OCCURRENCE AND INTERACTIONS OF MYCOBIOMES WITH THE *Anopheles gambiae* HOST

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DECLARATION

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

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DEDICATION

To myself and those who through interactions willingly or/and unknowillingly ensured I developed the "The muscle and spirit of endurance, resilience training on how to deal with failure, life challenges, unexpected turn of events, discrimination, rejection and cruelty as a final rite of passage" My appreciation of life has never been this unusual. To the ***QUEEN*** who kept me going and parted my back when it was aching, ***Gladys Cherono***, a cheerleader of mine whom we also do life together, you are the best. You were tolerant to my absence and nurtured our stars (Habbil, Buyanzi and Indinda Jr), and this still gives me joy.

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MAY GOD BLESS YOU ALL

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LIST OF ACRONYMS/ABBREVIATIONS

ACT : Artemisinin-based Combination Therapy

AMPs : Antimicrobial Peptides

CDC : Centres for Disease Control

cDNA : Complementary Double Nucleic Acid

CFUs : Colony Forming Units

CRISPR : Clustered Regularly Interspaced Short Palindromic Repeats

ELISA : Enzyme-linked Immunosorbent Assay

Fbn9 : Fibrinogen-Related Protein Family

HEG : Homing Endonuclease

HRM-PCR : High Resolution Melting – Polymerase Chain Reaction

IMD : Immune Deficiency Pathway

IRS : Indoor Residual Spraying

ITNs : Insecticide-Treated Bednets

***i*ToC**: *icipe* Thomas Odhiambo Campus-Mbita

ITS : Internal Transcribed Spacer

KEMRI : Kenya Medical Research Institute

Lepto : *Leptosphaerulina* sp

LRIM1 : Leucine-Rich Repeat Proteins

Mab : Monoclonal Antibody

MEGA : Molecular Evolutionary Genetics Analysis

ML-EID: Martin Luscher Emerging and Infectious Diseases Laboratory

PCR : Polymerase Chain Reaction

PDA: Potato Dextrose Agar

PF : *Plasmodium falciparum*

PGRPLC : Peptidoglycan Recognition Protein LC

PRRs : Pattern Recognition Receptors

qPCR : quantitative Polymerase Chain Reaction

SIT : Sterile Insect Techniques

TEP1 : Thioester-Containing Protein 1

WHO : World Health Organization

YPDA : Yeast Peptone Dextrose Agar

ABSTRACT

Anopheles mosquitoes are colonized by diverse microorganisms that may influence hosts' biology and vectorial capacity. Resident *Anopheles* bacterial symbionts isolated from these hosts are known to influence traits such as generational fitness, survival, host seeking,and infectivity of pathogens such as*Plasmodium falciparum.*Eukaryotic symbionts such as fungi residing in the gut of *Anopheles gambiae*could confer similar phenotypes, but are undexplored. It was hypothesized that fungi associating with *Anopheles* could establish a stable and generational association with these mosquitoes thereby influencing their fitness and could offer protection against malaria parasites. The objective of this study was to characterize fungalsymbionts of *Anopheles gambiae*and establish their tripartite interactions with the parasite. *Anopheles* samples obtained from screen houses mimicking field environmental setting at *icipe*-Thomas Odhiambo Campus (*iTOC*) Mbita were used for fungal isolation, while field samples screened for the isolated fungi were collected from selected parts of Western, Central and Coastal Kenya. Using standard tissue culture techniques and rDNA-ITS sequencing, 25 fungi were isolatedand identified from developmental stages of semi-field mosquitoes. These isolates were found to belong to the genera*Aspergilus*, *Penicillium*, *Periconia*, *Epicoccum*, *Leptosphaerulina*, *Hyphopichia*, *Alternaria*, *Lichtheimia*, *Cladosporium*, *Hasegawazyma*, *Marasphaerium*, and *Dothideomycetes* sps.Eight of these isolates namely *Penicillium*, *Periconia*, *Epicoccum*, *Leptosphaerulina*, *Alternaria*, *Lichtheimia*, *Cladosporium*, and *Hasegawazyma*sp resided in both the midguts and ovaries, and were selected for re-introduction to the immature stages to establish symbiont-vector association. One of these isolates namely *Leptosphaerulinasp*. was found to stably associate with *An. gambiae*. This fungus undergoestransstadial transmission across developmental stagesof *An. gambiae* and was detected in their progeny. Field surveys indicated that it was present in the field-collected larvae and adult *Anopheles* sp. at moderate intensities across sampled geographical regions. The presence of *Leptosphaerulina* sp. in the infected host wasmanifested by deposition of a distinctive

melanin in their fat tissues (melanotic phenotype).It was suggested that melanosis contributed to the establishment and persistence of *Leptosphaerulina* in the host tissuessince symbiont was localized in the midgut and reproductive tissues. This colonization was found to elevate host basal immunity suggesting that systemic infectioncaused mild interference to the host's fitness by slowinglarval development and reduction in fecundity during the first generation of infection. Protein coding genes linked to KEGG pathways suggests that the presence of this fungus is likely to modulate host nutrient metabolism. However, *Plasmodium* challenge experiments with laboratory and field mosquitoes showed that *Leptosphaerulina* exhibits subtle effects on the development of *P.falciparum* sporozoites (Using unpaired t-test,*An. arabiensis*: t(98)=1.365, *p*=0.1755; *An. gambiae*: t(121)=1.955, *p*=0.0529; field collected *An. gambiae*complex: t(36)=0.5885, *p*=0.5599). This suggested the existence of a complex host-fungus-parasite tripartite interaction. Upon establishing that cultivable fungi utilize mixed routes of transmission, it was hypothesized that a non-culturable fungus of *Anopheles* namely*Microsporidia MB* previously identified through high throughput sequencing and found to protect hosts against*Plasmodiumfalciparum*could also exploit mixed modes of transmission.The rationale for developing a sustainable mode of spreading *Microsporidia MB*is based on the the need to increase field prevalence to a level that is sufficient in lowering malaria disease burden. Notably, it was established that the spread of *Microsporidia MB*was only found to occur between adult mosquitoeswith evidence between infected individuals of opposite sexes. Thissuggested that *Microsporidia MB* is transmitted through venereal/sexual means.Importantly, *Microsporidia MB*was observed in the male gonad and found to undergoa series of developmental stages in the ejaculatory duct and is secreted in the male seminal fluid. Observation of sperms in the spermathecae of females acquiring *Microsporidia* through insemination and a lack of transmission in same sex cohorts (through direct contact)signifythat this symbiont can be transmitted horizontally through sexual/venereal route. To conclude, the study identified cultivable fungi that associate

with various developmental stages of *Anopheles gambiae* species. One of these fungi namely *Leptosphaerulina* sp. was found to establish a stable co-existence across developmental stages of these hosts with minimal fitness cost and occurred naturally in the wild caught *Anopheles gambiae* complex. It was also found that *Microsporidia MB*exploitssexual transmission to spread through *Anopheles* populations.It is recommended that a sequencing approach that targets uncultured fungi could reveal mycobiomes that were not identified in this study. It would also be important to study mechanisms utilised by these symbionts to undergo maternal transmission. These fungi could be secreting secondary metabolites which ought to be studied in relation to host fitness and *Plasmodium* blocking. To utilise *Leptosphaerulina* in paratransgensisis it would be important to transform it with effector molecules. A large scale trial of the properties that enhance males' competence in the dissemination of *Microsporidia MB*is important for successful field trial implementation.Taken together, these findings suggest that fungal symbionts establish stable association with *Anopheles* hosts and could be utilized to either deliver anti-*Plasmodium* factors or used directly to block transmission of *Plasmodium* thereby contributing to the current modus operandi ofintegrated malaria control strategies.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Malaria remains a huge burden especially in the Sub-Saharan Africa. The *Anopheles gambiae* complex is the primary malaria vector transmitting *Plasmodium* sp. a parasite causing the disease with devastating consequences, and this transmission occurs when an infected female *Anopheles* mosquito bites naïve humans(Yadav *et al*., 2016). Recent reports indicate stagnation and abandonment in the fight against malaria. For instance, the World Health Organization malaria report indicates that 229 million cases of malaria from 87 countries were reported in 2019 (WHO, 2020). This is against a backdrop of 212 million cases from 106 countries in the previous year. While the number of countries with malaria cases dropped from 106 to 87, the numbers of death report at 409,000 in 2019 signify stalled progress. Possible reasons for the stalled progress includes shunting of resources from malaria control programs, vectors becoming tolerant to existing control strategies and effective in disease transmission. Besides, recent emergence of diseases such as Covid-19 is likely to exacerbate the gains made in managing malaria because the disease is no longer attracting the effort and attention it requires. Many of the reported cases in 2019 (29 countries accounted for 95% of all cases) were in the sub-Sahara Africa as shownin **Figure 1.1**(WHO, 2020). The mainstay approaches for controlling the disease involve using antimalarials and

vector control (Huijben & Paaijmans, 2018). Over the past 15 years the reproductive rate of the main parasite *Plasmodium falciparum* has reduced due to integrated efforts of control strategies targeting both the parasite and the vector as previously shown (Bhatt *et al*., 2015). Reports indicate that vector control remains the primary way of lowering parasite burden(WHO 2009, 2018; 2020; Bhatt *et al*., 2015). Despite the contribution of these frontline control strategies in loweringincidences of the disease, they are being hindered by mosquitofactors such as changing genetic components of the vector, difficulty in accessing vector's habitat, and microbial composition within the vectors' midgut influencing their fitness(Coon *et al*., 2014, 2015, 2017). Coon attributes these factors to vector's competence since they influence mosquitoes' fitness properties such as ability to develop, reproduce, colonize a given environment, acquire, and transmit the pathogen(Coon *et al*., 2014, 2015, 2017). These observations subscribe to previous findings that incriminate resident microbes to influence vector competence by conferring significant changes to the vectors' physiology (Ramirez *et al*. 2014). Correspondingly, future control strategies would need to incorporate methods that exploit mosquito associated factors such as uncharacterized microbial residents and their role in vector density and competence.

