. Malaria is predominantly found in the tropical and subtropical regions of Africa, Asia and the South America (Figure 1). There were 241 million reported cases and 627, 000 deaths in 2020 with women and children (below 5 years) being the most vulnerable (World Health Organization, 2021). In sub-Saharan Africa, malaria is caused predominantly by *Plasmodium falciparum* (Breman, Alilio and Mills, 2004; Snow *et al.*, 2005) whereas *P. vivax* is predominant in America and South-East Asia (Guerra *et al.*, 2010). Both *P. falciparum* and *P. vivax* cause majority of the malaria cases across the word (Snow *et al.*, 2005). *Plasmodium ovale* and *P. malariae* are responsible for a few cases globally (Gebru *et al.*, 2017). Although *P. knowlesi* was initially considered a rare zoonotic species, it is now known to infect humans (Singh and Daneshvar, 2013; Vythilingam *et al.*, 2014; World Health Organization, 2019). Another species, *P. cynomolgi* has also recently been found to cause malaria in human hosts (Ta *et al.*, 2014; Hartmeyer *et al.*, 2019; Imwong *et al.*, 2019).

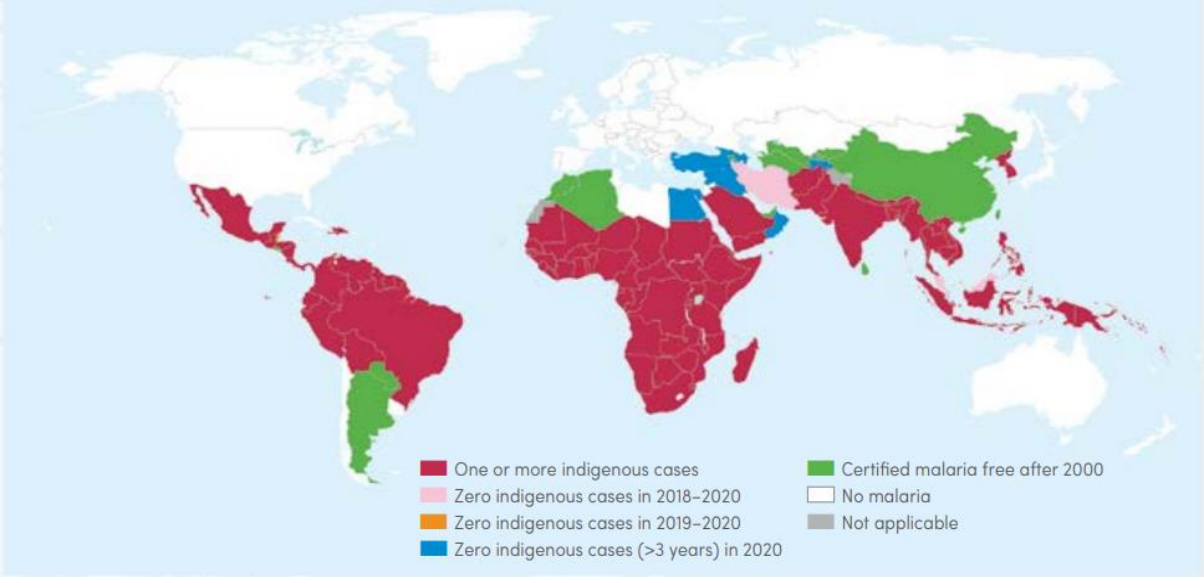

Figure 1: Global distribution of malaria. Most malaria cases occur in Africa with a few cases in America and South-East Asia. (Source: World Malaria Report 2021).

2.2 Transmission of malaria

The lifecycle of the *Plasmodium* parasite involves an invertebrate vector and a vertebrate host. *Anopheles* mosquitoes are the only efficient vectors of malaria and transmit the disease from person to person through their bites. Malaria infections may be acquired via blood transfusions or the use of contaminated needles. However, transfusion-transmitted malaria (TTM) is rare with the risk of infection greatly reduced by the screening of blood donors (Owusu-Ofori *et al.*, 2013; Verra *et al.*, 2018). The female *Anopheles* vector ingests male and female gametocytes from an infected human host during a blood meal. The chemical and physical environment in the mosquito midgut immediately triggers gametocyte transformation into gametes (Billker *et al.*, 1997, 1998) which fuse to form zygotes that develop into motile ookinetes within the vector midgut within 24 hours post-ingestion (Yassine and Osta, 2010). These motile forms (ookinetes) cross the peritrophic matrix surrounding the blood meal (Weiss *et al.*, 2014) and transverse the midgut epithelium to form oocysts at the basal lamina. After 7 to 10 days the oocysts eventually develop into the sporozoites (Yassine and Osta, 2010) that spread through

the haemolymph to all parts of the vector including the salivary gland (Smith, Vega-Rodríguez and Jacobs-Lorena, 2014; Sierra *et al.*, 2015). Infective sporozoites (about 20-200) (Kappe, Kaiser and Matuschewski, 2003) are injected into the vertebrate host dermis during the mosquito's subsequent blood feeding process. The sporozoites stay within the dermis for at least 5 minutes (Matsuoka *et al.*, 2002; Amino *et al.*, 2006; Yamauchi Lucy M. *et al.*, 2007) and they travel to the liver to invade the hepatocytes (Figure 2). An asexual exo-erythrocytic cycle is set up in the liver cells where merozoites are subsequently released after 2-16 days (depending on the parasite species) (Cox, 2001; Sturm *et al.*, 2006; Tarun *et al.*, 2006) into host circulation following the rapture of infected hepatocytes (Cowman, Berry and Baum, 2012; Cowman *et al.*, 2016). The released merozoites attach to and invade erythrocytes. Within the red blood cell, the merozoite is transformed into a ring or early trophozoite form, which in turn develops into a mature trophozoite that undergoes asexual multiplication to form a schizont containing numerous merozoites. The erythrocytic schizont ruptures, releasing merozoites that re-invade uninfected host red blood cells, thereby completing the erythrocytic cycle (Cowman, Berry and Baum, 2012). After several rounds of asexual replication in the host, lasting between 24 and 72 hours, (Venugopal *et al.*, 2020), ring-stage parasites commits to gametocyte production by expressing gametocyte exported proteins on the surface of the infected red blood cells (Tibúrcio *et al.*, 2015). The early stages of the gametocyte (Stages I-IV) take about 7-10 days to mature (Gardiner and Trenholme, 2015) and they remodel the surface structure of infected red blood cells to sequester in internal organs such as the heart, brain, spleen, gut and bone marrow (Aguilar *et al.*, 2014; Joice *et al.*, 2014) but the mature forms are found in the bloodstream after 12 days (Tibúrcio *et al.*, 2015; Neveu and Lavazec, 2019).

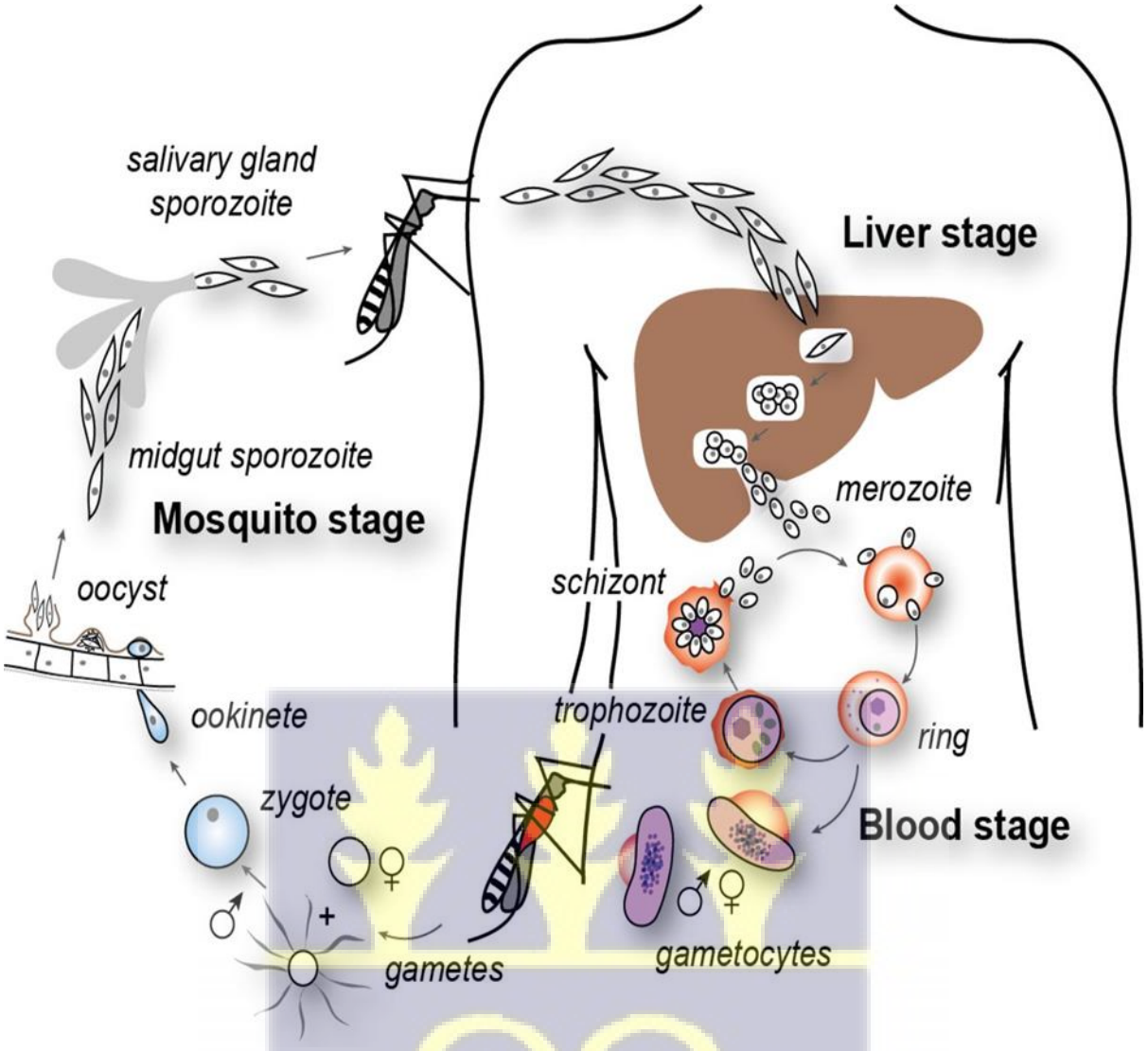

Figure 2: The life cycle of *Plasmodium falciparum* (Source: (Cowman, Berry and Baum, 2012)). *Plasmodium* sporozoites injected into the host's bloodstream travel to the liver to invade host hepatocytes and develop into merozoites that are released into host circulation following the rapture of the infected hepatocytes. Released merozoites invade uninfected host red blood cells, develop into trophozoites and finally maturing into schizonts. Raptured schizonts release merozoites that re-invade uninfected host red blood cells setting up an asexual blood stage cycle. Gametocytes, developing from the asexual blood-stage are picked by female *Anopheles* mosquitoes and transformed into gametes in the mosquito midgut yielding zygotes. Emerging zygotes develop into ookinetes, oocysts and finally sporozoites that migrate to the salivary gland.

2.3 The *Plasmodium* parasite

Plasmodium sp. are obligate intracellular apicomplexan parasites that infect primates and other mammals such as birds, and reptiles. There are currently over 200 species identified (Watson, 1967) but only six of these; *P. falciparum, P. vivax, P. malariae, P. ovale, P. knowlesi and P. cynomolgi*) infect humans (Cowman *et al.*, 2016). *Plasmodium* parasites have a diverse morphology and a very complex life cycle. They grow and develop in both the invertebrate vector and the vertebrate host (Venugopal *et al.*, 2020), the sexual life stage occurs in the invertebrate host. In extreme cases, the parasites can be found in several organs such as bone marrow, heart and spleen (Aguilar *et al.*, 2014; Joice *et al.*, 2014). The blood-stage forms of *Plasmodium spp.* are responsible for the non-flu like symptoms of malaria (Maitland and Marsh, 2004; Arévalo-Herrera *et al.*, 2015, 2016; Escalante *et al.*, 2015)

2.3.1 *Plasmodium falciparum*

Globally, this parasite is responsible for the majority of malaria cases and is the deadliest of all the *Plasmodium* species (Snow *et al.*, 2005). *P. falciparum* invades red blood cells of all ages (Simpson *et al.*, 1999) and the asexual blood stages exhibit a characteristic 48-hour cycle or tertian pattern (Ziegler, 1980; Barnes, 1986). It takes approximately 9 to 14 days after *Plasmodium falciparum* infection for the symptoms to manifest. Characteristic to *P. falciparum* infections, infected red blood cells adhere to the endothelial linings of blood vessels and sequestration in the cerebral microvasculature causes cerebral malaria. Cerebral malaria is associated with impaired consciousness and in severe cases coma (Idro *et al.*, 2010; Rénia *et al.*, 2012). The parasite is the most studied and several strains of *P. falciparum* have been adapted for *in vitro* blood cultures (Trager and Jensen, 1976).

2.3.2 *Plasmodium vivax*

The parasite is transmitted globally in the tropics except for West Africa. This is because the West African human population lacks the Duffy binding receptor which *P. vivax* requires to establish infection (Guerra, Snow and Hay, 2006; Culleton *et al.*, 2008). Recent reports of *P. vivax* infections have, however, been made on Duffy negative individuals (Michon *et al.*, 2007; Guerra *et al.*, 2010). The parasite infects reticulocytes (young red blood cells) (Kitchen, 1938; Vryonis, 1939; Simpson *et al.*, 1999; Tjitra *et al.*, 2008; Poespoprodjo *et al.*, 2009). The symptoms of *P. vivax* infection has a characteristic tertian pattern or 48-hour cycle (Ziegler, 1980; Barnes, 1986; McKenzie, Jeffery and Collins, 2002) and incubation period of 12 to 18 days (Powell, 1986; Yeom and Park, 2008; Brasil *et al.*, 2011; Kim *et al.*, 2013). In endemic areas, the parasites undergo relapse every three weeks (Hill and Amatuzio, 1949; Coatney, Cooper and Young, 1950; Anstey *et al.*, 2012).

2.3.3 *Plasmodium malariae*

Plasmodium malariae is globally distributed and coincides with *P. falciparum* infection. They are widespread throughout sub-Saharan Africa, Southeast Asia, South America and Europe (Collins and Jeffery, 2007). The parasite infects old red blood cells (normocytes) in primate hosts (Simpson *et al.*, 1999; Ashley and White, 2014). *Plasmodium malariae* has a 72-hour cycle (quartan) (Powell, 1986) and it takes 18 to 40 days after infection for the symptoms of the disease to manifest (Ziegler, 1980; Barnes, 1986). The sporozoites of *P. malariae* takes about 1 hour to get to the liver and they mature in about 15 days. There are no quiescent liver stage forms (hypnozoites) or recrudescence as is the case in human *P. vivax* and *P. ovale* infections (Collins and Jeffery, 2007).

2.3.4 *Plasmodium ovale*

This parasite is found predominantly in tropical regions of Africa and Asia (Collins and Jeffery, 2005; Mueller, Zimmerman and Reeder, 2007; Smith *et al.*, 2008). The global burden of *P. vivax* infection is often underestimated due to the difficulty in the detection of the parasite using microscopy especially at low parasitaemia (Rousset, Couzineau and Baufine-Ducrocq, 1969; Faye *et al.*, 1998, 2002; Mueller, Zimmerman and Reeder, 2007). There are two distinct species of *P. ovale* parasites; *Plasmodium ovale curtisi* (classic type) and *Plasmodium ovale wallikeri* (variant type) and they both infect reticulocytes with a characteristic tertian or 24-hour cycle (Powell, 1986; Sutherland *et al.*, 2010; Ngotho *et al.*, 2019). *P. ovale* has an incubation period of 12 to 18 days and has the potential to form hypnozoites extending for not less than 10 months (Collins and Jeffery, 2005; Richter *et al.*, 2010).

2.3.5 *Plasmodium knowlesi*

This parasite is naturally known to infect long-tailed and short-tailed macaques (Butcher, Cohen and Garnham, 1970). The first case of zoonosis was discovered in Kapit Division of Sarawak, Malaysian Borneo (Singh *et al.*, 2004). The parasite has since then successfully infected people in the Asian territories (Cox-Singh and Singh, 2008) and currently considered the fifth *Plasmodium* parasite to naturally infect man (White, 2008). The life cycle of the parasite is similar to that described in *P. falciparum* (Fig. 1) and it is transmitted by *Anopheles leucosphyrus* mosquitoes (Peyton, 1989; Sallum, Peyton and Wilkerson, 2005). No clinical symptoms are associated with parasite development in the liver. However, the development of the parasite in the erythrocytes comes with unique symptoms that follow a 24-hour (quotidian) cycle (Watson, 1967). The parasite is morphologically similar to *P. malariae* (Cox-Singh and Singh, 2008) but distinguishable using molecular techniques. The incubation period of the

parasite is between 11 to 12 days (Watson, 1967) and *P. knowlesi* infections are readily treatable when diagnosed early.

2.3.6 *Plasmodium cynomolgi*

Macaques are the natural host of this *Plasmodium* parasite (Butcher, Cohen and Garnham, 1970). However, *P. cynomolgi* has been found to also infect humans (Imwong *et al.*, 2019) where it invades the reticulocytes (Russell and Cooke, 2017; Chua *et al.*, 2019). The parasite has remarkable morphological and biological similarities to *P. vivax* which is traceable to their genetic make-up (Tachibana *et al.*, 2012; Pasini *et al.*, 2017). The life cycle of the parasite is similar to other *Plasmodium* species (Fig. 2). *Plasmodium cynomolgi* also has dormant forms (hypnozoites) that may be activated to cause relapse infections weeks to months after the primary infection (Krotoski *et al.*, 1982; White, 2011; Joyner, Barnwell and Galinski, 2015; Joyner *et al.*, 2016). With *P. cynomolgi* infection, clinical symptoms are often mild but may result in severe complications which can quickly lead to death (Rahimi *et al.*, 2014). The parasite plays a vital role in scientific research where they are used as models for studying *Plasmodium* (Pacheco *et al.*, 2011; Fonseca *et al.*, 2017; Voorberg-van der Wel *et al.*, 2017; Chua *et al.*, 2019).

2.4 The *Anopheles* vector

Mosquitoes are vectors life-threatening infectious diseases such malaria, lymphatic filariasis, dengue and chikungunya virus (Gendrin and Christophides, 2013). The prevalence of these infectious diseases relies on the distribution, abundance and vector competence of mosquitoes (Kibuthu *et al.*, 2016). Approximately 40 *Anopheles* species are competent vectors of malaria. *Anopheles* mosquitoes belong to the Order *Diptera*, Family *Culicidae* and Subfamily *Anophelinae*. They are found all over the world except in the Antarctica (Ebenezer *et al.*, 2014).

The predominant mosquito species in Africa are *Anopheles arabiensis*, *An. funestus*, *An. gambiae*, *An. melas*, *An. merus*, *An. moucheti* and *An. nili* (Sinka *et al.*, 2010). In Ghana, *Anopheles gambiae s.l* are the predominant mosquito species. *Anopheles funestus,* and *An melas* are also present and their distribution is ecological (Appawu *et al.*, 2004; de Souza *et al.*, 2010; Tuno *et al.*, 2010).

2.4.1 Mosquito life cycle

The *Anopheles* mosquito has four developmental stages namely; egg, larva, pupa and adult (Rozendaal, 1997). The early developmental forms (eggs, larvae, pupae) are aquatic and are often found in sunlit pools (Rozendaal, 1997), often man-made habitats such as burrow-pits, drains, brick-pits, hoof-prints, and permanent habitats like water holes or rainwater collecting in natural depressions (Gimnig *et al.*, 2001; Imbahale *et al.*, 2011; Mattah *et al.*, 2017). Anophelines survive and thrive in habitats with optimum environmental conditions like temperature and humidity (Ndoen *et al.*, 2010; Beck-Johnson *et al.*, 2013). Their abundance and distribution are seasonal (Amaechi *et al.*, 2018; Sanei-Dehkordi *et al.*, 2019) and are most prevalent with high rainfall periods (Mahgoub, Kweka and Himeidan, 2017). The increase in mosquito population during rainy seasons could explain the high number of malaria cases recorded in the rainy seasons across sub-Saharan Africa (McMichael, 2013; M'Bra *et al.*, 2018).

Anopheles mosquitoes lay eggs in singles on the surfaces of water (Fig. 3). The eggs hatch in 1 to 2 days and it takes about 10 days for the larvae to progress across all four developmental phases (first instar to fourth-instar) at optimum temperatures between 26-28 ºC (Impoinvil *et al.*, 2007). Fourth (4^{th}) instar larvae take 2-3 days to emerge into pupae at an optimum temperature between 25-28 ºC and the pupal-to-adult transition takes 1-2 days depending on environmental cues such as temperature and average humidity (Bayoh and Lindsay, 2003;

Kirby and Lindsay, 2009). Besides the morphologically distinguishable features between male and female adults (Fig 4), these also differ in terms of nutrition and dispersion. Generally, both male and female adult mosquitoes feed on plant nectar 24-36 hours post-emergence for all their energy requirements (Foster and Takken, 2004; Manda *et al.*, 2007; Barredo and DeGennaro, 2020). The adult male mosquitoes continue to feed on the sugar meals to maintain their reproductive abilities (Gouagna *et al.*, 2014). After 5 days of feeding on nectar, gravid adult female mosquitoes feed on blood for the development of their fertilised eggs (Foster and Takken, 2004; Hansen *et al.*, 2014). Adult female mosquitoes rely on cues such as carbon dioxide (Gillies, 1980; Erdelyan *et al.*, 2012), exhaled chemicals (lactic acid) (Acree *et al.*, 1968) and a range of odourants (acetates, alcohols, and ketones) to locate their primate hosts (Hallem *et al.*, 2004; Potter, 2019). The digestion of the blood meal is temperature-dependent and takes about 2-3 days in the tropics and 5-8 days in temperate environments (Paskewitz, 1995). Generally, *Anopheles* mosquitoes feed only once per oviposition or gonotrophic cycle, however, newly emerged mosquitoes may feed two or more times during their first oviposition cycle (Venkat Rao, 1943; Thomson, 1948; Edman, Webber and Kale, 1972; Ezemuoka *et al.*, 2020).

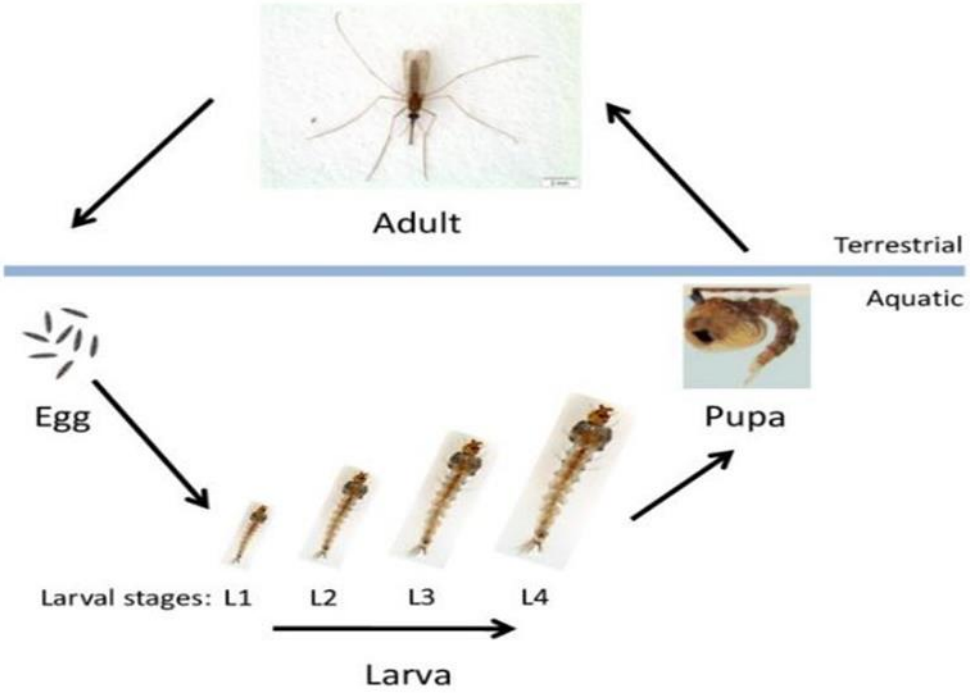

Figure 3:The life cycle of the *Anopheles* mosquito.
The female adult *Anopheles* vector lays eggs on water surfaces. The eggs hatch into larvae, develop to pupae and emerge as adults. Eggs, larvae and pupae are found in water. Adult mosquitoes are terrestrial. (Photo source: (Williams and Pinto, 2012).

Figure 4:Adult male and female Anopheles mosquitoes. Distinctive features of adult male and female *Anopheles* mosquitoes. Adult male mosquitoes have feathery antennae that help them sense their potential mates' wingbeats. However, female mosquitoes have uniquely plain antennae with mouthparts adapted for blood feeding. Their proboscis is long, slender relatively smooth compared to the males (Photo source: (*Penn Vet | Nikon SMZ 1000*) accessed at https://www.vet.upenn.edu/research/core-resources-facilities/imaging-core/instruments-applications/nikon-smz-1000)

2.5 Control of malaria

2.5.1 Diagnosis of malaria

The early and accurate diagnosis of malaria is a committed step in the efforts deployed to control the disease. The clinical or presumptive diagnosis of malaria makes use of the signs and symptoms of the disease. However, the accuracy and specificity of this method have been extremely low. This means that exclusive dependence on clinical features or symptoms for malaria diagnosis results in abuse of anti-malarial drugs, under treatment and/or overtreatment (Pillay *et al.*, 2019). Because of this, the World Health Organization (WHO) recommends that parasitological tests should be carried out for all suspected malaria cases using rapid diagnostic

tools (RDT) and/or microscopy before commencing treatment (World Health Organization, 2019).

2.5.1.1 Microscopy

Microscopy is the gold standard for the detection of the malaria parasite in peripheral blood smears (Kilian *et al.*, 2000; Njama-Meya, Kamya and Dorsey, 2004; Sousa-Figueiredo *et al.*, 2012). However, this technique is time-consuming and requires technical expertise. The specificity and sensitivity of microscopy are also subjective (Pillay *et al.*, 2019). The detection of malaria parasites at a very low parasitaemia using microscopy is often underestimated and even gets complicated in cases of mixed infections (Kilian *et al.*, 2000; McKenzie *et al.*, 2003; Singh *et al.*, 2004). Despite these challenges, microscopy is inexpensive and allows for the identification and quantification of *Plasmodium species* where the expertise is available (Kilian *et al.*, 2000). Molecular diagnostic methods including PCR and isothermal assays, and rapid diagnostic test kits (RDTs) (Lucchi *et al.*, 2013) are highly sensitive and specific to parasite detection techniques compared to microscopy (Okell *et al.*, 2009; Berzosa *et al.*, 2018; Pillay *et al.*, 2019).

2.5.1.2 Rapid Diagnostic Tests (RDTS)

RDTs are immunochromatographic tests that detect specific parasite antigens such as histidine-rich protein 2 (HRP2), *Plasmodium* lactate dehydrogenase (pLDH) or aldolase, which are often produced during the erythrocytic cycle (Ugah *et al.*, 2017). This diagnostic technique gives reliable results (Bharti *et al.*, 2008) but the sensitivity wanes with parasitaemia below 300–500 parasites/µL (Kim *et al.*, 2008). Moreover, some parasites have undergone mutations by deleting their histidine-rich protein 2 and 3 (*PfHRP2* and *PfHRP3*) making them evade

detection by the RDTs (Mariette *et al.*, 2008; Koita *et al.*, 2012; Wurtz *et al.*, 2013; Amoah, Abankwa and Oppong, 2016).

2.5.1.3 Polymerase chain reaction (PCR)

Polymerase chain reaction detects parasite DNA and can identify infections below the detection limits of microscopy and RDTs (Rogawski *et al.*, 2012).

The gene targets used in PCR include the 18S ribosomal RNA gene and *cytochrome b* gene which give reliable results (Steenkeste *et al.*, 2009). However, conventional PCR is not applicable as point-of-care diagnostic tools in many malaria-endemic areas because they are expensive, and require a high level of skills (Steenkeste *et al.*, 2009; Rogawski *et al.*, 2012).

The loop-mediated isothermal amplification (LAMP) is also a type of PCR which gives accurate, specific, and sensitive results (Cuadros *et al.*, 2017; Piera *et al.*, 2017; Vásquez *et al.*, 2018). LAMP is a simple molecular diagnostic tool hinged on the principle of isothermal amplification, which requires little or no special equipment or laboratories and provides results in about 1 hour (Hopkins *et al.*, 2013). LAMP requires a small amount of blood sample and it is also tolerant to inhibitory substances present in blood samples (such as haemoglobin and immunoglobulin) making it convenient for use in low- resourced settings (Port *et al.*, 2014; Yongkiettrakul *et al.*, 2014).

2.5.1.4 Future of malaria diagnostics

There are attempts to automate the detection methods to allow for a quicker, simpler and cost-effective diagnosis of the disease (Laroche *et al.*, 2017; Poostchi *et al.*, 2018). The presence of an automated system will provide adequate information on infected red blood cell count of patients to enable the monitoring of parasite post-treatment and provide an early marker of drug resistance. It will also provide an opportunity to identify asymptomatic cases, and potentially

preventing the spread of blood transfusion-related malaria (Purwar *et al.*, 2011; Abbas *et al.*, 2018; Pillay *et al.*, 2019).

2.5.2 Treatment of malaria

2.5.2.1 Chemotherapy

Malaria treatment is a core component of the interventions championed by the WHO to facilitate the control and eradication of malaria. The major objective of malaria treatment is to alleviate the symptoms, prevent relapses and block the spread or transmission of the disease. Therefore, antimalarial drugs target asexual erythrocytic stages, liver or tissue forms and gametocytes (Fig. 2) with much attention given to the drugs that target the blood-stage forms (Delves *et al.*, 2012; Bosson-Vanga *et al.*, 2018).

Generally, the kind of treatment regimen deployed depends on the degree of severity of the disease. For example, case management for uncomplicated and severe malaria involves the use of ACTs to facilitate parasite clearance. For fatal cases of uncomplicated malaria, patients are immediately placed on fluids for the resuscitation of patient and preventing their possible death as a result of dehydration and followed with supportive care (Maitland and Marsh, 2004; Hodgson and Angus, 2016). In Southeast Asia, multidrug-resistant *P. falciparum* is treated with combinations of artemisinin derivatives and mefloquine or atovaquone plus proguanil (Kain, 1996). However, Chloroquine is used to mainly treat infections with *P. vivax*, and in some cases *P. malariae* and *P. ovale* (Kain, 1996; Olliaro and Mussano, 2009). Currently, WHO strongly recommends the use of ACTs for the treatment of uncomplicated *P. falciparum* malaria in children and adults (except pregnant women in their first trimester) (White, 2004; Dondorp *et al.*, 2009; World Health Organization, 2022).

In *P. falciparum* endemic areas, pregnant women (in their first trimester) are put on quinine and clindamycin (or Sulfadoxine-Pyrimethamine (SP) in areas where SP is still effective)

medications during antenatal visits to prevent from malaria-related maternal anaemia, deaths and low-birth weight infants (Shulman, 1999; Nzila, Okombo and Molloy, 2014; World Health Organization, 2022). This therapy, generally called intermittent preventive treatment (IPT), is also applicable to infants who form the majority of the at-risk individuals (Schellenberg *et al.*, 2001; Greenwood, 2006). IPT schemes have been extensively studied in infants (IPTi), children younger than 5 years (IPTc) and pregnant women (IPTp) and have been demonstrated to be protective against malaria and its related adverse outcomes (Schultz *et al.*, 1994; Shulman, 1999; Greenwood, 2006; McGready, 2009; Bojang *et al.*, 2010; Konaté *et al.*, 2011; Matangila *et al.*, 2015). It has also been demonstrated that Artemisinin combination therapies (ACTs) (SP, DP, SP + AS, AQ + AS) when used in IPT, had acceptable protective efficacy (PE) against clinical malaria (Matangila *et al.*, 2015; Al Khaja and Sequeira, 2021). In early and late pregnancy, intravenous artesunate is the drug of choice for treating complicated malaria during early and late pregnancy, respectively (Al Khaja and Sequeira, 2021).

People traveling to and returning from malaria-endemic areas are advised to take chemoprophylactics (Wickremasinghe *et al.*, 2017) and this approach is targeted at reducing cases of imported malaria. Chemoprophylaxis treatment regimens administer drugs such as chloroquine, doxycycline and atovaquone-proquanil.