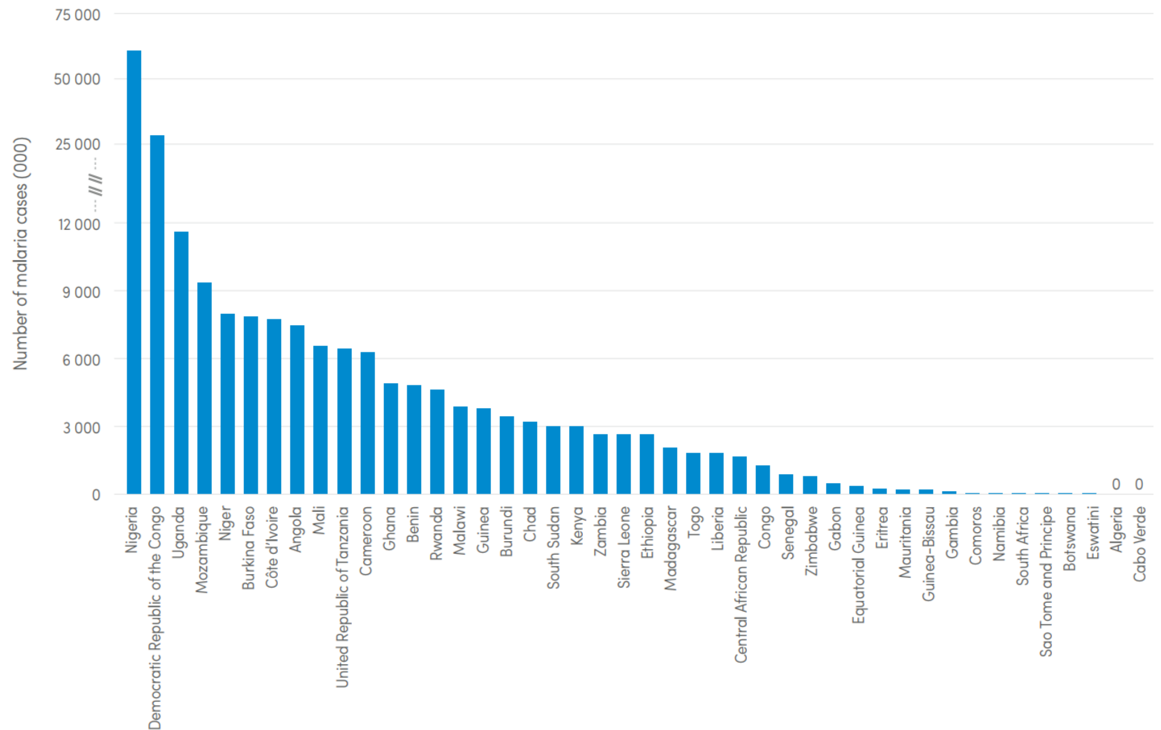


Figure 1.1: Malaria cases in the African region (WHO, 2020)

Mosquitoes colonize environments that provide appropriate living conditions. During the early stages of larval and pupae development, mosquitoes live in aquatic environment, and terrestrial locations at adult stages. Water provides shelter and food for the larvae while adults feed on plant nectars. These habitations put mosquitoes in close proximity to microbes such as bacteria, viruses, spirochetes, and fungi. It is believed that *Anopheles* acquire surveyed microbes from these environments (Dong *et al*., 2009). It is proposed that these environments provide access to microbes required for functions such as nutrition and digestion (reviewed in Dillon & Dillon, 2004; Russell & Moran, 2005),

reproduction, development (Valzania *et al*., 2018; Coon *et al*., 2014), and protection against pathogens (Cirimotich et al., 2011b; Cirimotich, *et al*., 2010; Dong *et al*., 2009). Some of the acquired microbes have co-evolved with these vectors to establish symbiotic relationships (Ott*et al*., 2014). Some of the symbiotic microbes harboured by the insects are heritable, i.e., they are passed on from mother to the offspring by vertical transmission, for instance,*Asaia* sp. and *Serratia* sp. (Favia *et al*., 2007). Other microbes are horizontally transmitted, and this happens when infected host comes into direct contact with the uninfected vector or through contaminated environment (Coon *et al*., 2104; 2016). There are also incidences of mixed transmissions where both vertical and horizontal modes are utilized for the passage of microbes to and from one generation to another and through contact concurrently (Kwan *et al*., 2017; Ruiz-guzma *et al*., 2016). However, to establish persistence and promote fidelity between the associations, microbes ought to undergo vertical transmission (Favia *et al*., 2007). Isolation of novel microbes and studying their modes of transmission could inform their utilization in paratransgenesis or if they posses refractoriness phenotypes against pathogens associated with human diseases they could be utilized directly to block disease transmission.

BacteriaSymbionts of*Anopheles* sp. have received immense attention over the last decade. The focus has been to identify bacterial microbes through culture dependent and culture independent methods, ascertain if they stably associate with the vector and evaluate their functional roles in these vectors(Favia *et al*., 2007). It has been established that some bacteria symbionts in *Anopheles* sp. help the host assimilate nutrition, required

for development, and confer pathogen protection (Cirimotich *et al*., 2011a, b; Cirimotich, Ramirez, *et al*., 2011). For instance, anopheline mosquitoes harbouring *Enterobacter* sp. and *Serratia* sp. have been shown to possess resistant phenotypes against *Plasmodium* parasite, and it was proposed that microbes either elicitthe host immune system or produce antimicrobial peptides with anti-plasmodial activities (Cirimotich *et al*., 2011; Clayton*et al*., 2013). Reduction or elimination of these bacteria by feeding the vector with antibiotics resulted in the development of susceptibility to both *Plasmodium* and dengue infection, suggesting that the presence of symbionts conferred protection against invasion (Dong *et al*., 2009; Xi, Ramirez et al., 2008; Meister *et al*., 2009).The discovery of *Wolbachia* as a non-chemical tool that lessens the burden of Zika and dengue transmission(Iturbe-Ormaetxe *et al*., 2011), informed prospects of identifying this symbionts in *Anopheles* and determine whether it could be exploited for similar strategy to eliminate or reduce malaria transmission. *Wolbachia* is an intracellular bacteria that infects most arthropods with high prevalence on the reproductive tissues(Iturbe-Ormaetxe*et al*.,2011; Mousson *et al*., 2012; Nguyen *et al*., 2015; Ye *et al*., 2015). Localization on the sex tissues enhances its transovarial transmission, and researchers have exploited these traits in arthropods of medical importance that are not naturally infected with *Wolbachia* sp. For instance, the infection of *Aedesaegypti* with this symbionts has demonstrated immense reduction in the transmission of both Zika and Dengue viruses both in Brazil and Australia (Kambris *et al*., 2010;Iturbe-Ormaetxe *et al.*, 2011; Mousson *et al*., 2012; Nguyen *et al*., 2015; Ye *et*

al., 2015). However, infection of *Anopheles* sp. with *Wolbachia* sp. was transient, and this made it difficult to exploit its transmission blocking in the management of malaria (Burt, 2014; Caragata *et al*., 2015). Besides, other bacteria isolates from the *Anopheles* sp. have proved challenging in establishing strategies such as paratransgenesis for the management of malaria (Wilke & Marrelli, 2015). Perhaps an alternative to these challenges is the isolation of a non-bacterial gut components such as fungi and evaluate their role in vectors with respect to fitness cost and pathogen transmission.

Recently, efforts to harness eukaryotic symbionts such as fungi that are linked to anophelines indicate that they could be enhancing the growth of larvae or controlling the male reproductive system (Cappelli *et al*., 2014; Valzania *et al*., 2018). Other studies demonstrate that the *An. gambiae*midgut is inhabited by ascomycete fungus such as *Penicillium chrysogenum* and *Talaromyces*sp. species that confers modulation to parasite infection and renders *Aedes* vectors more susceptible to Dengue infection (Angleró-Rodríguez *et al*., 2016, 2017). It is not clear whether the effects on *Plasmodium* or Dengue were a result of compounded effect on the host. Pathogenic fungi such as *Candida parapsilosis* isolated from the wild mosquitoes signifying the possible role these vectors could be playing in the dissemination of pathogenic fungi (Bozic *et al*., 2017). Besides, families of Culicidae have been shown to co-exist with several members of filamentous fungi (Pereira *et al*., 2009). Whether these isolates impart any biological fitness on the host remains unexplored. Novel mycobiomes could be those that persist along the transtadial phases of development and passed on to their

subsequent progeny for sustainable utilisation. The presence of fungi in mosquitoes is not striking because these vectors dwell in environments that put them in close proximity to a consortium of microbes ranging from unicellular to eukaryotic cells. Besides, fungi have been isolated from other arthropods such as beetles (Hu *et al.*, 2015), carnivorous insects such as *Pantalaflavescens*(Shao *et al*., 2015), and from mosquitoes (Pereira *et al*., 2009; Jaber *et al*., 2016). These examples are indicative of theimportance to bioprospect for novel mycobiomes that could be exploited in the management of malaria parasite thereby adding arsenals to the toolbox of malaria control. Of importance would be the non-entomopathogenic fungal symbionts that are stably associated and transmitted along the mosquito developmental stages, which could be harnessed for downstream experiments to establish their role in blocking pathogen transmission or utilised in paratrangenesis.

This study aimed to: (i) isolate and characterize fungi from the immature stages, midgut and reproductive tissues of semi-field adult *Anopheles* sp; (ii) investigate the effects of selected isolates on the host fitness traits such as development, survival and fecundity as well as whether this symbiont is established in these host upon infection; (iii) determine mechanisms utilised by selected fungus to establish within these host by examinging tissue distribution of the isolates and their role in host immunity; (iv) determine the effects of selected isolates on the transmission of *Plasmodium falciparum* and establish genomic features from whole genome sequences; (v) establishing

alternative self sustained propagation routes of disseminating of a non-cultivable symbiont *Microsporidia MB* in *Anopheles* mosquitoes.