2.5.2.2 Vector control

Mosquito vector control, a means of disrupting malaria transmission, is the second pillar in the global efforts by the WHO to achieve total elimination and eradication of malaria. The World Health Organization recommends integrated vector management (IVM) techniques to control the vector through chemical, biological and genetic strategies. For these interventions to be effective, they must be tailored to meet the local contexts of the countries in which they are being implemented. Adult vector-control interventions involve the deployment of synthetic

products to kill the adult mosquitoes by direct contact sprays or indirectly as preventive methods in indoor residual spraying (IRS) and long-lasting insecticide-treated bed nets (LLINs) Kokwaro (2009). In larval control, the breeding sites of the mosquito are targeted and can include clearing of aquatic weeds and filling of man-made and permanent habitats of the mosquitoes. Larvicides are also applied to water bodies to kill the immature stages of the mosquitoes (larvae or pupae) and they could act as mosquito stomach toxins, contact larvicides, surface agents, biological larvicides and insect growth regulators (Lacey, 2007). Other biological interventions make use of enthomopathogenic fungi, sterile insect technique (SIT), and engineered mosquitoes and new larval control tools currently being evaluated include lethal ovitraps and acoustic larvicide systems (Kokwaro, 2009; Abdul-Ghani, Al-Mekhlafi and Alabsi, 2012).

Transgenesis and paratransgenesis are promising genetic vector-control strategies that hold promise for the effective control of malaria. Transgenesis involves genetic techniques that modify mosquito vectors to release anti-parasite effector molecules, making them refractory to parasites (Kokwaro, 2009; Abdul-Ghani, Al-Mekhlafi and Alabsi, 2012). Pararatransgenesis, on the other hand, involves the modification of mosquito microbiome to confer parasite resistance to the vector (Wang and Jacobs-Lorena, 2013). The prospect of paratransgenesis is hinged on the fact that the enteric microbes of mosquitoes are symbiotic and obtained mainly from the environment (Romoli and Gendrin, 2018). These approaches have been successful in the laboratory (Wang and Jacobs-Lorena, 2017) and yet to be rolled out on a global scale because of the varied and dynamic environmental conditions in the wild.

2.6 Challenges with current control strategies

2.6.1 Antimalarial drug resistance

The WHO and its partners intend to reduce malaria case incidence and malaria mortality by 90% before 2030 (WHO, 2015). Therefore, the reduced sensitivity of *Plasmodium* parasites to antimalarial drugs is a phenomenon that is frustrating global efforts to achieve this. Antimalarial drug resistance occurs when there is widespread exposure of parasites to antimalarial drugs often via poor compliance or inadequate treatment (Payne, 1988; White, 2004; Kokwaro, 2009; Maude *et al.*, 2009). Monotherapy has also contributed to the increase in antimalarial drug resistance thereby justifying the need for combination therapy (Rodrigues Coura, 1987; Dondorp *et al.*, 2009; Da Silva and Benchimol, 2014). Antimalarial drug resistance is confirmed via standard testing methods such as *in vitro* studies of resistance, detection of molecular markers of resistance and therapeutic drug efficacy studies; the latter remains the gold standard for determining drug resistance, decreased drug efficacy and for guiding drug policy (Talisuna, Bloland and D'Alessandro, 2004; Achan *et al.*, 2011; WHO, 2017).

Resistance to Chloroquine (CQ) was reported a few years after it was rolled-out in early 1950 which led to the resurgence of malaria in the 1980s (Antony and Parija, 2016; Blasco, Leroy and Fidock, 2017; Wotodjo *et al.*, 2018). It is believed that *P. falciparum* resistance to chloroquine is due to mutations at position 76 of the *Plasmodium falciparum* chloroquine resistant transporter (*PfCRT*) gene. *Plasmodium* sp. with this point mutation pump out chloroquine at much higher rates than the wildtype (Chen *et al.*, 2003; Valderramos and Fidock, 2006; Sá *et al.*, 2009). Another point mutation in a related gene, *Plasmodium falciparum* multi-drug resistant transporter gene, *pfMDR1*, has also been implicated in chloroquine resistance (Foley and Tilley, 1997; Djimdé *et al.*, 2001; Dorsey *et al.*, 2001; White, 2004). Later,

Sulphadoxine-Pyrimethamine (SP) replaced CQ for the treatment of CQ resistant parasites (Roper *et al.*, 2004; Abiodun *et al.*, 2011). *Plasmodium* parasites that are resistant to antifolates have point mutations in the Dihydro folate reductase (DHFR) and Dihydropterate synthase (DHPS) genes which reduce the affinity of the DHFR and DHPS enzyme complex to antifolates (Imwong *et al.*, 2003; Amaratunga *et al.*, 2016).

More recently, evidence was reported of artemisinin-resistant *P. falciparum* in the Greater Mekong Subregion and Africa (Dondorp *et al.*, 2009; Ashley *et al.*, 2014; Leang *et al.*, 2015; Antony and Parija, 2016; Phyo *et al.*, 2016; Lu *et al.*, 2017; Thanh *et al.*, 2017; Bopp *et al.*, 2018; Balikagala *et al.*, 2021). Resistance to artemisinin and its derivatives have been proposed to be due to mutation in the *kelch 13* gene (Bonnington *et al.*, 2017; Zaw, Lin and Emran, 2019; Siddiqui *et al.*, 2020; Uwimana *et al.*, 2020, 2021; Asua *et al.*, 2021; Balikagala *et al.*, 2021). Currently, there is the need for improved surveillance for drug resistance across Africa, and the use of different ACTs or triple ACTs to halt the emergence and/or spread of resistance to both artemisinin and key partner drugs (van der Pluijm *et al.*, 2020). This further supports the call for other control interventions to hasten parasite clearance and reduce the disease burden.

2.6.2 Vaccines

Vaccines have been very effective and efficient in the control and prevention of diseases like smallpox, poliomyelitis and measles (Rieckmann *et al.*, 2017). It is in this regard that several attempts have been made at developing potent vaccines for the control of malaria. Several vaccines targeting the different stages of the life cycle of the *Plasmodium* parasite are at various stages of clinical trials (Delves *et al.*, 2012). It has been extremely difficult obtaining an effective anti-malaria vaccine for global use because the biology of the malaria parasite has not been completely understood (Gardner *et al.*, 2002). There are a lot of parasite protein-coding

genes that have not been functionally annotated although the entire genome of the malaria parasite has been completely sequenced and this impedes the discovery of effective vaccine targets (Gardner *et al.*, 2002). The very few targets that have been found in the parasites are either polymorphic, redundant or do not elicit strong immune responses (Douglas *et al.*, 2019). For example, the WHO-recommended pilot implementation of the anti-malaria vaccine RTS,S/AS01 in some parts of Africa showed the vaccine has low prevention and efficacy indices (Moorthy and Okwo-Bele, 2015). It has also been reported that the efficacy of the RTS,S/AS01 vaccine declines over time (RTS, 2014; Olotu *et al.*, 2016). The host immune factors that offer protection against malaria is still under study (Taylor, Parobek and Fairhurst, 2012). However, the RTS, S/AS01 malaria vaccine research has made a lot of progress and is currently the vaccine of choice for children living in *P. falciparum* endemic (moderate to high transmission) areas (World Health Organization, 2022). It is highly likely that another vaccine with greater protective efficacy than the recommended RTS, S/AS01 may be approved (Datoo *et al.*, 2021).

Several attempts have been made to use the knowledge of the different stages of the *Plasmodium* parasite's life cycle to produce blood (erythrocytic) stage vaccines, pre-erythrocytic stage vaccines and transmission blocking vaccines (TBV) (Arama and Troye-Blomberg, 2014). Pre-erythrocytic vaccine strategy targets the liver stages of the malaria parasite and aims at preventing malaria infection to the host. Scientists have attempted the use of attenuated whole sporozoites vaccines for malaria control (WSV) (Nussenzweig *et al.*, 1967; Clyde *et al.*, 1973; Rieckmann *et al.*, 1974; Draper *et al.*, 2018). Despite the high level of protection recorded among volunteers treated with the attenuated form of the whole sporozoites, it was impossible to roll out this intervention on a larger scale (Draper *et al.*, 2018). Currently, whole sporozoite vaccines are being produced in large proportions. However, the

efficacy of these vaccines in recent studies in malaria endemic areas is low compared to has previous reports (Mwakingwe-Omari *et al.*, 2021).

The circumsporozoite surface protein (CSP) antigen on the surface of the sporozoite was targeted as a replacement for the attenuated form of the whole sporozoite (Draper *et al.*, 2018). A high level of protection was seen in mice immunized with CSP and this resulted in the development of sub-unit malaria vaccine, RTS, S (Stoute *et al.*, 1997; Birkett, 2010; Crompton, Pierce and Miller, 2010). However, subunit vaccines have recorded low efficacy, and this has revived the interest in whole sporozoite vaccine (WSV) strategy.

The blood stage vaccine approach aims to decrease the number of parasites in the blood in order to reduce the severity of the disease. This is premised on the fact that people who have constant exposure to malaria develop natural immunity to the disease over time (Marsh and Kinyanjui, 2006). It has been observed that vaccine containing proteins from the merozoite surface is feasible and can prevent malaria (Cohen, McGregor and Carrington, 1961; Sabchareon *et al.*, 1991).

The transmission blocking vaccine (TBV) strategies target the sexual stage of the malaria parasite and aims to prevent mosquitoes carrying malaria parasites from spreading them. TBV exploits antibodies to antigens such as Pfs25, Pfs48/45, Pfs28 and Pfs230 (Kaushal *et al.*, 1983; Nikolaeva, Draper and Biswas, 2015) to block the sexual stages and prevent transmission of malaria (Huff, Marchbank and Shiroishi, 1958; Carter and Chen, 1976).

Recently, an evidence of a multistage malaria vaccine has been successfully produced to a chimeric recombinant antigen composed of fragments of Pfs48/45, and the blood stage antigen glutamine rich protein (GLURP)(Theisen *et al.*, 2014). Another study has produced human monoclonal antibodies directed against a portion of the CSP and tested it in healthy human participants (Gaudinski *et al.*, 2021) This progress in relation to the expression of

immunogenic transmission blocking antigens presents a lot of promise towards the possibility of the development of a transmission blocking malaria vaccine.

2.6.3 Insecticide resistance

The fight to eliminate and eradicate malaria in most parts of the world especially, sub-Saharan Africa requires a combination of efforts chemotherapy (active antimalarial drugs), and effective vector-control methods. The use of chemical-based approaches for vector control is challenged by reduced efficacy of the currently recommended chemicals due to vector resistance to these synthetic compounds (Liu, 2015) which has occurred through constant exposure to agrochemicals (Nkya *et al.*, 2013, 2014) and modification of key detoxification enzymes (Chandor-Proust *et al.*, 2013).

The periodic emergence of anti-malarial drug resistance, lack of effective anti-malarial vaccines and failing vector-control methods continues to militate against the successful elimination and eradication of malaria (World Health Organization, 2019). This calls for the need for a paradigm shift to identify novel approaches for the control of malaria.

2.7 The *Anopheles* mosquito microbiota and parasite transmission

2.7.1 The *Anopheles* mosquito microbiota

In mosquitoes, symbiotic micro-organisms are found inhabiting organs such as midguts, salivary glands, and gonads (Pidiyar *et al.*, 2004; Rani *et al.*, 2009; Gusmão *et al.*, 2010; Noden *et al.*, 2011; Oliveira *et al.*, 2011; Zouache *et al.*, 2011; Strand, 2018). The diverse communities of microbes (bacteria, fungi and viruses) depends on ecological factors , diet and stage of insect development (Wang *et al.*, 2011; Akorli *et al.*, 2016; Benjamino *et al.*, 2018; Jiménez-Cortés *et al.*, 2018). Bacteria are the predominant community of the midgut microbial flora (Strand,

2018) and thus, the most commonly studied (Guégan *et al.*, 2018). Larvae have more diverse microbial communities than adults , as they feed directly on the micro-organisms in their aquatic environment (Wang *et al.*, 2011; Gimonneau *et al.*, 2014). Although, some bacteria are maintained through development from larvae to adults many are lost during metamorphosis (Lindh, Borg-Karlson and Faye, 2008). In *An. gambiae*, the number of operational taxonomic units (OTU) of bacteria are 3 times more in larval and pupal stages compared to the adult stages. *Enterobacteriaceae* and *Flavobacteriaceae* were present in all stages but the midgut of adult mosquitoes was mainly made up of *Proteobacteria* and *Bacteriodetes* (Moncayo *et al.*, 2005). A profile of the microbiome of *An. gambiae* larvae and pupae revealed a 40% composition with cyanobacteria (Wang *et al.*, 2011). The structural make-up of mosquitoes usually changes during metamorphosis. One key anatomical change that comes with moulting is the appearance of meconial peritrophic membrane (MPM1) at the pupal and adult emergence stages (Moncayo *et al.*, 2005). MPM1 is believed to sterilize the midgut of adult mosquitoes (Moncayo *et al.*, 2005). The microbes ingested by the mosquito larvae are sequestered by the MPM1 and eventually eliminated after the membrane is shed off when adult mosquitoes emerge (Moncayo *et al.*, 2005). This could account for the different proportions of bacterial communities between the early developmental forms and the adult stages. The common bacteria genera encountered in the different mosquito species include *Enterobacter, Serratia, Cedacea, Escherichia, Klebsiella, Pseudomonas,* and *Staphylococcus* (detailed review in (Gendrin and Christophides, 2013).

Bacteria found in mosquitoes influence various physiological functions including, nutrition, reproduction metabolism, and immunity (Jupatanakul, Sim and Dimopoulos, 2014). Microbes resident in mosquito habitats may influence the host-seeking behaviours of these disease vectors (Day, 2005; Verhulst *et al.*, 2009, 2010, 2011). For instance, the level of attractiveness of humans to mosquitoes is dependent on the composition of skin microbiota of the host and

the chemicals they secrete, and volatile compounds like lactic acid produced by *Corynebacterium minutissimum* attract *An. gambiae* (Verhulst *et al.*, 2010).

2.7.2 Role of mosquito microbiota in larval development

Bacterial microbiota plays an important role in mosquito larval development (Lindh, Terenius and Faye, 2005). Bacteria are a major food source for mosquito larvae (Merritt, Dadd and Walker, 1992). This was demonstrated when larvae of *Ae aegypti* survived for several weeks on fish meal whereas growth was halted in larvae that were reared in antibiotics-treated water (Merritt, Dadd and Walker, 1992). The growth of *Cx. quinquefasiatus* larvae is stimulated in the presence of *Pseudomonas aeruginosa* cultured in a phosphorus-rich medium. However, the development of the larvae of *Cx. tarsalis* was hindered in the same medium (Peck and Walton, 2006). The larvae-to-pupae transition of *An. gambiae* were significantly hindered after gentamycin and penicillin-streptomycin were introduced in the larval rearing water (Touré *et al.*, 2000). The introduction of Gentamycin into *An. gambiae* and *An. quadriannulatus* larval rearing water also tremendously affected the size of the larvae. Larvae in Gentamycin-treated larval rearing water were smaller in size compared to those reared in the absence of Gentamycin (Wotton *et al.*, 1997).

2.7.3 Role of mosquito microbiota on the nutrition of *Anopheles* mosquitoes

The composition of bacteria microbiota in adult mosquitoes is significantly affected by diet (Foster, 1995). Plant sap and nectar (sugar sources) are the main sources of food for adult mosquitoes. Nectar is primarily composed of carbohydrates and some traces of free amino acids (González-Teuber and Heil, 2009). Carbohydrates are used by mosquitoes to generate

energy for flight (Sacktor and Wormser-Shavit, 1966) and free amino acids (proline) are required to fuel flight (Scaraffia and Wells, 2003). Sugar meals are abundant in carbohydrates with little protein. Hydrolytic enzymes are secreted into the mosquito midgut to help digest the ingested sugar. This leads to selective pressure for bacteria resident in the mosquito midgut. Female mosquitoes require blood meal for the nourishment of eggs (Foster, 1995). Bacteria resident in female mosquitoes aid the digestion of blood (Gusmão *et al.*, 2010; Gaio *et al.*, 2011). Immune and oxidative stress responses are down-regulated due to a temperature burst that ensues post blood-feeding which triggers increased bacteria proliferation (Oliveira *et al.*, 2011) and influences bacteria diversity in the midgut (Wang *et al.*, 2011). Blood meal reduces the diversity of bacteria in the mosquito midgut. Bacteria proliferation is skewed in favour of *Enterobacteriaceace* following a blood meal (Wang *et al.*, 2011). This is because *Enterobacteriaceae* endure nitrosative and oxidative stresses that come with blood digestion. Thus, *Enterobacteriaceae* help maintain redox homeostasis (Wang *et al.*, 2011).

The midgut microbiome also enhances digestion via inhibition of enzymes necessary for nutrient absorption (Minard, Mavingui and Moro, 2013). *Enterobacter* and *Serratia* secrete hydrolytic enzymes which facilitate the digestion of blood in mosquitoes (Minard, Mavingui and Moro, 2013). Aseptic mosquitoes have reduced degradation of host red blood cells and low proteins (Gaio *et al.*, 2011). In *Ae. albopictus*, *Acinetobacter johnsonii* and *Acinetobacter baumanii* have been linked with both nectar assimilation and blood digestion (Minard, Mavingui and Moro, 2013). *Asaia bogorensis* from *An. stephensi* was found to replenish the invertebrate host with vitamin B proving that gut microbiota of blood sucking dipterans could supply their host with essential nutrients (Douglas and Smith, 1989; Damiani *et al.*, 2010).

2.7.4 Role of mosquito microbiota in vector competence

Vector competence is the ability of a vector to carry and spread pathogens (Lambrechts and Scott, 2009). Therefore, interventions that militate against pathogen transmission help reduce vector competence. The immune system of mosquitoes is a major player of vector competence. Mosquito midgut microbiota significantly influences the immunity of insect vectors (Gonzalez-Ceron *et al.*, 2003). Mosquito midgut microbiota may secrete chemicals that have a direct or indirect impact on disease-causing agents (Habib *et al.*, 2001; Chaudhary *et al.*, 2013). Some gut resident bacteria like *Enterobacter* and *Serratia* have a direct impact on mosquito vector competence by inhibiting the maturation of *Plasmodium* and other pathogens such as fungi and viruses (Gonzalez-Ceron *et al.*, 2003; Cirimotich *et al.*, 2010; Cirimotich, Ramirez and Dimopoulos, 2011; Weiss *et al.*, 2019). *Enterobacter* (*Esp_Z)* isolated from wild *Anopheles* populations in Zambia has been found to suppress *Plasmodium* parasite from developing before they are able to invade the midgut epithelium by exerting oxidative pressure through the secretion of reactive oxygen species (ROS) (Kumar *et al.*, 2003; Molina-Cruz *et al.*, 2008; Cirimotich *et al.*, 2010; Dennison *et al.*, 2016; Coon *et al.*, 2017). An increase in the number of bacteria particularly after blood meal boosts the innate immune responses of the mosquito to control the bacteria load (Cirimotich *et al.*, 2010). Generally, mosquitoes lack adaptive immunity (Garver *et al.*, 2012).

2.8 Paradigm shift to bacteria-mediated malaria control strategies

Several studies are currently being carried out to aid our understanding of the contribution of mosquito midgut microbiome to the disruption of pathogen transmission. In insects, the impact of *Wolbachia* on vector competence has been studied extensively over the years. *Wolbachia* are maternally acquired, obligate endosymbionts of many arthropods (Kittayapong *et al.*, 2000;

Moreira *et al.*, 2009; Iturbe-Ormaetxe, Walker and O' Neill, 2011; Walker and Moreira, 2011; Herren *et al.*, 2013). *Wolbachia* can influence insect reproduction via parthenogenesis, feminization and cytoplasmic incompatibility (CI) which enhance reproduction in infected insects. Cytoplasmic incompatibility ensures that the progeny requires *Wolbachia* (Dobson, Fox and Jiggins, 2002). *Wolbachia*-mediated cytoplasmic incompatibility resulted in the development of efficient vector control strategies such as incompatible insect technique (IIT) (Werren, Baldo and Clark, 2008; Atyame *et al.*, 2011). *Wolbachia* also inhibits the multiplication and transmission of pathogens in various mosquito species. The pathogen intensity and lifespan of *Ae*. *aegypti* and *Anopheles* were greatly reduced in mosquitoes that did not have naturally resident *Wolbachia* species (Walker and Moreira, 2011). *Wolbachia* can also stimulate oxidative stress that suppresses the multiplication of dengue virus in *Ae*. *aegypti* (Pan *et al.*, 2012).

A major bottleneck of the *Plasmodium* life cycle, gametocyte-to ookinete-to oocyst transition (Fig 5), occurs 24-26 hours after blood-meal (Siciliano *et al.*, 2020). Midgut bacteria species such as *Asaia* produce anti-*Plasmodium* effector proteins that confer refractoriness to mosquitoes (Damiani *et al.*, 2010). Trans-infection of mosquitoes with bacteria such as *Pantoea agglomerans* also effectively inhibited the maturation of *Plasmodium falciparum* and *P. berghei* by 98% (Wang *et al.*, 2012). The technique used *P. agglomerans* as a vehicle to convey antimalarial gene products to the lumen of the midgut of the mosquito vectors. (Wang *et al.*, 2012).

An *Enterobacter* species (*Esp_Z*) found in field-caught mosquitoes from Zambia fully or partly inhibited the establishment of ookinete, oocyst and sporozoite (Cirimotich, Ramirez and Dimopoulos, 2011). The *Enterobacter* sp. suppressed the development of the parasite before invading the midgut lumen of the mosquitoes. Co-infection of mosquitoes with *Esp_Z* and *Plasmodium* gametocytes resulted in reduced infection levels, but aseptic mosquitoes were less

resistant. Similarly, the co-infection of *An. albimanus* with *Serratia marcescens* and *P. vivax* resulted in 1% oocysts infection, compared to 71% infection observed in aseptic mosquitoes (Gonzalez-Ceron *et al.*, 2003). The significant association seen between the high level of *Enterobacteriaceae* and infection with *Plasmodium* parasites suggests that bacteria-parasite associations can be explored to identify novel strategies to combat malaria and other mosquito-borne diseases (Boissière *et al.*, 2012).

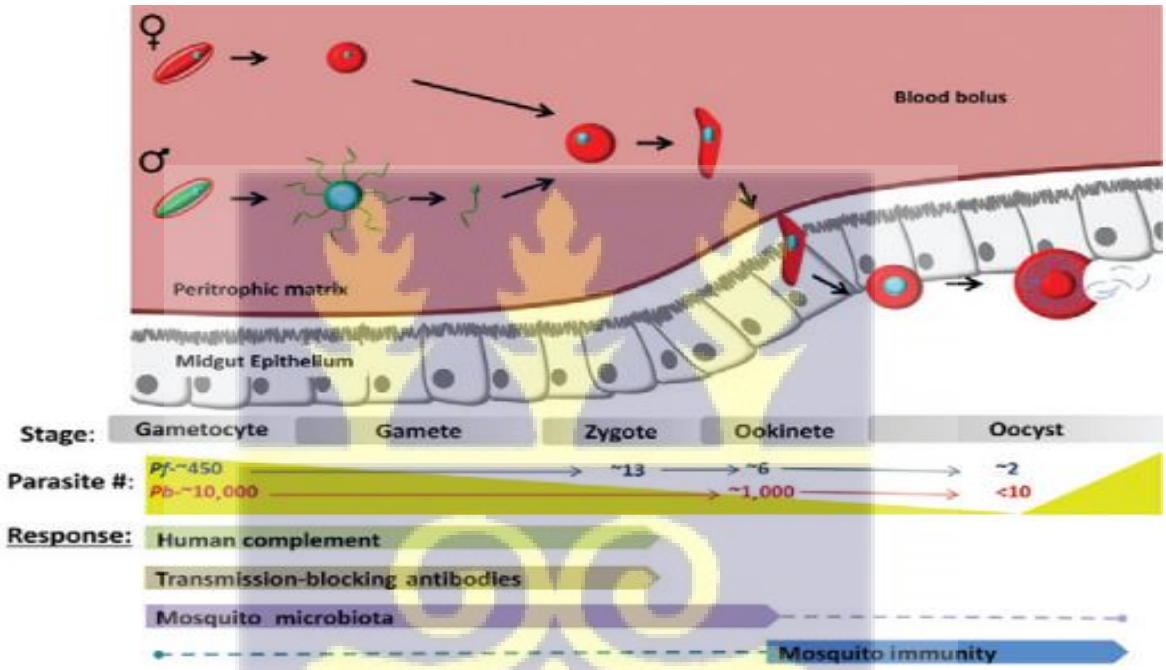

Figure 5: *Plasmodium falciparum* developmental changes as they travel through the mosquito gut. *Plasmodium* gametocytes develop into ookinetes that traverse the midgut epithelium to transform into oocyst and eventually sporozoites. (Source: Smith et al. 2014).

2.8.1 Paratransgenesis

Synthetic insecticides are often used for the control of mosquitoes. The resistance of mosquitoes to insecticides, accompanied by environmental pollution and the potential harm to non-target organisms have triggered concerns about the continuous use of chemical based-

approaches for mosquito control (David *et al.*, 2010). Bacteria-mediated mosquito approaches have become a promising intervention (Lindh, 2007). Paratransgenesis is a technique that employs symbiotic organisms to transfer anti-pathogenic gene molecules to vector populations thereby, reducing the ability of the vectors to transmit pathogens (Beard, Cordon-Rosales and Durvasula, 2002). Successful paratransgenic interventions require that the bacteria be closely associated with the vector and the pathogen, microbiota must be cultivable and must be genetically transformable, the modified bacteria must be fit, modified bacteria must have an appropriate technique for reintroduction, and the modified bacteria species must be easy to disseminate in the vector population (Durvasula *et al.*, 1997; Beard, Cordon-Rosales and Durvasula, 2002; Riehle and Jacobs-Lorena, 2005). Paratransgenesis has been beneficial in the control of American trypanosomiasis. The endosymbiont of *Rhodnius prolixus* (Tsetse vector) was modified to express a toxic peptide, cecropin A, which significantly inhibited *Trypanaosma cruzi* (pathogen) development (Durvasula *et al.*, 1997). *Asaia* and *Pantoea h*ave also been successfully exploited in paratransgenic interventions (Dinparast Djadid *et al.*, 2011). *Escherichia coli* was used as a vehicle to express two-anti-*Plasmodium* gene products (SM) and (PLA2) which suppressed the maturation of *P. berghei* confirming that paratransgenesis can be explored to curb malaria (Riehle *et al.*, 2007).

2.8.1.1 Challenges with paratransgenic interventions

The route for the re-introduction of the modified bacteria into wild mosquitoes is a major problem in paratransgenesis. Habitats of mosquito larvae are often used to transport transformed symbionts into adult mosquitoes but transtadial bacteria transfer is not efficient. Only about 0.7% of total bacteria is transferred from the fourth instar (larvae) to adult mosquitoes (Moll *et al.*, 2001). The huge loss of larval microbiota in adult mosquitoes is due to the shedding of MPM1 and the intake of moulting fluid which contains antibiotics (Moll *et al.*, 2001). Oviposition sites can also be treated with transformed endosymbionts (Riehle and

Jacobs-Lorena, 2005). However, creating oviposition sites in and around houses may lead to an increase in the number of mosquitoes in domestic areas. The artificial egg-laying sites may not be used by adult female mosquitoes especially during the rainy seasons due to the multiplicity of oviposition sites (Riehle and Jacobs-Lorena, 2005; Riehle *et al.*, 2007). Adult mosquitoes feed on nectar (sugar sources) after they emerge from the pupae (Impoinvil *et al.*, 2004). Treatment of sugar sources with transformed endosymbionts may serve as a way to re-introduce bacteria into mosquitoes. Attractants can be employed to enhance the efficacy of the bait (Foster and Takken, 2004).

2.8.2 Transgenesis

Transgenesis describes interventions that kill adult mosquitoes by transforming the mosquito vectors. It involves the use of mosquito lethal molecules. Some microbes such as *Clostridium bifermentans serovar* Malaysia and *Bacillus substilis* subspecies *substilis* can secrete insecticidal compounds (Yiallouros *et al.*, 1994). *Bacillus thuringiensis* serovar israelis produces toxins which are encoded by the mosquito lethal genes, *Cry* and *Cyt* (Gonzalez, Brown and Carlton, 1982). It has been demonstrated that the *Cry* toxins are harmful to large insects belonging to orders Coleoptera, Lepidoptera, Hymenoptera and Diptera. However, only dipteran insects are susceptible to *Cyt* toxins (Schnepf *et al.*, 1998). Death of mosquito larvae occurs due to breakdown of the midgut membrane of the larvae after the toxins have been activated by high pH in the midgut of the larvae (Bravo, Gill and Soberón, 2007). Similarly, *Mts* and *Bin* toxins secreted by *Lysinibacillus sphericus* are also extremely lethal to mosquito larvae (Berry, 2012). These toxins offer a lot of hope in our quest to fight malaria. They have been successfully experimented in the wild to kill *An. gambiae* mosquitoes in The Gambia and Ghana (Majambere *et al.*, 2007; Nartey *et al.*, 2013).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Reviving of archived bacteria isolates

Preserved *Enterobacter cloacae* and *Serratia marcescens* previously isolated from the midgut of wild female *Anopheles gambiae* mosquitoes and characterized by MALDI-TOF and sequencing of bacterial 16S small subunit ribosomal ribonucleic acid (SSU rRNA) (Ezemuoka *et al.*, 2020) were used for this study. Bacterial isolates were removed from cold storage and thawed to room temperature on a BIO LAB NS-8A clean bench (Bio Laboratories, France). The thawed bacteria samples (100µL) were spread on fresh LB agar plates and incubated overnight at 37ºC to resuscitate the dormant bacteria cells. Subsequently, a colony was streaked on an LB agar plate and re-streaking was repeated to obtain pure/single bacteria colonies. Single colonies of *Enterobacter cloacae* and *Serratia marcescens* were aseptically transferred into 50mL sterile LB broth and incubated overnight at 37ºC with shaking at 120rpm to obtain the stock bacterial cultures.

3.2 Growth curve estimation of *Enterobacter cloacae* and *Serratia marcescens*

One millilitre (1mL) of *Enterobacter cloacae* (OD_{600}= 0.64) and *Serratia marcescens* stock cultures (OD_{600}= 0.59) were each transferred into 49mL sterile LB broth under aseptic conditions and incubated at 37ºC with shaking at 120 rpm to obtain overnight cultures.

Approximately 1mL of bacteria overnight culture i.e *Enterobacter*: OD_{600} = 0.55 or *Serratia*: OD_{600} = 0.50, was aliquoted into each of 15 Falcon tubes containing 50mL sterile LB broth and incubated as previously described. After 30 minutes, the first tube was removed from the incubator, placed on ice and optical cell density (OD_{600}) was measured in triplicate. 1mL portion of the content of the Falcon tube (that has been cultured for 30-minutes) was serially

diluted (10-fold dilution) in 1X PBS to 10^{-10}-10^{-18} and 100µL of each final dilution was spread on LB agar plates in triplicates. The plates were placed in a 37°C incubator for overnight culturing. The procedure described above was repeated at 30-minutes intervals until the remaining 14 tubes had been assayed. The counting of single bacteria colonies was done manually using a counting chamber. The counting was done thrice per plate and an average was taken. OD_{600} was plotted against time using Graphpad prism v8 to obtain the growth curve for each bacterium. The number of colonies was also plotted against OD_{600} to estimate the concentration of bacteria cells.

3.3 Preparation of bacteria culture cell-free spent medium

Five hundred microliters (500µL) each of *Serratia marcescens* (OD_{600}= 0.68) and *Enterobacter cloacae* (OD_{600}= 0.84) were grown in 50mL sterile LB broth for 0.5, 2.5, 4, 8, and 12 hours at 37°C with shaking at 120 rpm and optical cell density measured in triplicate. Cultures at each time point (OD_{600} for *Serratia* and *Enterobacter* were adjusted so that the starting concentration were similar at each time-point) were centrifuged at 14,000 rpm to separate the bacteria cells from the spent medium. The spent medium was further filtered using a 0.22-micron filter (Sigma Aldrich, USA) to remove excess cells. The optical cell density of the filtered spent medium was measured to confirm the exclusion of cells. About 500mL of each bacteria filtrate/extract (cell-free spent medium at different incubated time points) was freeze-dried and stored at 4°C until used to treat *Plasmodium falciparum*.

3.4 Malaria parasite culturing

3.4.1 Thawing of laboratory parasite isolates

Cryopreserved parasites (3D7 and Dd2) were retrieved, thawed at 37ºC for 2 minutes and transferred into 15mL tubes. The parasites were pelleted by centrifuging for 10 minutes at 1500 rpm and the supernatant containing 4.2% sorbitol (Sigma Aldrich, USA) and 0.9% NaCl (Sigma-Aldrich, USA) was discarded. 1mL of complete parasite medium (CPM) was added, mixed well and centrifuged at 1500 rpm for 10 minutes. The supernatant was discarded and washing with CPM was repeated. CPM was again added to the pelleted parasites and mixed. The content was transferred into a T-25 culture flask (Thermo Fisher, USA) containing 5mL complete parasite medium and 200µL packed red blood cells. The culture flask was flushed gently with mixed gas for about 45 seconds, tightly closed and carefully placed in the incubator at 37ºC.