1.2Statement of the problem

According to the previous and recent World Health Organization Malaria Reports(WHO, 2018, 2019, 2020), malaria remains a menace across the globe despite the efforts in the past 15 years that reduced the reproductive rate of the main vector *Anopheles* mosquitoes (Bhatt *et al*., 2015). The report indicates high cases of malaria treatment failure to the main drug artemisinin-based combination therapy (ACT) in Mekong sub-regions of Cambodia, Thailand, and Lao Republic (WHO, 2018). Besides, the report documents incidences of vector resistance to the main insecticides such as pyrethroids (78% cases of confirmed resistance) and organochlorines (58%), carbamates (31%) and organophosphates (19%) in Sub-Saharan Africa (WHO, 2018). These cases signify an increase in the burden of controlling malaria when compared to previous report indicating consorted efforts in lowering the burden of infection and effective treatment of cases using ACTs (WHO, 2016). These developments denote that control of malaria could have stalled and other possible causes could be attributed to changes in the biology of both the vector and parasite, hence the need to identify alternative strategies that could complement what are currently used in malaria control toolbox.

The use of bacteria symbiont called *Wolbachia* to control vectors and/or parasites have been demonstrated in arboviruses such as dengue and zika viruses in the field trials

with immense success rate (Hoffmann *et al*., 2011; Frentiu *et al*., 2014; Nguyen *et al*., 2015).Similar effects could not be established in *Anopheles* mosquitoes. Kambris *et al*. (2010) and Thomas *et al*., (2011)suggestedthat the problem with *Wolbachia* is its inability to naturally infect *Anopheles* sp. Therefore, identification of microbes that co-exist with *Anopheles* and could confer similar phenotypes to *Wolbachia* remains a priority to establish candidates that could equally be useful in malaria management. While studies on bacterial symbionts associating with anopheline mosquitoes are extensive, studies on eukaryotic symbionts such as fungi are limited. Recently, fungal symbionts associating with *Anopheles* mosquitoes have been isolated from dead mosquitoes (Jaber *et al*., 2016), reproductive tissues and midgut of *Anopheles stephensi*(Ricci *et al*., 2011), and field collected larvae (Pereira *et al*., 2009). These studies propose that fungi co-exist with anopheline, but it is not clear whether the isolated fungi were true symbionts of anopheline mosquitoes and whether they could influence host fitness traits in a beneficial way to justify their suitability in developing additional tools of management.Importantly, a more robust RNA shortgun metagenomic sequencing confirmed the presence of 13 fungal families, viruses, and bacteria that co-exist with *Anopheles* mosquitoes (Chandler *et al*., 2015). Whether these candidates establish within the gut or whether they alter the host fitness or interact with malaria parasitesremain unexplored. The establishment of fungal symbionts that co-exist with anopheline mosquitoes are necessary for the identification of strains that could be

exploited directly in malaria transmission blocking or indirectly in a paratransgenesis approach as previously suggested(Chavshin *et al*., 2014; Wilke & Marrelli, 2015).

It is plausible that fungi could be conferring beneficial attributes such as digestion, nutrition, enhancement of development, and protection against pathogens in a similar way that bacterial counterparts do as previously shown (Coon *et al*., 2016, 2014; Douglas, 2011, 2015; Minard *et al*., 2013; Valzania *et al*., 2018). Other studies have demonstrated that feed regimens play an essential component in the acquisition of microbes both by immature and adult stages(Coon *et al*., 2014). It must be mentioned that vectors acquire microbes *per os* from their environment and develop reduction mechanisms for maintaining beneficial microbes, some of which were found to co-evolve with the host vector(Lindh *et al*., 2008; Ott *et al*., 2014). Besides, it is suggested that the quantities of acquired microbes decreases when these vectors develop from larvae to adult so that adults posses reduced taxa and that some of the taxa retained play important roles in the host development (Coon *et al*., 2014). These observations demonstrate that it is possible for *Anopheles* mosquitoes and other vectors to acquire microbes through feeding and only retain the important ones as they develop. Correspondingly, these observations indicate that *Anopheles* harbour fungi, but their roles in these hosts are not clear. Despite the existence of knowledge on the presence of fungi in mosquitoes,whether they establish stable and intimate association, their influence on host fitness traits, and interaction with parasite remains largely unexplored. Therefore, the identification of fungal isolates and elucidating their roles in host fitness,

interaction with the parasite and whether their association with the vector is a stable co-existence remains important.

1.3 Justification of the study

The realization that current malaria management programs are faced with challenges is enough motivation for continuedsearchofemerging tools that could be incorporated into the existing control strategies. Use of native symbionts isearmarked as a potential tool for the development of alternative sustainable and safer methods to either replace or complement the conventional vector/parasite control strategies. Vector control remains the most effective way of tackling malaria burden (Bhatt *et al*., 2015). With the recent increase in mlaria caseload to 229 millions (WHO, 2020), previous reports acknowledge that current vector control methods could have reached their carrying capacity hence the need to scale up the search for alternative/complementary strategies (WHO, 2018). In particular, the increase in the incidences of resistance to insecticides are attributed to the ineffectiveness of the current frontline strategies (WHO, 2018).

Challenges associated with insecticide resistance informed the development of non-chemical approaches using midgut microbes to control both the vector and/or parasite by exploiting their tripartite interactions (Bian *et al*., 2013). When strains of *Wolbachia* symbionts (*w*MelPop and *w*Mel) were discovered and a proof of concept established on their utilization to block dengue and zika viruses in field trials (Hoffmann *et al*., 2011; Frentiu *et al*., 2014; Nguyen *et al*., 2015), it was expected that the same

knowledge could be reciprocatedin the management of malaria. However, trials of the concept on malaria control yielded transient effect because it was believed the bacteria could not infect *Anopheles* sp nor establish a stable association of high infection intensities(Kambris *et al*., 2010; Thomas *et al*., 2011). Notably, these findings informed the need to identify native microbes that readily and naturally associate with *Anopheles* sp. with the anticipation that they could be exploited in the direct management of vector population or parasite through paratransgenic and transmission-blocking approaches. Fungi are poised as an important component of insect midgut since they provide active products needed by the insects (Shao *et al*., 2015). It is expected that the identification of midgut fungi associating with *Anopheles* mosquitoes could be an additional tool to the alternative approaches of parasite control if such isolates possess *Plasmodium*blocking phenotypic traits. Whether fungi play beneficial/adverse role on mosquitoes fitness indicators such as development and survival remains largely unexplored (Chandler *et al*., 2015). Understanding the association between native fungi and the host will inform the selection of candidates that could be exploited for the delivery of anti-plasmodial factors if such isolates form intimate relationship that is not virulent to the vector and does not possess transmission blocking traits. Besides, the isolated fungi would help in establishing candidate isolates based on their physiological and biological roles across generation and transstadial life cycle. It is expected, therefore, that novel fungal candidates that associate with the vectors shall possess minimal fitness cost, persist in these host, and be passed transstadially to their subsequent life cycle stages and

progenyand to inhibit the development of the parasite.It is possible that a symbiont establishing such association could have co-evolved with the host and whole genome sequences could shed more clues on their evolutionary characteristics.

1.4 Objectives

1.4.1 General objective

The primary aim was to characterize fungi associating with *Anopheles gambiae* and establish their tripartite interactions with the parasite.

1.4.2 Specific objectives

1. To isolate fungifrom *Anopheles gambiae*developmental stages obtained from semi-field environment.
2. To investigate the effects of selected isolates on the *Anopheles gambiae*host fitness.
3. To determine the mechanisms utilised by the selected fungi to establish within the *Anopheles gambiae* populations.
4. To determine the effects of selected isolate on *Plasmodium falciparum* transmission and its genomic features
5. To establish a self sustained propagation strategy for field dissemination of the novel fungal symbiont of *Anopheles arabiensis*

1.5Research Questions

Question 1: Which fungi are found in the different developmental stages of *Anopheles gambiae* host?

Question 2: What fitness effects do the isolated fungi impose on the *Anopheles gambiae* hostupon re-introduction?

Question 3: What mechanisms do these fungi utilize to co-exist with the *Anopheles gambiae*host?

Question 4: Does the fungus protect these hosts against infectionby *Plasmodium*parasite?

Question 5: What is the most sustainable strategy that can be employed to spread novel fungus in the *Anopheles arabiensis* populations?

1.6Scope, assumptions and limitation of the study

Fungal symbionts utilized in this study were isolated from *Anopheles gambiae* maintained in the screen houses (semi-field environment) at the *icipe*-Thomas Odhiambo Campus (*iTOC*) Mbita station. This is a laboratory setting that mimics a natural environment. It was assumed that such a set-up enabled the host to select mutualistic taxa of mycobiomes that are essential for their development. Fungi isolation was done using standard tissue culture technique and rDNA-ITS sequencing. It is possible that these hosts could be harboring non-cultivable mycobiomes that were not targeted by the culturing technique. A non-culturing approach such as deep sequencing of 18s gene

using host tissues could have revealed additional taxa not identified in this study. Curing of fungi in the eggs of *Anopheles* to establish fungus-free host colonies for studying candidate fungus did not eliminate bacteria symbionts, which may have influenced establishment and propagation of the candidate fungus isolates under study thereby impacting on the host fitness. While melanosis and boosting of host immunity was linked to the establishment and persistent of *Leptosphaerulina* sp, it is possible that this fungus could be secreting secondary metabolites, which were not profiled in this study. To implicate *Leptosphaerulina* sp for paratransgenic approach, the fungus would need to be transformed and cloned with anti-effector molecules. While it was established that *Microsporidia MB* can spread horizontally through sexual route, it would be useful to try spreading the symbiont in a system that simulate field environment before it can be deployed for field trial.