3.4.2 Washing of human O+ erythrocytes (RBCs)

Human O^+ and sickle cell negative blood was collected into sterile vacutainer tubes containing citrate phosphate dextrose and stored at 2-8ºC for 48 hours. Plasma was collected and the blood was transferred to a sterile 15mL tube using a sterile pipette and centrifuged at 2000 rpm for 10 minutes. The serum and buffy coat which contains white blood cells was removed and 10mL of parasite washing media (PWM) was added to the tube, well mixed and centrifuged for further 10 minutes at 2000 rpm. The supernatant was aspirated, and the washing process was repeated twice with parasite washing media and stored at 2-8ºC.

3.4.3 Culturing *Plasmodium falciparum in vitro*

Plasmodium falciparum Chloroquine-sensitive strains (3D7) and Chloroquine resistant laboratory strains (Dd2) were cultured in RPMI 1640 (Sigma Aldrich, USA) at 4% haematocrit and synchronized with 5% sorbitol (Sigma Aldrich, USA) to obtain rings, according to the malaria parasite culture SOP (Department of Parasitology, NMIMR) adapted from Trager and Jensen (1976). The gametocyte-stage of the parasites was purified with Percoll (Sigma Aldrich, USA). The parasite cultures were monitored every 48 hours by preparing thin smears, fixing in 100% methanol, staining with 10% Giemsa and viewing under the Leica microscope at 100X. The parasite culture medium was also changed and gassed with malarial gas (2% O_2, 5.5% CO_2, and 92.5% N_2).

3.5 Growth inhibition assay (GIA)

About 0.5g of the freeze-dried spent media was dissolved in 1mL of distilled water and centrifuged at 14000 rpm for 30 minutes. The supernatant was filtered with 0.22µ filter into a 1.5mL tube to obtain the stock extract solution. Two-fold serial dilution of the stock extracts were prepared using RPMI 1640 (Sigma Aldrich, USA) to get the working concentration of the extract. Ten microliters (10µL) of each serial dilution were aliquoted in triplicates into a 96-well culture plate containing 90µL of parasite (Dd2 or 3D7) culture at 1% parasitaemia and 2% haematocrit. To account for parasite growth, the negative control wells contained 100µL of only uninfected RBCs in complete parasite medium at 2% haematocrit and positive control wells contained 100µL of parasite mixture with no extract. The extract control wells had 100µL lyophilized LB medium added to 90µL of parasite (Dd2 or 3D7) culture at 1% parasitaemia and 2% haematocrit. The culture plates were subsequently gassed and incubated at 37°C for 72 hours in a modular incubator chamber (Billups-Rothenberg, Inc.). After 72 hours of incubation,

the culture plates were removed from the incubation chamber and 100µL of SYBR Green-1 fluorescent (MSF) lysis buffer was added to each well and mixed by pipetting up and down a few times. The plates were then wrapped with aluminium foil and incubated in the dark at room temperature for an hour. The SYBR Green fluorescence intensities were measured with a Varioskan LUX multimode microplate reader (Thermo Fisher Scientific, USA) at excitation and emission wavelengths of 485nm and 530nm, respectively. Negative control wells of the parasites were used to blank the data generated from microplate reader by subtracting the average values of the control wells from the average values from the other wells. A graph of percentage parasitaemia versus logarithmic concentration of the extract was generated and IC_{50} values extrapolated on Graphpad Prism version 8.

3.6 Expression of *GAMER* gene following co-culture of *P. falciparum* gametocytes and cell-free bio-products of *E. cloacae* and *S. marcescens*

3.6.1 Co-culture of *P. falciparum* parasites with cell-free bio-products of *E. cloacae* and *S. marcescens*

To investigate the expression of *GAMER* gene following treatment of gametocytes with bacteria extracts, IC_{50} concentration of each extract was prepared and used to perform GIA on asynchronous parasite culture with gametocyte parasitaemia of 0.09%. Briefly, 10µL of each IC_{50} concentration of extracts was aliquoted into 96-well culture plates containing 90µL/well Dd2 and 3D7 cultures at 1% parasitaemia and 2% hematocrit. Negative control wells contained 100µL of only uninfected RBCs in complete parasite medium at 2% hematocrit to normalize the effect of the experiment on red blood cells) and positive control wells contained 100µL of RBCs and parasites (infected red blood cells) in complete parasite medium at 2% hematocrit with no extract or cell-free spent medium to cater for parasite growth in the absence of the

effect of bacteria cell-free spent medium). The extract control wells contained 100µL lyophilized LB medium added to 90µL of parasite (Dd2 or 3D7) culture at 1% parasitaemia and 2% haematocrit (to normalize the effect of cell-free LB medium on parasite growth). One hundred microliters (100µL) of the content of each well were taken at 0, 1, 6, 8, and 12 hours after co-culture into 1.5mL microcentrifuge tubes containing 1mL RNA later (Fisher, USA). The samples were stored at -80^{o}C until RNA extraction.

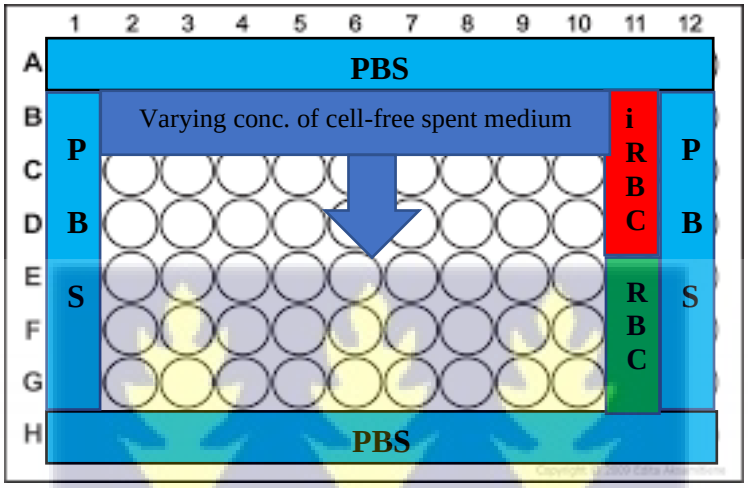

Figure 6: Plate map for the design of the growth inhibition assay of *Plasmodium falciparum* 3D7 and Dd2 strains.

3.6.2 Total RNA extraction from malaria parasites

RNA was extracted using a Trizol protocol developed by Maria Del Pilar Quintana (https://www.researchgate.net/post/Could-anyone-tell-me-which-is-the-best-method-to-extract-total-RNA-from-in-vitro-cultured-Plasmodium-falciparum-strains) with a few modifications (Quintala, 2015). Briefly, the frozen samples were allowed to thaw to room temperature and lysed with zirconia beads by vortexing briefly and centrifuging at 13, 600rpm for 3 minutes. The supernatant was collected into a sterile 1.5 mL tube and 200uL of chloroform was added. The tube was shaken vigorously by vortex, incubated at room temperature for 3

minutes and centrifuged at 13,600 rpm for 15 minutes at 4ºC. About 600µL of the upper aqueous phase was carefully removed and placed in a new tube. 500µL of 100% isopropanol was added and carefully mixed by pipetting up and down, followed by incubation at room temperature for 10 minutes. The sample was centrifuged at 10400 rpm for 10 minutes at 4ºC. The supernatant was carefully removed leaving the RNA pellets. The RNA pellet was washed with 1mL of cold 75% ethanol, vortexed briefly and centrifuged at 6500 rpm for 5 minutes. The supernatant was carefully removed, and the pellet air-dried by heating at 56ºC for 10 minutes. The pellet was resuspended in 30µL of RNAse-free water and incubated for 10 minutes at 55ºC. The concentration was measured using Invitrogen Qubit 2.0 fluorometer (Life Technologies).

3.6.3 Complementary DNA (cDNA) synthesis

cDNA was synthesized using High-Capacity cDNA reverse transcription kit (Thermo Scientific, USA) following the manufacturer's protocol. Briefly, the kit component was allowed to thaw on ice and the required number of reactions were prepared in a master mix. 10µL of 2X master mix was pipetted into each well of a 96-well reaction plate and 10µL of RNA sample was added into each well and properly mixed. The tubes were sealed and briefly centrifuged to spin down the content of the tubes and eliminate any air bubbles. The tubes were placed in a thermocycler and the reverse transcription run started. The PCR products were quantified using Qubit 2.0 fluorimeter (Life Technologies).

3.6.4 Gene expression analysis using RT q-PCR

Quantitative RT-PCR (RT-qPCR) was employed for the expression analysis of the *GAMER* gene. Amplification and quantification of the GAMER gene were performed using the Power Up SYBR Green Universal Two-Step RT-qPCR kit (Applied Biosystems) according to the

manufacturer's instructions using Quant Studio™ 5 Real-Time PCR Systems (ThermoFisher Scientific, Singapore). Each sample was run in triplicate at a total volume of 20µL per reaction. Each reaction contained 2X PowerUp SYBR Green Master Mix, 800nM each of the forward and the reverse primers and 2µL of the cDNA template using the following thermocycling conditions: 2 minutes at 95°C, 40 cycles of 15 seconds at 95°C and 1 minute at 55.1°C. The specificity of the resulting products was analyzed using are melting curve. Gene expression was estimated using the $2^{-\Delta\Delta Ct}$ method of relative quantification. The primers used targeted *GAMER* gene, and *st-RNA* was used as the endogenous control.

The data generated were analyzed using the Quant Studio™ 5 Real-Time PCR Systems (ThermoFisher Scientific, Singapore).

3.7 Data analyses

Absorbance readings were recorded on an Excel spreadsheet (Microsoft Excel 2016). Bacterial growth curves (Figure 6) were analyzed with GraphPad Prism v8.01 and paired sample t-test was used to compare the growth curve of *S. marcescens* to *E. cloacae*. The relationship between bacterial concentration and mean absorbance (Figure 7) were determined using Spearman correlation and unpaired sample t-test was used to compare the two regression lines. Mean absorbance was expressed ± the standard error of the mean (SEM).

IC_{50} curve plots were generated in Graphpad Prism v8.01. The IC_{50} of *Plasmodium falciparum* 3D7 and Dd2 respectively screened with bacteria cell-free spent medium (*Ec*-0.5, *Ec*-2.5, *Ec*-4, *Ec*-8, *Ec*-12; *Ec*-24. *Sm*-0.5, *Sm*-2.5, *Sm*-4, *Sm*-8, *Sm*-12, *Sm*-24) were compared using paired sample t test. The IC_{50} (Table 1) and RI values (Table 2) were compared using one-way ANOVA (Figure 8 – Figure 14, Table 3).

The expression of *GAMER* gene was analysed using Quant studio software version 3 and the data was compared using one-sample t test.

CHAPTER FOUR

4.0 RESULTS

4.1 Growth curve of *Enterobacter cloacae* and *Serratia marcescens*

The growth dynamics of *Enterobacter cloacae* and *Serratia marcescens* were determined after culturing in LB broth for 7 hours. This was done to quantify the bacteria cells that will be used in the inhibition assays. Both bacteria were observed to have similar growth dynamics (Figure 7). An exponential growth of both bacteria was observed over the first 2.5 hours of culturing and there was a steady growth for another 2.5-3 hours after which bacteria growth became constant (Figure 7). The growth curves of the two bacteria species reveal that *E. cloacae* and *S. marcescens* grow differently when cultured in LB broth (p=0.003). However, both bacteria species exhibit a sigmoidal pattern of growth.

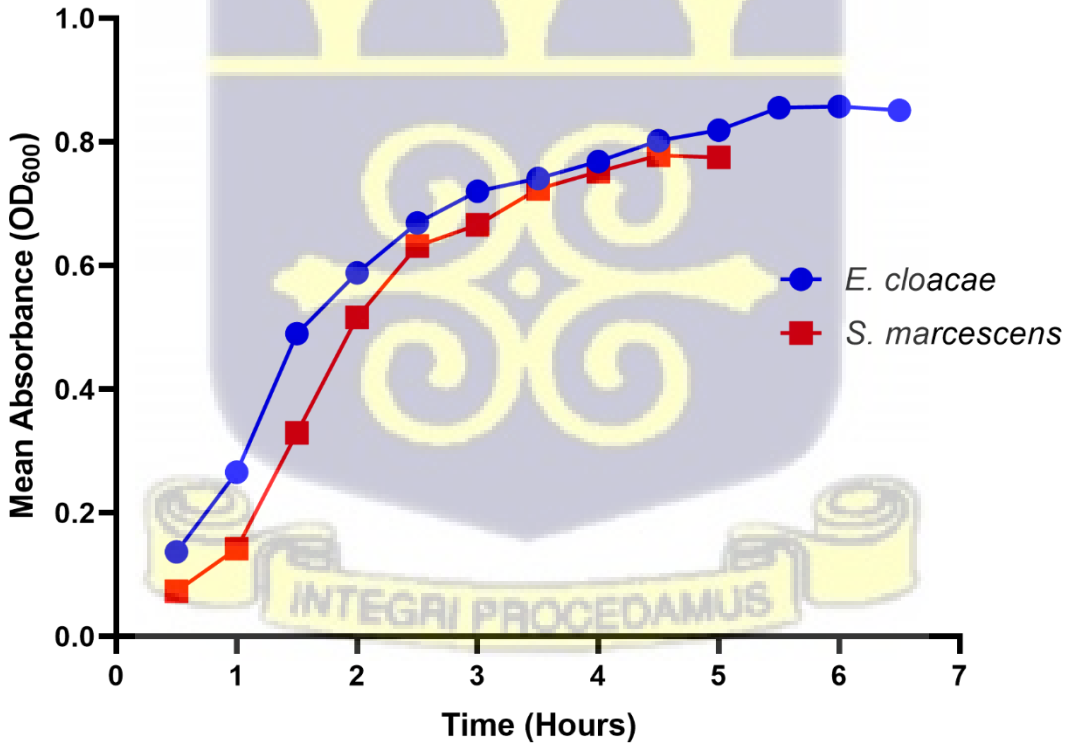

Figure 7:Growth curve of *Enterobacter cloacae* and *Serratia marcescens.*

The optical cell densities were measured at 600 nm and the absorbances (OD_{600}) values are mean of three biological replicates ± SEM (n=3).

4.2 Standard curve for *Enterobacter cloacae* and *Serratia marcescens*)

Bacterial concentration (CFU/mL) was plotted against OD_{600} (Figure 8). *Serratia* appears to have higher concentration of cells than *Enterobacter* at lower OD values. For instance, there were 10^6 cells/mL (antilog of 12) *S. marcescens* cells present in 50mL LB broth at OD_{600}=0.1 while there were 10^4 cells/mL (antilog of 11) *E. cloacae* cells present in the same volume of media at the same OD. However, both bacteria when left with time to proliferate in the same media similarly with time and, OD values and concentration differed significantly between the two bacteria species at higher ODs. Correlation between the colony forming units and mean absorbance was 0.74 (p-value=0.002) and 0.57 (p-value=0.0019) for *E. cloacae* and *S. marcescens*, respectively (**Figure 8**).

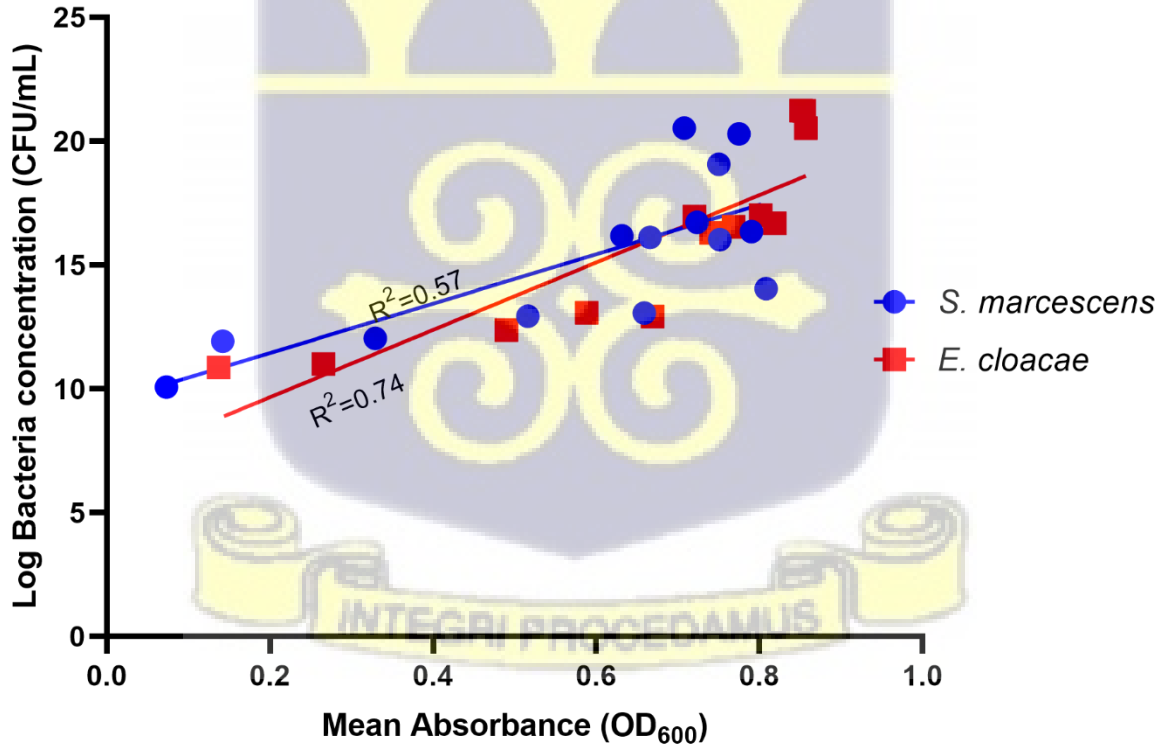

Figure 8:Concentration of *Serratia marcescens* (log transformed) versus mean absorbance (OD600).

The bacteria cells were incubated overnight at 37°C and optical cell density was measured at 600nm. Mean Absorbance (OD_{600}) are averages of three biological replicates ± SEM (n = 3).

4.2.1 Effect of bacteria cell-free spent medium on *P. falciparum* 3D7 and Dd2 strains

Plasmodium falciparum 3D7 and Dd2 parasites were treated with filtered bacterial cell-free spent medium (extracts). The ODs of the extracts were determined to confirm the exclusion of cells (Supplementary Table 1 and 2). The growth of parasites following exposure to bacteria cell-free spent medium was compared to parasites that had no-bacteria extract treatment. The results from parasites cultured in no-bacteria cell-free spent medium was used to normalize cells cultured in bacteria cell-free extracts. Parasites grown in cell-free LB broth control extract was used to normalize for the effect of the bacteria-derived components on growth of parasites accounting any interactions from the LB medium which were mixed with the extracts.

After normalization with results from parasites cultured in cell-free spent media, *Enterobacter cloacae* cultured for 8 hours (*Ec*-8) was the most effective at inhibiting *P. falciparum* 3D7 parasite growth while *Ec*-2.5 was the least active. *Ec*-4 was the most active at killing Dd2 parasites while *Ec*-0.5 was the least active (Figure 9, Table 6). As observed with *E. cloacae*, 3D7 strains were also most affected with 8 hours spent medium of *S. marcescens* (*Sm*-8) (Figure 10). A more concentrated *S. marcescens* spent medium (*Sm*-12) was required to achieve effective inhibition of Dd2 strains. Contrary to *E. cloacae* where 4-hour spent culture exhibited the strongest killing effect, bio-products from *S. marcescens* cultured from the same period (*Sm*-4) was the least active against Dd2 strains (Figure 10, S3 Table).

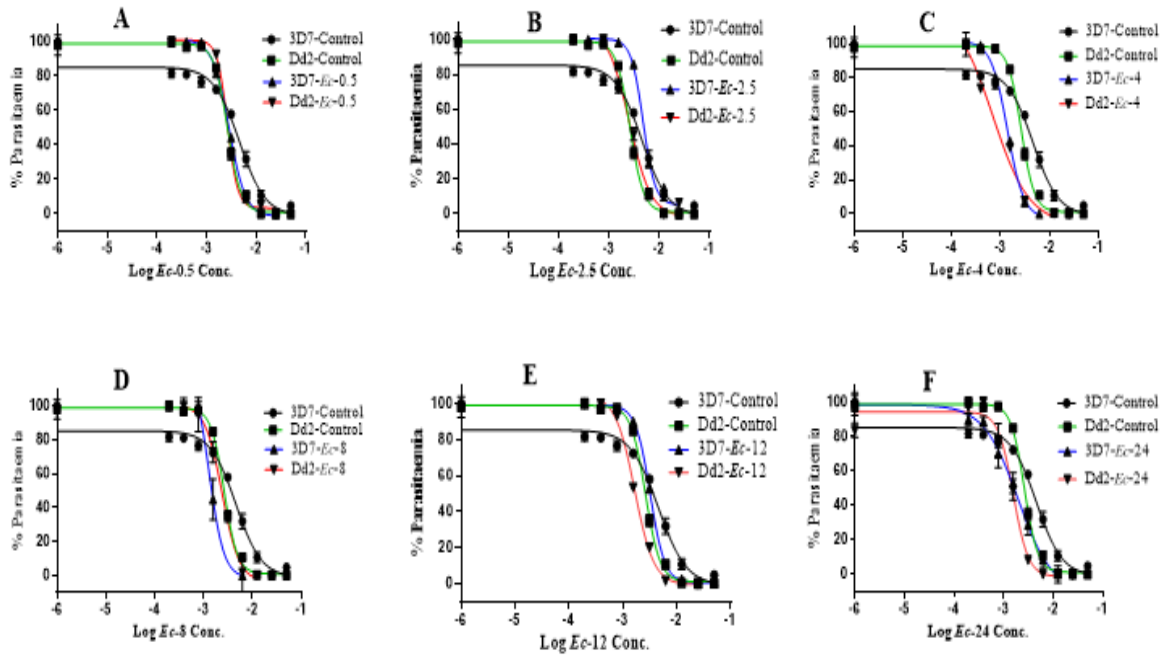

Figure 9: Growth curves of *P. falciparum* 3D7 and Dd2 strains treated with cell-free spent medium produced by *Enterobacter cloacae* cultured for 24 hours.
3D7-Control represent *P. falciparum* 3D7 treated with LB broth control. Dd2-Control represent *P. falciparum* Dd2 treated with LB broth control. **A**. Growth curves of *P. falciparum* 3D7 and Dd2 strains treated with cell-free spent medium produced by *Enterobacter cloacae* cultured for 0.5 hours. **B**. Growth curves of *P. falciparum* 3D7 and Dd2 strains treated with cell-free spent medium produced by *Enterobacter cloacae* cultured for 2.5 hours. **C**. Growth curves of *P. falciparum* 3D7 and Dd2 strains treated with cell-free spent medium produced by *Enterobacter cloacae* cultured for 4 hours. **D**. Growth curves of *P. falciparum* 3D7 and Dd2 strains treated with cell-free spent medium produced by *E. cloacae* cultured for 8 hours **E**. Growth curves of *P. falciparum* 3D7 and Dd2 strains treated with cell-free spent medium produced by *Enterobacter cloacae* cultured for 12 hours. **F**. Growth curves of *P. falciparum* 3D7 and Dd2 strains treated with cell-free spent medium produced by *E. cloacae* cultured for 24 hours

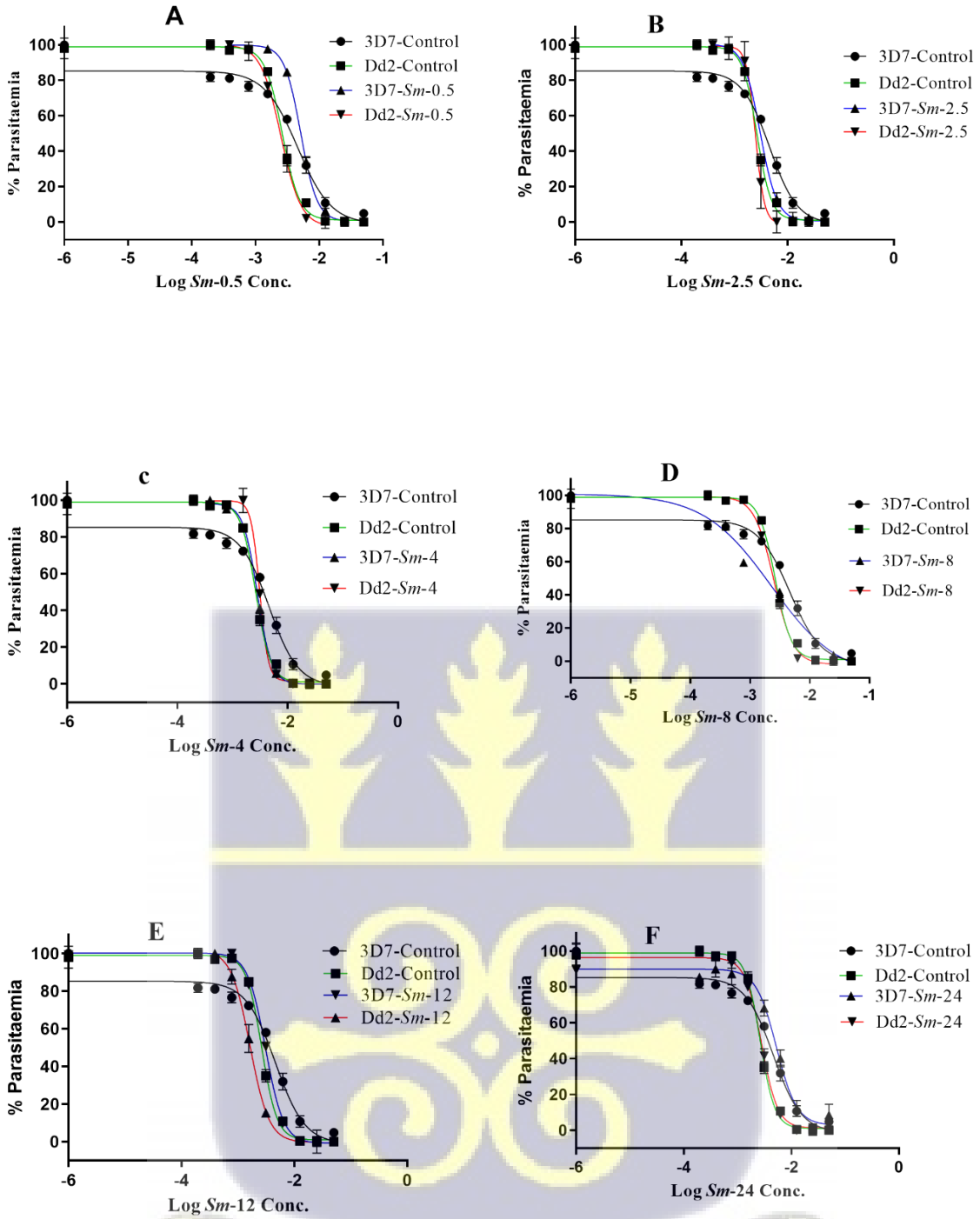

Figure 10: Growth curves of *P. falciparum* 3D7 and Dd2 strains treated with cell-free spent medium produced by *Serratia marcescens* cultured for 24 hours.

3D7-Control represent *P. falciparum* 3D7 treated with LB broth control. Dd2-Control represent *P. falciparum* Dd2 treated with LB broth control. **A**. Growth curves of *P. falciparum* 3D7 and Dd2 strains treated with cell-free spent medium produced by *S. marcescens* cultured for 0.5 hours. **B**. Growth curves of *P. falciparum* 3D7 and Dd2 strains treated with cell-free spent medium produced by *S. marcescens* cultured for 2.5 hours. **C**. Growth curves of *P. falciparum* 3D7 and Dd2 strains treated with cell-free spent medium produced by *S. marcescens* cultured for 4 hours. **D.** Growth curves of *P. falciparum* 3D7 and Dd2 strains treated with cell-free spent

medium produced by *S. marcescens* cultured for 8 hours. **E**. Growth curves of *P. falciparum* 3D7 and Dd2 strains treated with cell-free spent medium produced by *S. marcescens* cultured for 12 hours. **F**. Growth curves of *P. falciparum* 3D7 and Dd2 strains treated with cell-free spent medium produced by *S. marcescens* cultured for 24 hours.

Table 1: Average IC_{50} (mg/µL) following *in vitro* treatment of *Plasmodium* with spent media from *Enterobacter cloacae* and *Serratia marcescens*

Enterobacter cloacae			***Serratia marcescens***		
	P. falciparum strain			*P. falciparum* strain	
Spent medium	3D7	Dd2	**Spent medium**	3D7	Dd2
***Ec*-0.5**	2.83	2.63	***Sm*-0.5**	5.14	2.50
***Ec*-2.5**	4.83	2.55	***Sm*-2.5**	3.05	2.48
***Ec*-4**	1.38	0.69	***Sm*-4**	2.83	3.10
***Ec*-8**	1.29	2.40	***Sm*-8**	2.28	2.51
***Ec*-12**	3.23	1.67	***Sm*-12**	3.08	1.64
***Ec*-24**	1.97	1.58	***Sm*-24**	5.28	2.77
LB-Control	4.61	2.63	**LB-Control**	4.61	2.63

The IC_{50} of *P. falciparum* 3D7 screened with the various *E. cloacae* extracts (*Ec*-0.5, *Ec*-2.5, *Ec*-4, *Ec*-8, *Ec*-12 and *Ec*-24) were individually compared to the control treatment (LB-Control) using paired sample *t*-test. The IC_{50}s of extracts *Ec*-0.5, *Ec*-2.5, *Ec*-8, *Ec*-12 were effective at inhibiting *P. falciparum* 3D7 growth compared to the control (p= 0.03, 0.008, 0.003 and 0.02 respectively). When one-way ANOVA was applied to compare all the IC_{50}s of all the

E. cloacae extracts that were used to screen *P. falciparum* 3D7, only *Ec*-2.5 (p=0.01) and *Ec*-8 (p=0.005) showed significant *P. falciparum* 3D7 inhibition abilities relative to the control. Similarly, comparing the respective IC_{50}s of *E. cloacae* extracts (*Ec*-0.5, *Ec*-2.5, *Ec*-4, *Ec*-8, *Ec*-12 and *Ec*-24) that were used to screen *P. falciparum* Dd2 using paired sample t-test, the extracts (*Ec*-4, *Ec*-12 and *Ec*-24) showed more *P. falciparum* Dd2 inhibitory activity compared to the control with p-values 0.04, 0.05 and 0.02 respectively. However, analysis with one-way ANOVA to compare collectively the IC_{50}s of all the *E. cloacae* extracts on *P. falciparum* Dd2 reveal that the LB control and all the *E. cloacae* extracts had comparable *P. falciparum* growth inhibition potentials (p=0.65).

Screening of *P. falciparum* 3D7 with *S. marcescens* cell free spent media (*Sm*-0.5, *Sm*-2.5, *Sm*-4, *Sm*-8, *Sm*-12 and *Sm*-24) produced IC_{50}s which were independently compared to the IC_{50} of the control treatment (LB-Control) using paired sample t-test. The results revealed that IC_{50} values of all the *S. marcescens* extracts except *Sm*-24 (p=0.65) were significantly lower than that of the control implying the potency of *S. marcescens* cell-free spent media in affecting the parasite growth had been lost after 24 hours of culture. One-way ANOVA was also employed to compare the IC_{50}s of all the *S. marcescens* extracts when they were screened on *P. falciparum* 3D7and the results showed that all the *S. marcescens* cell free spent media (*Sm*-0.5, *Sm*-2.5, *Sm*-4, *Sm*-8, *Sm*-12 and *Sm*-24) had significantly lower IC_{50}s (p<0.0001) relative to the control. In summary, all the extracts (cell free spent media of *S. marcescens*) were more effective at inhibiting *P. falciparum* 3D7 growth compared to the control. On the other hand, when *P. falciparum* Dd2 was screened with the *S. marcescens* extracts and the IC_{50}s compared using paired sample t-test, only *Sm*-0.5 showed a better inhibitory effect (p=0.04). The comparism of the IC_{50}s of *P. falciparum* Dd2 screened with the *S. marcescens* extracts using one-way ANOVA showed that none of the extracts inhibited the growth of *P. falciparum* Dd2 compared to the control (p>0.99).

4.2.2 Resistance indices of *E. cloacae* and *S. marcescens* cell-free spent medium on *Plasmodium falciparum*

Resistance index (RI) is a measure of the efficacy of each cell-free spent medium (extract) compared to control. It is calculated as the ratio of the IC_{50} of the test extract to the IC_{50} of the control. A ratio of 1.0 represent the same activity of the cell-free spent medium as the control, a ratio <1.0 implies that the cell-free medium is more active at inhibiting parasite growth compared to the control, and >1.0 imply the cell-free metabolite is less active at killing parasites compared to the control.

A summary of the RI values reveal that *Ec*-8 and *Sm*-8 were the most effective cell-free spent media against the drug sensitive *P. falciparum* 3D7. However, the drug resistant *P. falciparum* Dd2 strains were most affected by *Ec*-4 and *Sm*-12.