CHAPTER TWO

LITERATURE REVIEW

2.1 Mosquitoes transmit human disease pathogens

Mosquitoes are categorized under Culicidae family. This family is grouped into sub-families namely: Toxorynchitinae, Culicinae, and Anophelinae(Osei-Poku, 2012). The Culicinae and Anophelinae are the most important groups because they transmit human diseases, however, Toxorynchitinae do not bite and are known to predate on the larvae of other mosquitoes (Zuharah, *et al*., 2015). Although there are close to 3500 species of mosquitoes, comprising of 41 genera, only a few species are known to transmit human diseases (CDC, 2010). Species such as *Anopheles sp* transmit malaria, while *Aedessp* transmit arboviruses (yellow fever, dengue, chikungunya. Rift Valley fever, zika virus) as illustrated by Osei-Poku (2012) as shown(**Table 2.1**). The list comprises of other genera such as *Culex*sp, and *Mansonia*sp, and the diseases they transmit. These genera comprise of complexes that make polytypic subspecies, which exhibits variations in transmission dynamics (Osei-Poku, 2012). For instance, the *Anopheles gambiae sensulato* is a complex comprising of primary (e.g. *An. gambiae s.s*) and secondary (*An. funestus*) malaria vectors. While the primary vector is responsible for the transmission of malaria (McCann *et al*., 2014; WHO, 2018), secondary vector has been shown to emerge as a threat in reverberating transmission dynamics. Several factors are attributed to successful transmission dynamics of these vectors.

Table 2.1: Summary of main mosquitoes and diseases transmitted to humans

Genera	Complex	Species	Disease transmitted
Anopheles	*gambiae* Giles *s.l*	*An. gambiae s.s*	malaria, bancroftian filariasis
		An. arabiensis	malaria, bancroftian filariasis
		An. melas	malaria, bancroftian filariasis
		An. merus	malaria, bancroftian filariasis
		An. bwambae	malaria
	-	*An. stephensi*	malaria
	funestus s.l.	*An. funestus Giles*	malaria, bancroftian filariasis
Aedes	*simpsoni*	*Ae. bromeliae*	yellow fever
	-	*Ae. aegypti*	dengue, yellow fever, chikungunya
	scutellaris	*Ae. albopictus*	dengue, chikungunya
		Ae. polynesiensis	bancroftian filariasis
Culex	*pipiens*	*C. pipiens*	bancroftian filariasis, West Nile and Rift Valley fever
		C. quinquefasciatus	bancroftian filariasis
Mansonia	-	*M. uniformis*	brugian filariasis

(Osei-Puko *et al*., 2012)

Changes in biological phenotypes such as development of multiple mechanisms toward insecticide resistance (Riveron *et al*., 2015), outdoor biting behaviors (Moiroux *et al*., 2012), and a shift towards diurnal activities (Sougoufara *et al*., 2014) signify evolutionary adaptations by the secondary vector that could complicate their management. Besides, conditions such as ambient temperatures and vegetation in the sub-Sahara regions provide suitable breeding sites that increases vector density thereby predisposing human population to life-threatening diseases (Murdock *et al*., 2015; WHO, 2018).

The burden of malaria remains a challenge that requires scientific attention and innovation of alternative control tools. Sub-Saharan Africa still accounts for >90% of the annual malaria reported deaths (212,000 and 409,000) and cases (>200 millions) between 2018 and 2019 worldwide (WHO, 2018; 2020). While there has been remarkable progress in the efforts towards reducing vector densities and competence over the past 15 years (Bhatt *et al*., 2015), recent reports indicate that the progress has stalled (WHO, 2018; 2020). Thiswas attributed to challenges associated with emerging difficulties in controlling vectors. In Kenya, for instance, the burden is not uniform since some endemic regions such as coastal, western, and parts of Baringo in Kenya (accounting for 29% population 2018) have been reporting high rates of transmission (U.S. President's Malaria Initaitve Kenya, 2018), while other parts report declining rates of infection. A nationwide survey showed that malaria accounts for 16% of outpatient visitation to hospitals (U.S. President's Malaria Initaitve Kenya, 2019), and is mostly

associated with the vector *Anopheles gambiae* complex (i.e. *An. gambiae s.s*., *An. arabiensis, An. merus*), and *An. funestus*(U.S. President's Malaria Initaitve Kenya, 2018). These reports underpin the importance of controlling these vectors as a sustainable way to achieve complete elimination (U.S. President's Malaria Initaitve Kenya, 2018; 2019). Ostensibly, control strategies have been the most effective ways of managing malaria over the last two decades as shown by Bhatt *et al*. (2015) (**Figure 2.1**). However, the *Anopheles* have developed resistance to insecticides, changes in their biting behaviours leading to a magnitude of outdoor transmission has necessitated the importance of alternative control methods (Degefa *et al*., 2017). Some of the emerging strategies were informed by the realization that environment of the vector, the diet, and feeding behavior could be exploited in mining for new methods. Basically, malaria is transmitted by an infected female whilst blood feeding on human host thereby injecting infectious sporozoites (Wang & Jacobs-Lorena, 2013). However, male *Anopheles* mosquitoes entirely depend on plant nectar (Coon *et al*., 2016). Although females also feed on nectar early on in life, blood meal is important for the production of eggs. Correspondingly, the larvae feed on small invertebrates, microorganisms, and detritus in their aquatic environments (Coon *et al*., 2016). It is hypothsized that these feeding dynamics cause modulation of microbes in the host digestive tracts where the malaria parasite develops. From this modulation most of the parasites are killed in the midgut and only a few proceed to the subsequent stages largely as a result of the effects of microbes residing in the midgut (Wang & Jacobs-Lorena, 2013). It is possible that

candidate microbes residing in the gut and establishing a stable association with the host could be identified and earmarked for either direct *Plasmodium*blocking or paratransgenesis to complement conventional methods.

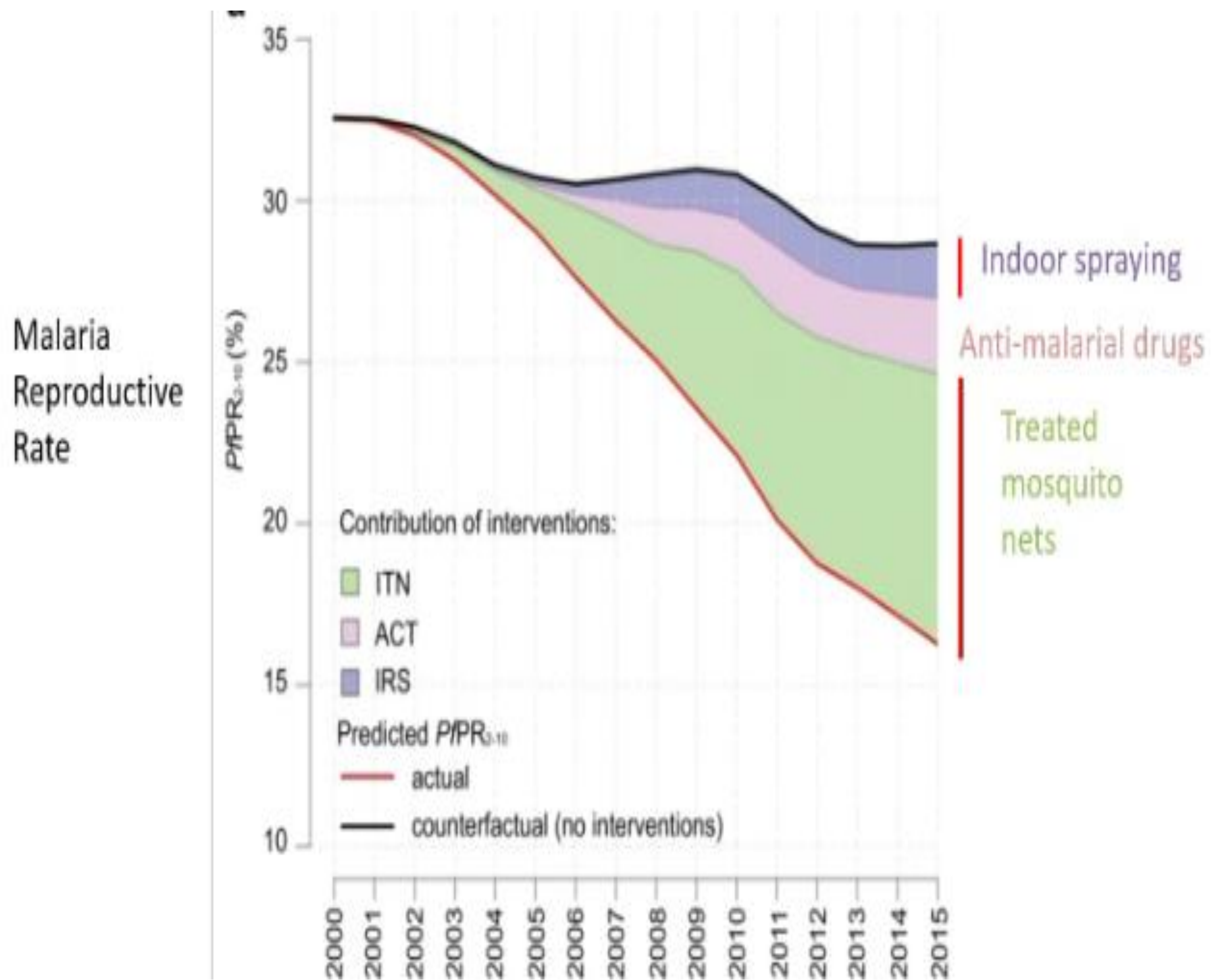


Figure 2.1: Vector control is associated with the decline of malaria cases over the last 15 years (Bhatt *et al*., 2015)

2.2Vector control strategies

2.2.1 Conventional vector control strategies are partially successful

In the absence of vaccines, malaria has largely been contained by targeting the main vector associated with its transmission, mosquitoes. In 1930s, dichloro-diphenyl-trichloroethane (DDT) was immensely used to fumigate the environment in an effort to eradicate vector-borne diseases such as malaria. However, the compound was withdrawn after it was found to exhibit toxicity to non-target species (Kawada *et al*., 2011). Besides, its persistence in nature and effects on human led to its withdrawal from the market despite its efficiency in eradicating vectors. Thus far, the strategies employed include indoor residual spraying, fumigation, removal of breeding habitats, using screens, and sleeping under insecticide treated bed nets (**Figure 2.2**). Increasingly, the use of bednets impregnated with insecticide formulations over the last two decades has lowered incidences of malaria since they repel mosquitoes that would otherwise bite at night and possibly transmit malaria if carrying infectious sporozoite (Mitraka *et al*., 2013). It is estimated that insecticide treated nets averted 68% of malaria cases, thereby contributing immensely towards curbing the disease (Bhatt *et al*., 2015). Besides, indoor residual spraying has made important contribution towards reducing parasite transmission by curbing incidences and prevalence when implemented at scale levels (Bhatt *et al*., 2015). In fact, combination of indoor residual spraying (IRS – 11%) and insecticide-treated bednets (ITNs – 68%) is thought to have lowered the levels of transmission by 79% from 2000 to 2015 (Bhatt *et al*., 2015). While the gold standard

malaria medication, Artemisinin-Combination Therapies (ACTs), played important role in averting severe diseases, reducing transmission and uncomplicated cases has largely relied on vector control (**Figure 2.1**).