Table 2*:* Resistance indices of *Enterobacter cloacae* and *Serratia marcescens* cell-free spent medium on *Plasmodium falciparum* 3D7 and Dd2 strains

Enterobacter cloacae			***Serratia marcescens***		
	P. falciparum			*P. falciparum*	
Spent medium	3D7	Dd2	**Spent medium**	3D7	Dd2
***Ec*-0.5**	0.61	1.00	***Sm*-0.5**	1.12	0.95
***Ec*-2.5**	1.05	0.98	***Sm*-2.5**	0.66	0.94
***Ec*-4**	0.30	0.26	***Sm*-4**	0.61	1.18
***Ec*-8**	0.28	0.91	***Sm*-8**	0.49	0.95
***Ec*-12**	0.70	0.63	***Sm*-12**	0.67	0.62
***Ec*-24**	0.43	0.60	***Sm*-24**	1.14	1.08
LB-Control	1.00	1.00	**LB-Control**	1.00	1.00

4.2.3 Pattern of the anti-Plasmodial effects of bacteria cell-free spent media

Screening of bacteria cell free spent media against 3D7 and Dd2 *P. falciparum* strains using the by-products of *Enterobacter cloacae* and *Serratia marcescens* revealed a wave-like pattern (Figures 11 and 12). Although the trend of the anti-*Plasmodium* effect of *E. cloacae* by-products on 3D7 and Dd2 were similar, *E. cloacae* cell free spent medium was more effective on Dd2 compared to 3D7. This is depicted by the generally lower IC_{50} values across the different concentrations of *E. cloacae* extracts on Dd2 compared to 3D7 (Figure 10). Similarly, IC_{50} was generally lower in Dd2 than in 3D7 when treated with by-product of *Serratia marcescens*. However, the *S. marcescens* appeared to perform better on 3D7 with lowest IC_{50} at 8 hours culture (4×10^{12} CFU/mL) and lowest for Dd2 at 12 hours culture (3×10^{20}CFU/mL) (Figure 12).

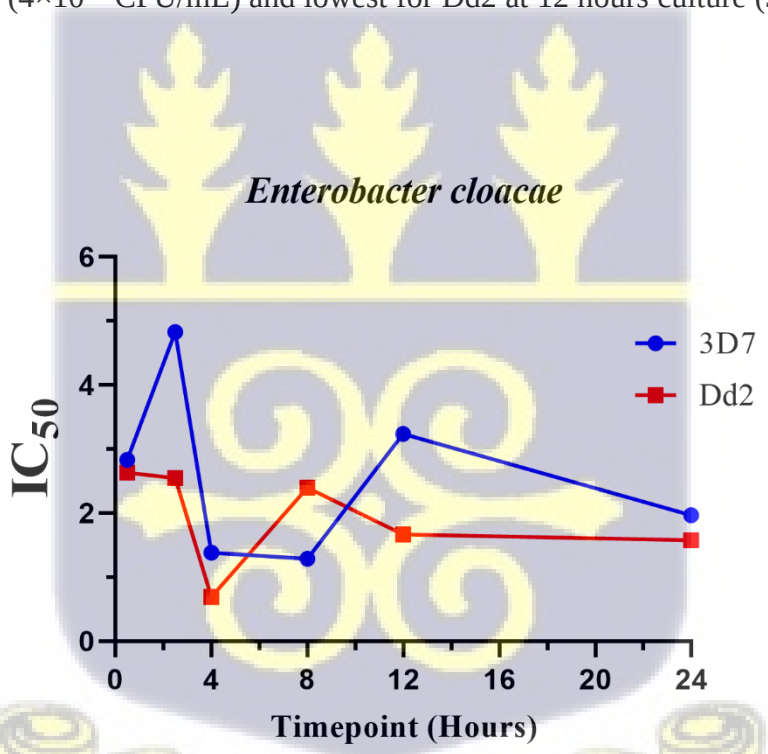

Figure 11: *P. falciparum* 3D7 and Dd2 strains treated with cell-free metabolites produced by *Enterobacter* cultured for 24 hours. Values are mean of five replicates ± SEM.

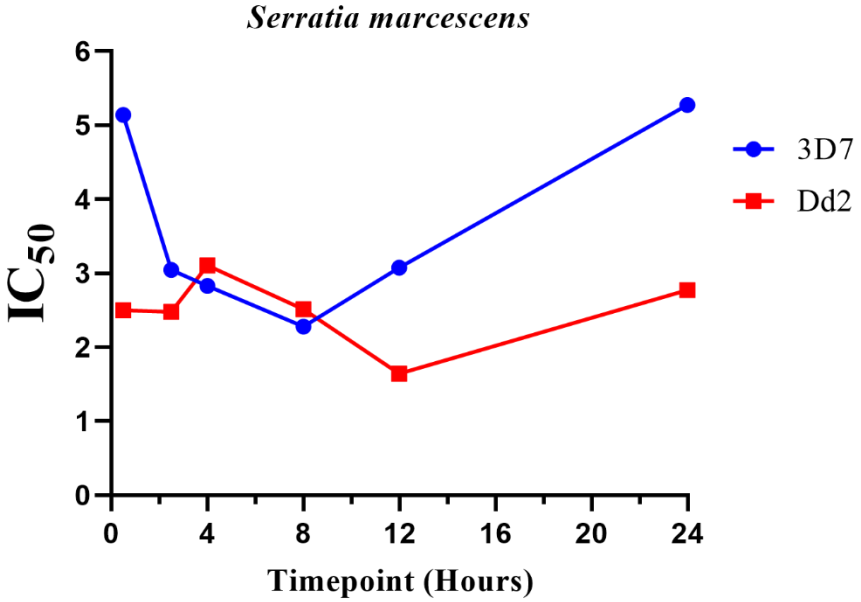

Figure 12: *P. falciparum* 3D7 and Dd2 strains treated with cell-free metabolites produced by *Serratia* cultured for 24 hours. Values are mean of five replicates ± SEM.

4.3 Expression of *GAMER* gene following exposure of *Plasmodium* to bacteria cell-free spent medium

Optimization of qPCR conditions and the efficiency of the primer was tested by performing a standard curve for the endogenous gene (*STRNA*) (Figure 13) and the target gene (*GAMER*) (Figure 14). The efficiency of the primers to *STRNA* and *GAMER* genes were 104.3 and 111.7 respectively.

To determine whether *GAMER* gene is affected by bacteria by-products, asynchronized cultures of 3D7 were screened with the most effective IC_{50} concentration of *E. cloacae* by-product (*Ec*-8) and parasites material collected after 0, 1, 6, 8 and 12 hours. RNA was extracted from these parasites to determine the expression pattern at the different time points (S6 Table). The expression of *GAMER* increased by 1-fold after 1 hour and remained unchanged after 6 hours and 12 hours of co-culture (S6 Table).

The absolute fold differences recorded after screening *P. falciparum* 3D7 strains over 12 hours were plotted against the extracts used (Figure 15). The expression of the *GAMER* gene was generally seen to be upregulated when *P. falciparum* 3D7 was treated with extracts prepared from LB broth compared to the untreated (No drug control). Similarly, a higher level of the *GAMER* gene expression was observed after *P. falciparum* 3D7 was screened with *Ec*-8 relative to the untreated control. However, the expression of the *GAMER* gene in parasites treated with *Ec*-8 was relatively low compared to those treated with LB.

The expression of *GAMER* after 1-hour, 6 and 12 hours of co-culturing *P. falciparum* 3D7 and LB broth and *E. cloacae* spent medium (*Ec*-8) relative to the control was compared using one-sample t-test. The results (S12 Table) showed that there was a significant change in the expression of *GAMER* (p <0.0001) compared to the control.

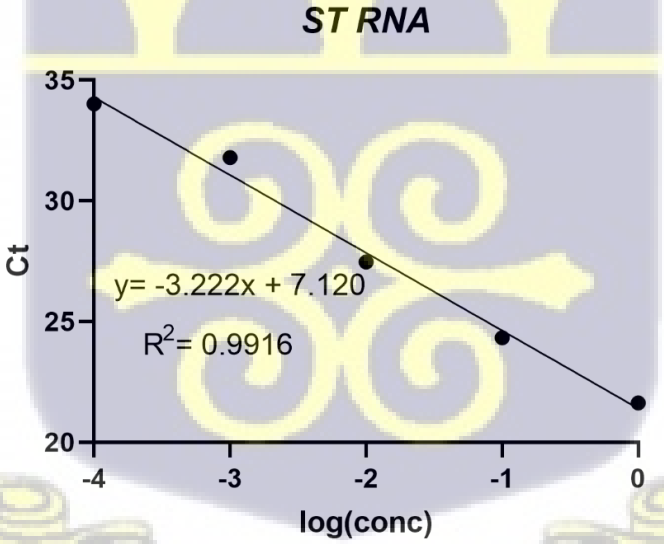

Figure 13: Standard curve of endogenous control.

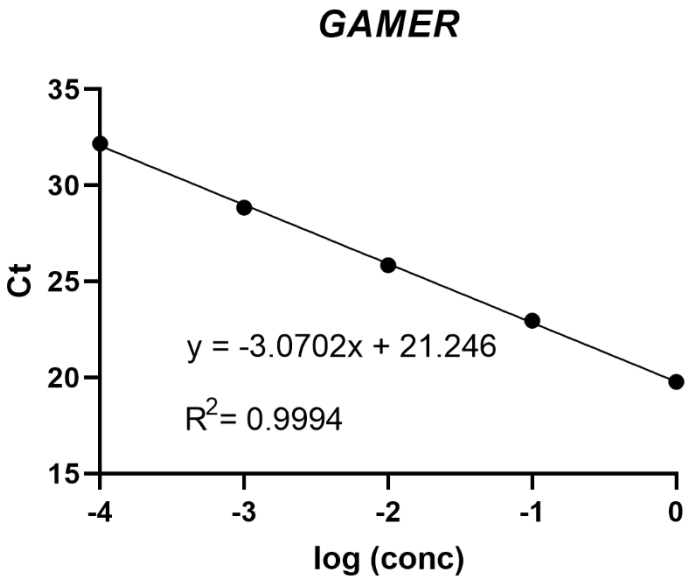

Figure 14: Standard curve of target gene.

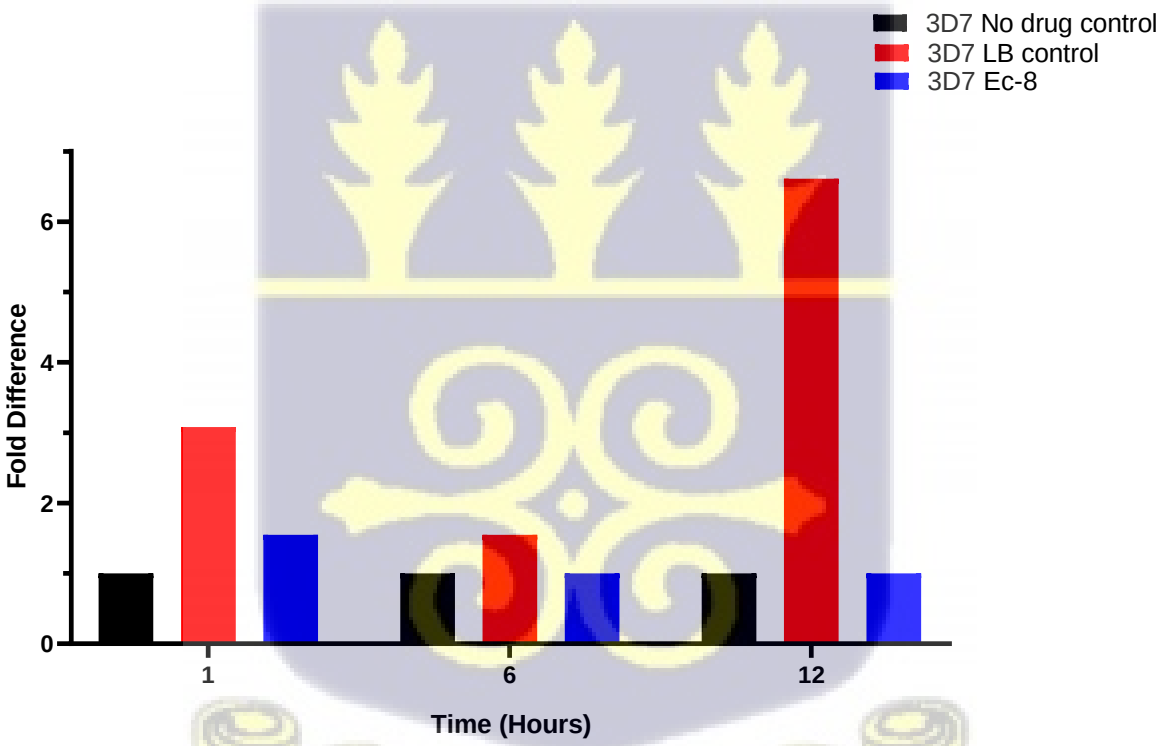

Figure 15: Expression of *GAMER* is upregulated after an hour of screening *P. falciparum* 3D7 with *Ec*-8. The gene expression analysis of *GAMER* at different time points between the different controls 3D7 No drug control (1hr), 3D7 LB control (1hr) and 3D7 *Ec*-8 (1

hr) and the same treatment after 6 hours and after 12 hours with the fold change in expression plotted against the extract treatments.

CHAPTER FIVE

5.0 DISCUSSION

Malaria continues to be a disease of global concern despite continuous efforts for eradication and elimination. The periodic emergence of anti-malarial drug resistance, lack of effective anti-malarial vaccines and failing vector-control methods impede the fight to successfully control the disease (World Health Organization, 2021). It has been demonstrated that an *Enterobacter sp*. isolated from wild *Anopheles* mosquitoes was able to make the vector refractory to *Plasmodium falciparum* infection via a mosquito-independent mechanism with the parasite that involved the secretion of reactive oxygen species (Cirimotich *et al.*, 2011). This suggests that the effects of bacteria on parasites in mosquito vectors is not exclusively through an immune-dependent mechanism and could be a direct interaction between bacteria and parasite. Therefore, this study investigated the lethal effect of cell-free secreted bio-products of *E. cloacae* and *S. marcescens* on a key *Plasmodium* parasite developmental gene (*GAMER*) for its potential as a target for malaria transmission-blocking.

Microorganisms found in the midguts of insects play diverse roles to support the general wellbeing of their insect hosts (Pidiyar *et al.*, 2004; Douglas, 2015). They are major players in physiological processes such as larval development, nutrition, digestion and immunity (Garver *et al.*, 2012; Coon *et al.*, 2017; Weiss *et al.*, 2019). *S. marcescens* and *E. cloacae* belong to the family Enterobacteriaceae (Cirimotich, Ramirez and Dimopoulos, 2011; Wang *et al.*, 2011; Gendrin and Christophides, 2013; Gendrin *et al.*, 2015). Although the abundance of *E. cloacae* and *S. marcescens* in the midgut of mosquitoes generally increase after a blood meal (Cirimotich, Ramirez and Dimopoulos, 2011; Wang *et al.*, 2011; Gendrin and Christophides, 2013; Gendrin *et al.*, 2015), little is known about the growth dynamics of the two bacteria species within the mosquito midgut.

It was observed in this study that *E. cloacae* and *S. marcescens* have different growth characteristics in the same culture media. That is, both bacteria species grow differently when cultured in LB broth. There were more *E. cloacae* cells than *S. marcescens* cells for the same OD_{600}. This is indicative that *E. cloacae* probably forms more dense compact cells than *S. marcescens* and may further suggest that *E. cloacae* is more abundant compared to *S. marcescens* in mosquito midgut. However, both bacteria strains produced a similar sigmoidal growth curve when cultured in LB.

The transmission of *Plasmodium* parasites from the vector to the human host depend on the successful completion of the complex developmental cycle of parasites in the mosquito vector (Smith, Vega-Rodríguez and Jacobs-Lorena, 2014). Some bacteria species resident in the mosquito midgut have shown their ability to interrupt the development of *Plasmodium* parasites which makes the mosquito midgut a major bottleneck for the development of *P. falciparum* (Cirimotich, Ramirez and Dimopoulos, 2011; Boissière *et al.*, 2012; Smith, Vega-Rodríguez and Jacobs-Lorena, 2014). It was observed in this study that *E. cloacae* and *S. marcescens* cell free bio-products partially inhibited the growth of *Plasmodium falciparum*.

Inhibition of *P. falciparum* 3D7 and Dd2 growth by cell-free bio-products of *E. cloacae* and *S. marcescens* suggests a direct effect of bacteria metabolites on parasite development. This is in agreement with similar *Plasmodium* growth inhibition studies with *E. cloacae* and S. marcescens isolated from wild Anopheles populations (Bahia *et al.*, 2014; Ramirez *et al.*, 2014; Dennison *et al.*, 2016; Bai *et al.*, 2019).

It was observed that the IC_{50} concentrations of the cell-free spent media bio-products of *E. cloacae* and *S. marcescens* depended on the duration of culture of the two bacteria species. This may suggest that the production, release into media and degradation of the anti-*Plasmodium* components of the bacteria cell-free spent medium is time regulated. Thus, the

lethal bio-products may be released at certain time points during the growth of bacteria and degraded after some time has elapsed making them unavailable to eventually affect *Plasmodium* growth and development. This is in agreement with similar studies that have shown that bacterial products such as tetracycline and doxycycline are lethal to *Plasmodium falciparum* (Azambuja, Garcia and Ratcliffe, 2005; Dahl *et al.*, 2006; Gaillard, Madamet and Pradines, 2015; Javvadi *et al.*, 2018).

Several *Plasmodium* genes regulate the various developmental stages of *P. falciparum* within the vector and key amongst these genes is *GAMER* that regulates the release of male gametes thereby affecting the progression from gametocytes to ookinetes (Dong, Manfredini and Dimopoulos, 2009; Akinosoglou *et al.*, 2015; Cappelli *et al.*, 2019). It was seen in this work that cell-free bio-products in *E. cloacae* spent medium negatively affected the expression of *P. falciparum* GAMER gene. Although the exact mechanism of action of the *E. cloacae* cell free bio products on *GAMER* cannot be fully explained, there may be a direct effect of the cell-free spent medium on the parasites *GAMER* gene.

It was observed in this study that LB broth do not inhibit the expression of *GAMER*. The relatively high *GAMER* expression observed when *P. falciparum* 3D7 was co-cultured with *Ec*-8 for an hour, denoted 3D7 *Ec*-8 (1hr), may be due to residual amount of LB in the extract. Hence, purification of the extracts (bacteria cell-free spent medium) may enable the true or exact impact of the extract (bacteria cell-free spent medium) on the *GAMER* gene to be fully determined.

5.1 LIMITATIONS OF THE STUDY

The major challenge of this study was the difficulty in designing the experiment to replicate the conditions of the mosquito gut microbiome. For example, replicating the natural concentrations of *S. marcescens* and *E. cloacae* in the mosquito midgut to allow for the

determination of the effect of the bacteria metabolites on the *P. falciparum* growth and development. A liquid-based blood growth medium which would have been more suitable for culturing *S. marcescens* and *E. cloacae* and allow for the measurement of the bacteria growth in the same medium was unavailable. This would have better mimicked the blood-preferred environment of these Enterobacteriaceae.

The study was also limited by the undue delay of the ordered materials and reagents required for gene expression assays. There were numerous instances of contaminated culture flasks during the long-term propagation of *P. falciparum*. Gene expression profiles for all the extracts (cell-free spent media) of *S. marcescens* could not be generated after the completion of the growth inhibition assays. However, the limitations of this study did not in any way compromise the quality and the accuracy of the study.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1 Conclusion

It was established in this study that bacteria cell-free spent medium of *E. cloacae* directly inhibits the growth of asynchronous cultures of *Plasmodium falciparum* better than *S. marcescens*. It has also been shown that the *E. cloacae* cell-free spent media down regulates the expression of the *GAMER* gene in *P. falciparum* parasites. This gene, therefore, provides an effective target that can be further studied to help in the development of a novel malaria control intervention.

6.2 Recommendations

Further (*in vivo*) studies should be carried out to determine whether the effect of bacteria cell-free spent medium on *Plasmodium falciparum* development which was seen in this *in vitro* study is similar to what happens within the *Anopheles* mosquito.

The active components of the bacteria cell-free spent medium should be isolated and characterized and used for further studies to explore the efficacy and potency of the components. The purification of the extract will help remove any residual amount of LB from the bacteria spent media. In essence, the effect on the expression of *GAMER* which would be observed after the experiment is repeated with the purified extracts will reflect the true effect of the components of the bacteria spent medium on the gene.

The anti-Plasmodial effect of *E. cloacae* and *S. marcescens* cell-free spent medium on other *Plasmodium falciparum* strains such as *P. falciparum NF54*, *w2mef* etc. should be assessed to help understand better the strain specificity effects of the bacteria by-products as this will give a better picture of what happens in the wild.

The total transcriptome of the parasite on exposure to the cell-free spent medium would provide a detailed understanding of the genetic effect of these bacterial by-products.

The expression of *GAMER* should be repeated using *P. falciparum* Dd2 in order to broaden our understanding of the interactions between the parasite and the mosquito microbiome *in vitro.*

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APPENDICES

SUPPORTING INFORMATON

S1 Table. Measurement of Optical cell density (OD600) of *Enterobacter cloacae*

Enterobacter cloacae OD_{600} Measurement

Time	Bacteria 1			Bacteria 2			Bacteria 3			Average OD	SDEV SQ	Average Dev	SEM
30	0.154	0.136	0.137	0.127	0.133	0.138	0.134	0.147	0.126	0.1369	0.0006	0.0063	0.0030
60	0.273	0.253	0.265	0.26	0.282	0.263	0.26	0.269	0.265	0.2656	0.0005	0.0060	0.0027
90	0.47	0.485	0.488	0.479	0.485	0.508	0.491	0.495	0.509	0.4900	0.0012	0.0100	0.0042
120	0.583	0.59	0.586	0.577	0.592	0.592	0.594	0.584	0.598	0.5884	0.0003	0.0052	0.0022
150	0.662	0.651	0.642	0.692	0.691	0.684	0.662	0.659	0.68	0.6692	0.0026	0.0156	0.0060
180	0.71	0.726	0.731	0.697	0.723	0.704	0.718	0.733	0.742	0.7204	0.0017	0.0117	0.0049
210	0.741	0.747	0.753	0.726	0.73	0.738	0.752	0.737	0.746	0.7411	0.0007	0.0074	0.0031
240	0.779	0.785	0.794	0.733	0.741	0.751	0.762	0.783	0.789	0.7689	0.0040	0.0194	0.0075
270	0.823	0.899	0.849	0.762	0.769	0.768	0.775	0.772	0.8	0.8019	0.0175	0.0367	0.0156
300	0.753	0.785	0.752	0.844	0.86	0.89	0.82	0.829	0.838	0.8190	0.0178	0.0371	0.0157
330	0.835	0.85	0.873	0.86	0.861	0.875	0.838	0.853	0.851	0.8551	0.0015	0.0107	0.0046
360	0.76	0.759	0.763	0.786	0.774	0.806	0.76	0.749	0.737	0.7660	0.0033	0.0151	0.0068
390	0.862	0.852	0.897	0.813	0.815	0.847	0.863	0.881	0.885	0.8572	0.0068	0.0226	0.0098

42 0	0.8 94	0.8 94	0.9 44	0.7 69	0.7 82	0.8 32	0.8 49	0.8 48	0.8 5	0.85133 3333	0.024 2	0.039 6	0.018 3

S2 Table. *Enterobacter cloacae* colony counts

***E. cloacae* colony counts (cfu/mL)**					
Time (Hours)	Bacteria Concentration (cfu/mL)			Average Bacteria Conc. (cfu/ml)	Log Bacteria Conc. (cfu/ml)
0.5	4.73E+08	1.70E+10	2E+11	7.25E+10	10.8603
1.0	4.43E+08	7.00E+09	3.00E+11	1.02E+11	11.0106
1.5	6.16E+09	2.53E+11	6.83E+12	2.36E+12	12.3735
2.0	5.07E+09	4.13E+11	3.52E+13	1.19E+13	13.0745
2.5	3.88E+09	2.31E+11	2.44E+13	8.21E+12	12.9144
3.0	2.28E+13	2.58E+15	2.70E+17	9.09E+16	16.9584
3.5	3.67E+13	1.31E+15	5.77E+16	1.97E+16	16.2941
4.0	5.03E+13	1.73E+15	1.03E+17	3.49E+16	16.5432
4.5	8.06E+13	3.56E+15	3.03E+17	1.02E+17	17.0095
5.0	3.21E+13	2.83E+15	1.43E+17	4.86E+16	16.6868
5.5	6.36E+17	5.17E+19	5.00E+21	1.68E+21	21.2264
6.0	4.92E+17	2.51E+19	4.26E+21	1.43E+21	21.15489
6.5	3.51E+17	1.60E+19	1.01E+21	3.42E+20	20.5342
7.0	2.03E+17	4.64E+19	5.14E+21	1.73E+21	21.2378

S3 Table. Measurement of Optical cell density (OD600) of *Serratia marcescens*

Optical density (OD_{600}) of *Serratia marcescens*												
Time (Hours)	Bacteria 1			Bacteria 2			Bacteria 3			Av. OD_{600}	Std Dev.	SEM
0.5	0.049	0.077	0.077	0.07	0.079	0.071	0.078	0.078	0.078	0.0730	0.00965	0.0032
1.0	0.14	0.145	0.153	0.148	0.141	0.138	0.143	0.135	0.135	0.1420	0.0060	0.0020
1.5	0.331	0.325	0.334	0.328	0.321	0.324	0.335	0.322	0.343	0.3292	0.0072	0.0024
2.0	0.531	0.524	0.541	0.505	0.491	0.505	0.508	0.51	0.531	0.5162	0.0162	0.0054
2.5	0.602	0.61	0.605	0.662	0.637	0.7	0.693	0.693	0.73	0.6591	0.0476	0.0159
3.0	0.626	0.63	0.621	0.628	0.625	0.635	0.638	0.642	0.64	0.6317	0.00734	0.0025
3.5	0.641	0.644	0.642	0.681	0.651	0.672	0.69	0.682	0.692	0.6661	0.0214	0.0071
4.0	0.664	0.668	0.68	0.678	0.674	0.886	0.956	0.966	0.941	0.7903	0.1412	0.0471
4.5	0.724	0.724	0.736	0.722	0.713	0.73	0.726	0.711	0.729	0.7239	0.0079	0.0026
5.0	0.708	0.703	0.726	0.773	0.759	0.777	0.78	0.762	0.776	0.7516	0.0308	0.0103
5.5	0.702	0.692	0.764	0.78	0.778	0.783	0.758	0.746	0.75	0.7503	0.0330	0.0110
6.0	0.676	0.686	0.684	0.684	0.694	0.703	0.752	0.754	0.741	0.7082	0.0317	0.0106
6.5	0.753	0.752	0.755	0.811	0.82	0.823	0.842	0.847	0.874	0.8086	0.0452	0.0151
7.0	0.776	0.798	0.799	0.799	0.815	0.83	0.721	0.71	0.728	0.7751	0.0442	0.0147
control	0.018	0.017	0.008	0.02	0.021	0.017	0.022	0.023	0.022	0.0187	0.0046	0.0015
Blank	0.002	0.001	0.004	0	0.004	0	0.002	0.001	0.004	0.0020	0.0017	0.0006

S4 Table. *Serratia marcescens* colony counts

Serratia marcescens colony counts					
Time (Hours)	Bacteria Concentration (cfu/mL)			Average Bacteria conc. (cfu/mL)	log Average Bacteria conc (cfu/mL)
30	80000000	2333333333	33333333333	1.1916E+10	10.0761
60	480000000	41000000000	2.5E+12	8.4716E+11	11.9280
90	1046666667	78666666667	3.16667E+12	1.08213E+12	12.0343
120	7083333333	1.165E+11	2.56167E+13	8.58008E+12	12.9335
150	5103333333	1.83667E+11	3.44333E+13	1.15407E+13	13.0622
180	1.29E+13	1.29E+15	4.46667E+16	1.53232E+16	16.1853
210	2.35333E+13	1.75667E+15	3.73333E+16	1.30378E+16	16.1152
240	6.67333E+13	2.25E+15	6.5E+16	2.24389E+16	16.3510
270	1.73E+13	2.38E+15	1.58E+17	5.34658E+16	16.7281
300	3.4E+12	2.91333E+15	2.86667E+16	1.05278E+16	16.0223
330	2.32667E+17	1.8E+19	1.66667E+19	1.16331E+19	19.0657
360	1.55E+17	5.86667E+18	9.96667E+20	3.34229E+20	20.5240
390	3.33333E+14	0	0	1.11111E+14	14.04587
420	4.13333E+16	6.3E+18	5.9E+20	1.9878E+20	20.2984

S5 Table. Cell-free spent medium of *Enterobacter cloacae* cultured at selected time points

	Mean Absorbance (OD_{600})		
Spent Medium	Before filtration	After filtration	**Mass (g)**
LB-C	0.001	0.000	5.0813
Ec-0.5	0.056	0.003	8.0179
Ec-2.5	0.108	0.002	4.1299
Ec-4	0.529	0.001	8.4793
Ec-8	0.663	0.000	2.1106
Ec-12	0.776	0.000	1.354
Ec-24	0.889	0.003	5.3827

Ec-0.5: Cell-free spent medium of *Enterobacter cloacae* cultured in LB broth for 30 minutes. *Ec*-2.5: cell-free spent medium of *Enterobacter cloacae* grown for 1 hour in LB broth. Similarly, *Ec*-4, *Ec*-8, *Ec*-12 and *Ec*-24 are cell-free spent medium of *Enterobacter cloacae* cultured in LB broth for 4, 8, 12 and 24 hours respectively. LB-C represent LB broth which served as control.

S6 Table. Cell-free spent medium *of Serratia marcescens* cultured at selected time points

Spent Medium	Mean Absorbance (OD_{600})		Mass (g)
	Before filtration	After filtration	
LB-C	0.001	0.000	5.0813
Sm-0.5	0.075	0.000	8.4201
Sm-2.5	0.193	0.001	9.5686
Sm-4	0.567	0.001	5.5636
Sm-8	0.650	0.002	2.7153
Sm-12	0.692	0.000	1.4458
Sm-24	0.711	0.002	7.6256

Sm-0.5, *Sm*-2.5, *Sm*-4, *Sm*-8, *Sm*-12 and *Sm*-24 are cell-free spent medium of *Serratia marcescens* cultured in LB broth for 0.5, 2.5, 4, 8, 12 and 24 hours respectively. LB-C represent LB broth control.

S7 Table. Primer sequences used in this study

GAMER	
Forward (5' to 3')	TGGTTCCATCTTCTTGGTCA
Reverse (5' to 3')	TGCCTTTTCAGCAAAAACAC
ST RNA	
Forward (5' to 3')	AAGTAGCAGGTCATCGTGGTT
Reverse (5' to 3')	TTCGGCACATTCTTCCAT AA

S8 Table. RT-qPCR reaction set up

Component	Volume for 20µL reaction	Final concentration
PowerUp SYBR Green Master Mix (2X)	10µL	1X
Forward Primer	0.6µL	800nM
Reverse Primer	0.6µL	800nM
Template cDNA	1.0µL	2 ng
Nuclease free Water (Molecular Grade)	7.8µL	

S9 Table. RT-qPCR reaction conditions

Cycle step	Temperature	Duration	Cycles
UDG activation	55°C	15 seconds	1
Initial denaturation	95 °C	2 minutes	1
Denaturation	95 °C	15 seconds	40
Extension	60 °C	1 minute	
Melt curve	60-95 °C	various	1

S10 Table. cDNA reaction set up

Component	Volume for 10µL reaction	Final concentration
10X Reverse transcriptase Buffer	2.0µL	2X
25X dNTP mix	0.8µL	2X
10X Random primers	2.0µL	2X
Reverse transcriptase	1.0µL	
Template RNA	5µL	3µg
Nuclease free water	4.2µL	

S11 Table. cDNA synthesis reaction conditions

Cycle step	Temperature	Duration	Cycles
Step 1	25°C	10 minutes	1
Step 2	37 °C	120 minutes	
Step 3	85 °C	5 minutes	
Step 4	4 °C	Hold	

S12 Table. Comparing the differences in *GAMER* expression using one sample t test

t, df	t=8.441, df=8
P value (two tailed)	<0.0001
P value summary	****
Significant (alpha=0.05)?	Yes
How big is the discrepancy?	
Discrepancy	-0.8123
SD of discrepancy	0.2887
SEM of discrepancy	0.09623
95% confidence interval	-1.034 to -0.5904
R squared (partial eta squared)	0.8991

S13 Table. Fold changes involved in the expression of *GAMER* in *P. falciparum* 3D7 screened with LB and *Ec*-8

CODE	SAMPLE ID	FOLD DIFFERENCE	STANDARD DEVIATION
37	3D7 NO DRUG CONTROL (1 HRS)	1	0.618
40	3D7 LB CONTROL (1 HRS)	3.08	0.819
43	3D7 *Ec*-8 (1 HRS)	1.55	0.138
75	3D7 NO DRUG CONTROL (6 HRS)	1	1.349
76	3D7 LB CONTROL (6 HRS)	1.55	0.713
80	3D7 *Ec*-8 (6 HRS) 2	1	
145	3D7 NO DRUG CONTROL (12 HRS)	1	0.850
149	3D7 LB CONTROL (12 HRS)	6.610	0.983
152	3D7 *Ec*-8 (12 HRS)	1	2.005