Figure 2.2: Partially successful conventional control strategies are faced with immense challenges (Bhatt *et al*., 2015)

2.2.2 Limitations of conventional vector control strategies leveraging on alternative methods

Evidently, the available tools are not sufficient for the control of malaria. There are incidences of mosquitoes becoming resistance to the available insecticides organochlorides, organophosphates, carbamates, and pyrethroids (Mulamba *et al*., 2014; Ranson *et al*., 2011), and the parasite becoming resistant to the conventional anti-malarial drugs (WHO, 2018). Notably, over the last two decades, efforts to reduce transmission levels have largely been attributed to the vector management approach that utilize low cost strategies such as IRS and ITNs (Bhatt *et al*., 2015). The combination of vector control strategies such as sleeping under ITNs and IRS accounts for 79% reduction in reproductive rate of the main malaria parasite *Plasmodium* (**Figure 2.1**). These findings support the position that vector management is the most efficient strategy for the control of malaria cases.

However, these frontline control strategies are being hindered by changes in mosquito biology that enhance their tolerance to the insecticides, and challenges in accessing their habitats (Benelli, 2015; Ranson *et al*., 2011). The development of resistance among the major insecticides namely organochlorides, organophosphates, carbamates, and pyrethroids has been reported in many parts (WHO, 2018). According to Ranson *et al*. (2011) multiple incidences of resistance could be induced, these are not limited to metabolic mode of resistance associated with production of enzymes that

sequester and detoxify insecticides or phenotypic resistance that occurs when a resistant gene is expressed.

The gold standard drug against malaria, Artemisinin-Combination Therapies (ACTs), has saved many lives since the discovery. The ACTs replaced previously used drugs that parasites had developed resistance against, such drugs were quinine and sulfadoxine-pyrimethamine (SP) (Tse *et al*., 2019), which the WHO recommends for treating uncomplicated malaria among pregnant women in their first trimester (WHO, 2018). Despite the potency of ACTs against the parasite, there are emerging reports from Thailand-Colombia regions on the development of resistance against it yet it is the sole drug for the management of the parasite (WHO, 2018; 2020). This is a worrying trend since several strains of *Plasmodium* parasites that evolved to multidrug resistance have proved difficult to manage (WHO, 2018). Maintaining the current level of efficacy is a major concern given that *Plasmodium* and *Anopheles* could evolve to resistant strains owing to partial control (Burt, 2015). Other alternatives such as malaria vaccines remain in the developmental and clinical trial stage. As at the moment, phase III clinical trials of RTS, S/AS02 and RTS, S/AS01 in Kenya and other African countries (Mozambique, Tanzania, Gabon, Ghana, and Gambia) have shown a vaccine efficacy 95% CI ranging from 30% - 53% in Kenya and 34% - 65% in other African nations (Malaria Vaccines (JTEG) & WHO Secretariat, 2015). A follow up study indicated that using RTS, S/AS01 could be initiated at the sixth week among neonates to reduce adverse immune response (Witte *et al*., 2018). Others also noted a mismatch among different *P. falciparum* isolates

in Africa (Pringle *et al*., 2018). While these efforts are commendable, there is need to venture into new areas for alternative strategies that could complement or replace existing vector control strategies to ease the burden on treatment of patients.

2.3 Emerging vector control strategies to counter failures in conventional methodsraise concerns

Studying alternative ways of hindering mosquito'sability to support or transmit the parasite will play a major role in eliminating or reducing disease burden.The use of non-chemical methods is emerging as an alternative strategy that could complement or replace partially successful conventional methods, which are marred with challenges discussed previously (**Figure 2.2**). Exploiting the biology of the vector has been earmarked as the most potent way of managing both the vector and the parasite population. Sequencing of mosquitoes depict that they closely associate with microbes (Chandler *et al*., 2015). Microbes such as bacteria, fungi, yeast, protists, and viruses have been observed in deep sequencing of mosquitoes at different developmental stages, and it has been hypothesized that environment, diet, and feeding behaviours influence the composition and dynamics of these microbes(Chandler *et al*., 2015). While some proposed approaches rely on genetic modification of mosquito genes (Beaghton *et al*., 2019; Hammond *et al*., 2016; Nolan *et al*., 2011), some countries prohibit release of genetically modified organisms to the environment for fear of unknown safety to the non-target agricultural insects, lack of clear socio-economic and health consequences.

Another approach focusing on transforming microbes that stably associate with the vector so that these symbionts could act as vehicles for delivering antiplasmodial properties in what is termed as paratransgenesis(Hurwitz *et al*., 2011; Mancini *et al*., 2016). Others have proposed non-genetic approaches that harness the role of native gut microbeswith the aim of controlling either their reproduction or parasite development (Berasategui *et al*., 2016; Crotti *et al*., 2012; Moreira *et al*., 2009; Murphy *et al*., 2016). These proposed emerging vector control strategies have merits and setbacks that hinder their utilization hitherto.

2.3.1 Genetic manipulation of mosquitoes raises regulatory concerns

The search for new innovative tools that could impose mosquito's refractoriness to *Plasmodium* infection informed the emergence of genetic methods. Mosquito transgenesis involves engineering of the vectors so that when it encounters a parasite, it elicits the production of anti-*Plasmodium* molecules in the midgut (Ghosh, Ribolla, & Jacobs-Lorena, 2001; Ito *et al*., 2002). The pioneering proof-of-concept was investigated using *An.stephensi* whose midgut was genetically engineered to express salivary gland and midgut peptide 1 when invaded by the malaria parasite (Ghosh *et al*., 2001). These peptides could bind to receptors on the ookinete surface preventing their invasion of the midgut epithelium (Ghosh *et al*., 2001; Wang & Jacobs-Lorena, 2013). Transgenesis exploits the developmental stages of the parasite in the midgut, where fertilization of the macro and micro gametes takes place to form zygote that differentiates to ookinete and later to oocyte to cross the two epithelia (midgut and salivary gland) before forming

multiple sporozoites (Wang & Jacobs-Lorena, 2013). The infective stages, sporozoites, are released into hemocoel and invade the salivary gland in readiness for passage to the next human host (Wang & Jacobs-Lorena, 2013). Other approaches of modification have exploited parasite killing peptides such as cecropins, gambicin, and defensins that originate from the host innate immune system (**Table 2.2)**, and whose role is to lyse the parasite, but has no effect on the host (Kim *et al*., 2004; Kokoza *et al*., 2010). Modification of mosquitoes renders them less efficient in transmitting the parasite (Ito *et al*., 2002). Collectively, these studies provided proof-of-concept that engineering mosquitoes was the most potent way of controlling parasite transmission. In May 2021, Oxitec biotech firm was first to release genetically engineered mosquiotoes in Florida the United States after decades of seeking regulatory approval (Waltz, 2021). The fact that Oxitec managed to permeate through regulatory requirement in the USA is a big deal for the future utilisation of this technology in other countries. It is from this realization that mosquitoes engineered to express midgut effector genes that inhibit the development of the parasite could be a game changer if such studies would be able to navigate regulatory huddles.

Table 2.2: Summarized anti-*Plasmodium* molecules adapted from Wang & Jacobs-Lorena

Effector	Properties	Target parasite	Parasite stages	Function or mechanism
Parasite killing				
Scorpine	Scorpion *Pandinus imperator* venom peptide	*P. falciparum* *P. berghei*	Gametocyte to Ookinete	Cecropin and defensin-like lytic peptide.
Shiva1	Cecropin-like synthetic peptide	*P. berghei* *P. falciparum*	Gametocyte to Ookinete	Lyses parasites
Shiva-3	Cecropin-like synthetic peptide	*P. falciparum* *P. berghei*	Gametocyte to ookinete	Lyses parasites
Cec A	*A. gambiae* cecropin A	*P. berghei*	Ookinete	Lyses parasites
Cec B	*A. gambiae* cecropin B	*P. falciparum*	Oocyst	Lyses parasites
DEF1 A	*A. gambiae* defensin A	*P. falciparum* *P. berghei*	Ookinete	Lyses parasites
Gambicin	*A. gambiae* antimicrobial peptide	*P. falciparum* *P. berghei*	Ookinete	Lyses parasites
Angiotensin II		*P. gallinaceum*	Sporozoite	Lyses parasites
Magainins	Peptides from the African clawed frog *Xenopus laevis* skin	*P. falciparum* *Plasmodium knowlesi* *Plasmodium cynomolgi*	All mosquito stages	Lyses parasites
Gomesin	A antimicrobial peptide from Spider	*P. falciparum* *P. berghei*	All mosquito stages	Lyses parasites
CEL-III	Hemolytic C-Type Lectin	*P. falciparum* *P. berghei*	Ookinete, oocyst	Lyses parasites
TP10	Wasp venom peptide	*P. falciparum*	Gametocyte to ookinete	Lyses the parasites
AdDLP	*Anaeromyxobacter dehalogenans* defensin-like peptide	*P. berghei*	Ookinete	Lyses the parasites
Meucin-25	Scorpion *Mesobuthus eupeus* venom gland	*P. berghei* *P. falciparum*	Gametocyte	Anti-microbial linear cationic peptide
Drosomycin	An inducible antifungal peptide initially isolated from the *Drosophila melanogaster* hemolymph	*P. berghei*	Ookinete	Lyses the parasites

(Wang & Jacobs-Lorena 2013)

Of importance is that genetic methods are faced with immense challenges that have stalled their utilization in the field. For instance, it is difficult to devise means of achieving effective field/wild drive of anti-malarial effector molecules cloned in insectary mosquitoes especially if the symbiont is not readily co-existing with these hosts (Wang & Jacobs-Lorena, 2013). Wang and Jacobs-Lorena (2013) assert that mass

release of engineered mosquitoes is not a cost effective and sustainable way of managing transmission dynamics. The primary reason supporting inefficiency of transgenesis is that the genetically modified mosquitoes lack competitive advantage over the wild population, which lowers their competitiveness against the wild species leading to their elimination from the population (Moreira *et al*., 2004; Wang & Jacobs-Lorena, 2013). Besides, the regulatory requirement from most countries with high burden of malaria makes it difficult to test these techniques in those localities. Of importance, therefore, would be a simpler method that exploits mosquitoes' inherent/native molecules that could be utilized to either immunize the vector against parasite or prevent them from transmitting parasites.

An emerging technology referred to as homing endonuclease (HEG) is a synthetic approach that falls under gene drive system, which naturally occurs in fungi and other microbes (WHO, 2009). It is a system in which a gene exploits the mendelian inheritance and self propagation in a population(WHO, 2009). Naturally occurring homing could be utilized in gene drive system targeting functional attributes in mosquitoes such as biasing of subsequent generation to produce male offspring that would not bite humans nor carry disease. The system involves gene bias of own transmission in a selfish and sustainable manner to the subsequent generation (Burt 2003; Windbichler *et al*., 2011). Specifically, the endonuclease is encoded to cleave the chromosomes where they are absent so that they could be copied during the repair process resulting in heterozygous offspring (Windbichler *et al*. 2011). Unlike

Mendelian law, which involves inheritance of 50% of a trait to the progeny, HEG ensures bias resulting in 90% of the offspring acquiring the trait (WHO, 2009; Windbichler *et al*. 2011). The technique has been tried in mosquitoes to knock-out genes associated with female fertility, knocking out genes associated with parasite transmission or knocking in genes that could inhibit the transmission of parasite (WHO, 2009). For instance, Windbichler *et al*. (2011) successfully devised a homing endonuclease technique by putting *SceI* derived from yeast cell into *Anopheles* embryos, and found that the gene could spread rapidly in the *Anopheles* population. Similar system led to sex-ratio distortion through cleavage of X chromosomes thereby resulting in over-transmission of Y chromosomes, producing more sons (male mosquitoes) so that human biting and transmission of malaria is reduced or eliminated (Windbichler *et al*. 2011).

Homing has been associated with rapid spread of a trait in the mosquito population (Hammond *et al*., 2016). The HEG nuclease introduced in the germline of male mosquito and found to undergo high rates of inheritance, which was described as super-Mendelian inheritance owing to its rapid invasion >90% of the offspring inheriting the trait in subsequent generation (Hammond *et al*., 2016). This approach gained attention as a promising tool for population replacement because it outweighed the fitness cost that could limit its spread across the population (Hammond *et al*., 2016). The difficulties to address concerns on whether the homing gene could spill over to untargeted insects and whether it was possible to recall the technology if resulting

effects became harmful became the main undoing of this technology. Collectively, transgenetic methods are limited because it is difficult to retarget HEG in wild-type genomes that could cleave specific sequences, besides, there are fears that the effects could spill over to non-target species. Discussions to formulate regulatory frameworks that are specific for these techniques are still under development and may take a long time before they receive acceptance for the field trial.

CRISPR is a new gene drive control system that involves mutagenic chain reaction (Jinek *et al*., 2012), and this was recently shown to immensely contribute to insect control (Esvelt*et al*., 2014). It was demonstrated that a trait could be inherited with 97% transmission rate in *D.melanogaster*(Esvelt *et al*., 2014). The approach could be utilized in the control of *Anopheles* mosquitoes by targeting either genes that makes the female susceptible to malaria infection, genes needed to develop malaria parasite in the midgut, or targeting female fertility genes (Hammond *et al*., 2016). Despite its prospects in controlling mosquitoes, this technology raises safety and regulatory questionsakin to those observed in HEG that scientists in its fore-front are grappling with to justify its implementation.

2.3.2 Sterile insect techniques (SIT) is labor intensive and unsustainable

The SIT technology involves combining classical genetics and gamma radiation to sterilize male insects. Sterilized males are later released into the environment with the hope they outcompete the wild counterparts for the wild females (Benedict & Robinson, 2003; Nolan *et al*., 2011). The offspring emerging from this insemination have been

demonstrated to be sterile by several studies (Alphey *et al*., 2010; Krafsur, 1998; Nolan *et al*., 2011; Vreysen *et al*., 2000). The SIT technique aims to replace the wild population with a first generation of insects that are non-viable and incapable of transmitting malaria parasites(Nolan *et al*., 2011). The technology was proven a success in the control of vectors such as *Cochliomyiahominivorax*, a screwworm fly in South and Central America regions of Mexico and Libya(Reviewed in Benedict & Robinson, 2003). Mosquito studies on SIT technology have not yielded sufficient data to validate the concept of population elimination (reviewed in Benedict & Robinson, 2003). Importantly, the pioneering SIT success was based on the reduction of *Culex quinquefasciatus* in Myanmar, which utilized cytoplasmic incompatibility (Laven, 1967). This was followed by the release of sterilised males of *Anopheles albimanus* populations in El Salvado(Reviewed in Benedict & Robinson, 2003), and *Glossinaausteni* eradication from Unguja Island (Vreysen *et al*., 2000). Recent sequencing of *Anopheles* mosquito genome introduced insights that could help inform the generation of transgenic strains of *Anopheles* that are amenable or suitable for utilization in SIT technology (Nolan *et al*., 2011). Such strains would possess constructs that facilitate rapid induction of sterility thereby obviating the need for radiation. Sterilization of the male is a laborious process that requires immense capital to generate enough quantities of males that match with the numbers of wild females. Besides, to achieve good results, the technique requires that sterile males induct sterility and outcompete the wild males for mating, all these were challenges associated with its

implementation (Berasategui, *et al*., 2016; Crotti *et al*., 2012; Nolan *et al*., 2011). These challenges led to exploitation of alternative strategies that utilize native insect symbionts associating with these vectors (Berasategui *et al*., 2016; Crotti *et al*., 2012).

2.3.3 Harnessing mycobiomes as an alternative for controlling mosquitoes-borne-pathogens

There has been a growing proof of concept that insect specific microbes could be harnessed for combating pathogens they transmit. While most of such studies majored on understanding the role of bacteria communities inhabiting digestive tracts of insects such as *Aedes*(Gusmão *et al*., 2007, 2010), phlebotomines(Akhoundi *et al*., 2012), and Anopheline (Gimonneau *et al*., 2014; Tchioffo *et al*., 2016; Valzania *et al*., 2018) and the pathogens they transmit, research on non-bacteria members such as fungal mutualists hasbeen limited (Chandler *et al*., 2015; Jupatanakul *et al.*, 2014). It is hypothesized that fungi and yeast-like symbionts in insects could offer an attractive way of controlling pathogens such as *Plasmodiumfalciparum*owing to their potential to express multiple effector molecules with antiparasitic properties (Angleró-rodríguez *et al*., 2016; Valzano *et al*., 2016). Increasingly, fungal studies on insects focused on entomopathogenic fungi (e.g. *Metarhiziumanisopliae* and *Beauveria bassiana*) and their role in killing immature and late stages of development (Bukhari*et al.,*2011; Darbro*et al.,*2011; Fang *et al*., 2011), but little effort has been channelled towards isolating cultureable fungal mutualists in *Anopheles* and other vectors of medical importance. Interestingly, fungal mutualists were found in insects such as bark beetle and demonstrated to confer

nutritional functions (Gonzalez, 2014; Hu *et al*., 2015). Recently, midgut of *Anopheles* and carcasses were demonstrated to inhabit mycobiomes such as *Talaromycessp*(Anglerό-Rodríguez *et al*., 2017), *Penicillium sp*(da Costa & de Oliveira, 1998), *Wickerhamomyces*, and *Candidasps*(Ricci *et al*., 2011; Ricci *et al*., 2011), and other filamentous fungi (Pereira *et al*., 2009), which were hypothesized as novel fungal symbionts. Others have also used culture dependent methods to isolate non-pathogenic fungi from malaria vector (Gusmão *et al*., 2010), and culture-independent methods that identified diverse microbes ranging from protists, viruses, and fungi (Chandler *et al*., 2015). In summary, these studies do not document the effect of fungi on the host fitness. Besides, it is not apparent if these isolates established a stable association with the host malaria vector. Ideally, the two criteria of an ideal fungal symbiont is the ability to possess minimal effects on the host and establish an intimate association with the vector(Wang & Jacobs-Lorena, 2013). These properties have been demonstrated in ideal bacterial symbionts as important for studying *Plasmodium*blocking and paratransgenesis(Wang & Jacobs-Lorena, 2013). Few taxa of these symbionts form signature microbes since most studies acknowledge extensive diversity of isolated taxa from different regions owing to differences in habitation, nutritional, and different phases of host development (Tchioffo *et al*., 2016; Wang *et al*., 2011).

2.3.4 Fungal symbionts are suited to paratransgenesis

Interfering with parasite development and transmission using paratransgenesis is earmarked among novel strategies that could be optimized to control parasitic diseases

such as malaria. Unlike gene drive methods that engineers the vector, paratransgenesis involves genetic manipulation of microbes isolated from the mosquito midgut or those that could easily be re-introduced in the insect host midgut after genetic manipulation to express effector genes that targets to inhibit parasite development (Wang & Jacobs-Lorena, 2013). This was informed by the realization that microbes and *Plasmodium*predominantly share the midgut of the host during their early vulnerable phases of development. It was hypothesized that novel microbes that establish in the mosquito midgutscould be modified to carry and deliver effector molecules that target the parasite thereby providing an alternative control strategy(Hurwitz *et al*., 2011). Paratransgenesis relies on a number of features that makes it an attractive choice. These include: (i) ease of exposing developing parasite to effector molecules through a shared midgut environment with engineered microbes; (ii) dramatic increase in the population of the midgut microbes by 1000 folds or more upon ingestion of a blood meal; (iii) the midgut lumen provides a severe bottleneck for the development of parasite and exploiting symbionts could provide a prime intervention; (iv) the midgut houses a consortium of microbes making a conducive environment for their multiplication (Nolan *et al*., 2011; Whitten, Shiao, & Levashina, 2006).

Paratransgenesis has been demonstrated in the management of parasitic diseases, for instance, *Trypanosoma cruzi*, a parasitic agent that cause Chagas disease, which is a protozoan parasite transmitted by *Rhodniusprolixus*, a triatomid bug (Durvasula *et al*., 1997). Durvasula and colleagues demonstrated that genetic engineering of

Rhodococcusrhodnii to express *cecropin A*antimicrobial peptides that exerts antiparasitic properties in the bug led to a reduction in *T. cruzi* survival. Early reports on the fight against malaria have shown that engineering bacterium *Escherichia coli* to express either a single-chain immune-toxin peptide (Yoshida *et al*., 1999), modified phospholipase or a dimer of single chain toxin (Riehle*et al*., 2007), could significantly reduce the number of oocysts formed by *P. berghei* in *An. stephensi*. These applications were limited in two ways. Using an attenuated strain of *E. coli* originally maintained in the laboratory could have led to it surviving poorly in the midgut of mosquitoes. Limited diffusion of effector molecules within the midgut occurring from its attachment to the bacterial surface or formation of insoluble inclusion complexes contributed to shortcoming of paratransgenesis(Riehle *et al*., 2007; Yoshida*et al*., 2001). These observations informed the need to use microbe isolates obtained from field mosquitoes. A bacterium *P. agglomerans* isolated from field and laboratory mosquitoes was engineered to secrete molecules that exert anti-*Plasmodium*effects (Riehle *et al*., 2007; Wang *et al*., 2012). This isolate did not confer loss to host fitness and led to a strong inhibition of *Plasmodium*development measured by the number of oocyst per mosquitoes, besides few mosquitoes developed oocyst suggesting the prospect for reduced transmission (Wang *et al*., 2012). These findings point to the suggestions that paratransgenesis could as well work in the field since human and rodent parasite share immense similarities. However, for effective control, a microbe that is a true symbiont associating with all stages of the vector and their offsprings could provide a major breakthrough for

paratransgenesis approach that is self-sustainable. Besides, using a symbiont that express multiple effector molecules would provide an additive approach for enhanced killing mechanism. Bacteria symbionts are single cell organisms that can carry a limited number of effector molecules hence the need to bioprospect for eukaryotic symbionts, which could deliver multiple antimicrobial peptides and provide an additive effect towards *Plasmodium*killing or transmission-blocking. It is important to note that mining for non-bacterial symbionts is informed by the immense literature on bacterial symbionts, and to a lesser extend eukaryotic symbionts. Therefore, studies on bacteria symbionts lay the basis for studying other symbionts.

2.4 Mosquitoes acquire symbionts during development

Mosquitoes, just like other insects, acquire microorganisms such as bacteria, yeast, fungi, viruses and protists (termed as microbiota) from their immediate environment. Intensities, composition, and diversity of these microbes changes across developmental phases through the influence of niches colonized and ecological factors (Coon *et al*., 2016, 2014). Usually, mosquito vectors undergo four life cycle stages beginning with eggs in the aquatic environment, which hatches to larvae (Yordanova *et al*., 2018). The larval stage remains in the aquatic condition for a at least a week before pupation that eventually emerge into an adult within 36 hours(Short *et al*., 2018). During their development in the aquatic environs, larvae obtain nutrients from the surrounding media and vegetation, and these have been shown to harbor communities of microorganisms (Coon *et al*., 2016; Valzania *et al*., 2018). It has also been demonstrated

that some of the microbes ingested establish in the larval gut thereby constituting midgut symbiotic communities (Yordanova *et al*., 2018). The presence of microbiota has been determined using both culture dependent (Gusmão *et al*., 2010; Pumpuni *et al*., 1996; Ricci *et al*., 2011; Valiente Moro *et al*., 2013) and culture-independent methods such as shortgun and 16S rRNA deep sequencing (Chandler *et al*., 2015; Fauver *et al*., 2016; Mancini *et al*., 2018; Osei-Poku *et al*., 2012). However, as pupation takes place, the expulsion of peritrophic matrix and food bolus is accompanied byresident microbes, and this results in net loss of microbial community in what is termed as microbial reduction (Lindh et al., 2008; Yordanova et al., 2018).This could explain a shift in diversity from immature stages to later phases of development. Interestingly, some of the microbes acquired during larval stages persist to pupal and adult stages (Coon *et al*., 2016). It has been demonstrated that the transition from pupae to adult is enhanced by remodeling and stringent process of sterilization that leads to loss of resident microbe when adult emerge (Lindh, Borg-karlson, and Faye, 2008). It is believed that re-establishment of microbes by the adults of insects such as *Spodopteralittoralis*(Chen *et al*., 2016), Beetle *Nicrophorusvespilloides*(Yin Wang & Rozen, 2017), and mosquito is based on the fingerprint microbial communities that larvae were exposed to in their breeding habitats (Boissière *et al*., 2012; Coon *et al*., 2014; Gimonneau *et al*., 2014). Important to note is the observation that most microbes in the adult stages are acquired while they feed on plant sugarsin the terrestrial environment (Coon *et al*., 2016, 2014). From these studies, it is apparent that while most microbes are lost during metamorphosis, generally,

microbial composition and diversity in the larval stage resembles those of their adults and we hypothesize that novel fungal microbes could also be conserved and/or undergo similar processes evidenced previously in bacteria.

2.4.1 Fingerprint symbionts residing in *Anopheles* midgut are ideal targets

A candidate microbiome in controlling pathogen ought to meet a critical criterion (i.e. ability to colonize vector's compartments that provide a first stop for early stages of pathogen development). Apparently, in mosquitoes, the most vulnerable developmental stages of *Plasmodium* happens in the midgut (Wang *et al*., 2012; WHO, 2009). These stages include maturation of gametes, fusion of these gametes leading to fertilization, formation of zygotes, and their subsequent stages such as ookinetes and oocyst (Wang & Jacobs-Lorena, 2013). It is important to note that few parasites survive in the midgut because it was hypothesized that midgut residents' imposes a severe bottleneck that impedes parasite development(Abraham & Jacobs-Lorena, 2004; Drexler*et al.,* 2008). Increasingly, there are evidence that the midgut is inhabited by multi-taxon microbes making the tripartite interaction between host-parasite-microbe a complex phenomenon (Hegde *et al.,*2015; Ramirez *et al*., 2012; Romoli & Gendrin, 2018), and this makes it an interesting compartment for isolating prime candidates for interrogation. For instance, midgut microbiomes such as *Enterobacter*(Cirimotich *et al*., 2011)and*Chromobacterium*(Ramirez *et al*., 2014)were found to block early stages of malaria development, while mosquitoes deprivedof residentmicrobes were found to have increased susceptibility to malaria infection (Dong *et al*., 2009; Kalappa *et al*., 2018).

Others demonstrate their usefulness in the growth/development of the vector (Coon *et al*., 2016, 2014). It is important to note that microbial symbionts also reside in various organs such as the gut, ovaries, malpighian tubules, and hemocoel (Eleftherianos*et al.*, 2013), or vary across developmental stages(Rani*et al*., 2009). However, the midgut is considered of particular interest because it represents the first site of extensive exposure to pathogens (Wang & Jacobs-Lorena, 2013). Gut microbes are also considered suitable for the paratransgenesis owing to the ease of re-introducing cultivable isolates through oral ingestion or through horizontal transfer to the host (Engel & Moran, 2013). There is increasing evidence that gut microbes are considered beneficial to host mosquitoes because they help manipulate host immune responses, enhance tolerance to perturbations, and providenutritional supplements (Dong *et al*., 2006, 2009; Ramirez *et al*., 2014; Weiss & Aksoy, 2011). Others observed that midgut microbes are acquired throughinheritance and/or passed to subsequent progeny (Rani*et al*., 2009). While the midgut offer residence to varied microbes, whose interaction could be complex, it offers an opportunity for isolating candidates that could be useful for direct or indirect interference with the parasite. It was hypothesized that fungal residents of mosquito midguts could offer alternative isolates to the extensively studied bacterial symbionts in harnessing their usefulness in either parasite or vector control.

2.4.2 Cultureable mosquito symbionts are useful for studying interaction with host vector

Isolation of novel symbionts is an essential underpinning for studying the interaction with the host and discerning ways of exploiting the association for the control of parasite or the vector. While deep sequencing and other high throughput methods provide a GPS surveillance of the mosquito alimentary system and the dynamics of microbes across life history (Osei-Poku *et al*., 2012; Wang *et al*., 2011), culture-dependent isolation of interesting microbiome could provide avenues to understand and manipulate interactions with the host. It has been shown that cultivable yeast from mosquitoes could be easily re-introduced to their host since they easily re-colonize and re-establish in the host (Cappelli *et al*., 2014). While most cultivable symbionts reside in the midgut, it would be important to target themfor developing paratransgenic tools asmost are amenable to genetic manipulation (Engel & Moran, 2013). Culture methods provide an unprecedented resolution for utilizing paratransgenesis, an approachthat utilizes engineered symbionts to express anti-plasmodialeffector molecules in the midgut (Hurwitz *et al*., 2011). While most studies demonstratedthat phylotypes of symbionts isolated by culture-independent methods are more diverse and divergent as opposed to those isolated by culturing that identified few isolates (Chandler *et al*., 2015; Gusmão *et al*., 2010; Rani *et al*., 2009), culture option provides novel candidates that are easy to study and manipulate. Studies that combine both culture-dependent and culture-independent methods are driven by the desire to identify more genera from each sample

since a single approach limits the number of symbionts per sample(Gibson & Hunter, 2009; Rani *et al*., 2009; Moro *et al*., 2013).

2.4.3 Semi field rearing conditions provide less diverse symbionts compared to lab or field sources

Semi-natural conditions are the primary point for undertaking field trials on developed symbiont-based interventions. These are screen-houses designed to mimic natural conditions, but to some extent take into accountlaboratory conditions of rearing *An. gambiae* complex. From the observation that both field and laboratory reared colonies harbor few similar symbionts (Rani *et al*., 2009; Straif *et al*., 1998;Wang *et al*., 2011), it suggests that laboratory colonies retain symbionts that establish in their midguts. However, it has been demonstrated that few microbes are true symbionts that could be isolated either in the field, laboratory, or semi-natural environments (Gendrin & Christophides, 2013). It is from this realization that mimicking a field condition in the screen houses could provide a stable environment for established symbionts that are likely to persist in both environmental settings. To the best of my knowledge, there is hardly any study that has exploited mosquitoes from semi-field conditions for the isolation of non-bacteria eukaryotic symbionts.

Bacterial composition of mosquitoes sampled from natural habitats is highly variable, but often contain a number of core microbiome dominated by a small number of taxas that vary depending on the insect species, geographical origin, ecological niche, and source of food, as well as sex, as previously shown (Coon *et al*., 2016; Valzania *et*

al., 2018; Osei-Poku *et al.*, 2012;Boissiere *et al*., 2012). While there is agreement that diet and location influence gut communities associated with mosquitoes (Coon *et al*., 2016; Valzania *et al*., 2018), few taxa are shared among hosts and this signifies commonality in the few symbionts that could be playing important functional roles (reviewed in Engel & Moran, 2013). For instance, members of the *Proteobacteria* class are often the most abundant in adult *An. gambiae*mosquitoes, with species of the *Enterobacter*, *Serratia,* and *Asaia*families commonly present (Osei-Poku *et al.*, 2012; Boissiere *et al* 2012). Comprehensive surveillance of microbes across life history also signify varied symbiont structure that change with developmental phase with a few taxa conserved across the developmental stages(Wang *et al*., 2011). Many bacterial species mostly enterobacteria and grams-negative proteobacteria have been identified from field collected *Anopheles* midguts (Straif *et al*., 1998; Ying Wang *et al*., 2011). It has also been demonstrated that bacteria isolated from lab-reared mosquitoes havereduced but largely similar structural composition to those from the wild mosquitoes, suggesting that *Anopheles* mosquitoes are likely to select a few population of mutualistic symbionts that could be useful for their development(Straif *et al*., 1998; Wang *et al*., 2011; Rani *et al*., 2009). In Kenya and Mali, for instance, *Pontoeaagglomerans*, which is a non-pathogenic isolate was reported to be a dominant bacterium symbiont associating with mosquitoes (Straif *et al*., 1998), and was also isolated from mosquitoes maintained in the laboratory insectaries of *An. albimanus*, *An. gambiae*, and *An. stephensi*(Pumpuni *et al*., 1996). While these observations show that both field and lab reared mosquitoes inhabit

microbial taxa that may change depending on their *in situ* ecological environments, they serve to foretell a scenario that stable symbionts are obtained by chance. While these studies have been majored on bacterial symbiont, of interest would be establishing eukaryotic symbionts that stably associate with mosquitoes. Therefore, exploiting an environmental set-up that simulates the two ecological situations such as those reared in a semi-field condition could be ideal for isolating novel candidates.

2.5 Exploiting novel fungal symbionts: building on features of known *Anopheles* symbionts

Although microorganisms are ubiquitously present in almost all insect systems, studies on their interaction with insect hosts have largely focused on how bacteria influence vector biology and their tripartite association with invading parasites(Ramirez *et al*., 2012; Romoli & Gendrin, 2018). Notwithstanding, the realization that both bacteria and other systems such as eukaryotic fungi colonizes insect system (Chandler *et al*., 2015; Osei-Poku *et al*., 2012), isolation and utilization of fungal symbionts native to *Anopheles* is receiving attention owing to the realization that fungal systems provide large biomass amongst various insect systems (reviewed in Vega & Dowd, 2005). It is also possible that fungi could be playing a role in mosquito development, ecology, parasite transmission, and evolution, but these are gaps that require validation.

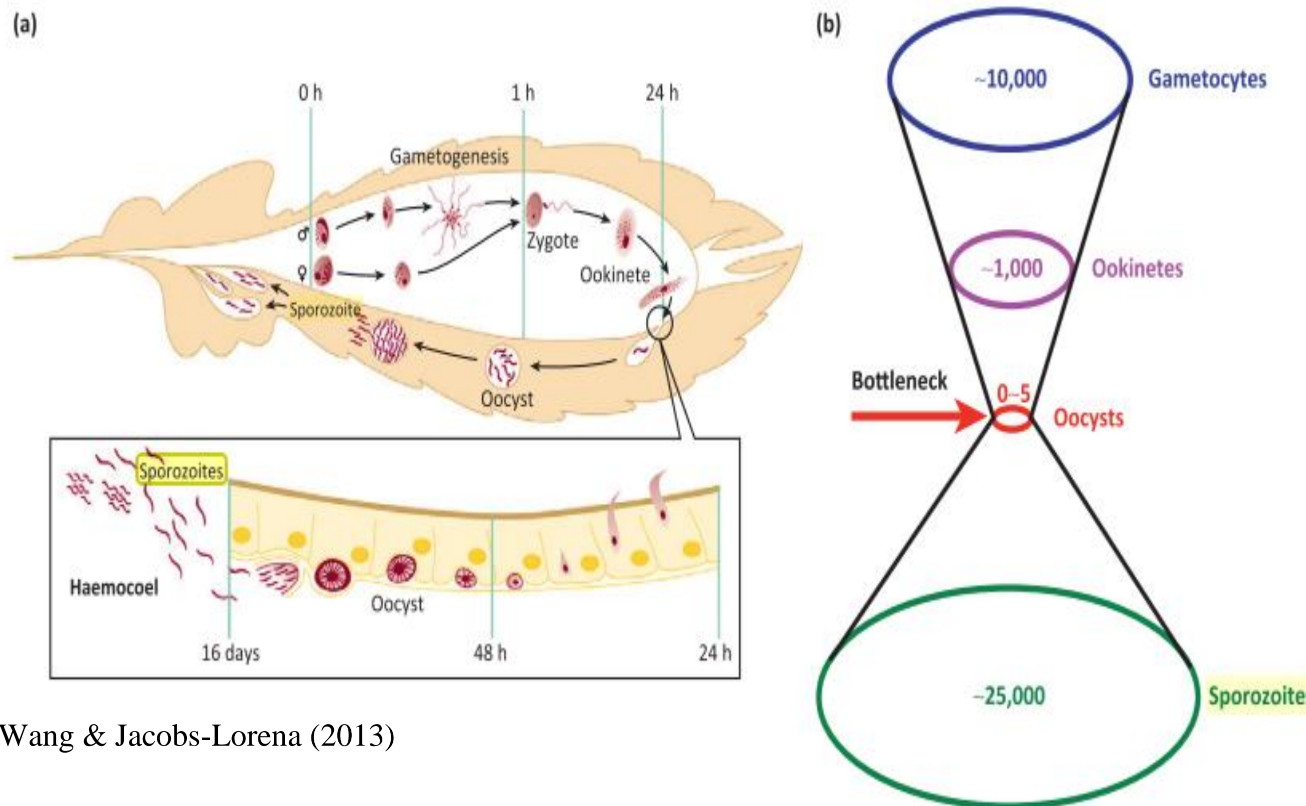
Some of the pioneering work in *Anopheles* microbiome sought to investigate the presence of fungal isolates in these hosts and their roles in blocking development and transmission of *Plasmodium*parasite. For instance, it has been demonstrated that

filamentous fungi such as *Fusarium*sp, *Aspergillus*sp and *Penicillium*sp(Pereira *et al*., 2009), *Wickerhamomyces*, and *Candidasp*(Ricci *et al*., 2011; Ricci *et al*., 2011), *Penicillium sp*(da Costa & de Oliveira, 1998), and *Talaromycessp*(Angleró-Rodríguez *et al*., 2017) were isolated from both the immature and mature stages of *Anopheles* and *Aedes* mosquitoes. Although these fungi were obtained using culture dependent strategies as previously detailed (Gusmão *et al*., 2010), culture-independent methods have also shown that non-bacteria symbionts in mosquitoes are prevalent (Chandler *et al*., 2015; Gusmão *et al*., 2010). Preliminary evaluation demonstrates that *Wickerhamomyces*sp forms a stable association with *An. stephensi*(Ricci *et al*., 2011), and produces killer toxin that could be detrimental to the development of malaria parasite (Cappelli *et al*., 2014), while *Talaromycessp*was demonstrated to infect *Ae. aegypti* where it augments dengue infection by releasing antitrypsin factors (Angleró-Rodríguez *et al*., 2017). The *Anopheles*-associated symbiont *Microsporidia MB* colonizes mosquito ovaries to undergo vertical transmission and can also block the transmission of malaria by *Anopheles* mosquitoes(Herren *et al*., 2020). The challenge with *Microsporidia MB* is that its field deployment to combat malaria would require rearing and releasing infected females with the hope that they populate and establish in the areas of release by out-competing to replace the wild species as previously shown in *Wolbachia* field experiments in Australia. However, *Microsporidia* does not utilise cytoplasmic incompatibility to invade the population suggesting there could be other mechanisms yet to be explored. Alternatively, it is hypothesised that infected males

could transmit infections to females during insemination and this could provide a sustainable approach of increasing its burden in the wild.

2.5.1 Mycobiome refractoriness to malaria

Mosquito midgut is the focal point from where vulnerable stages of malaria development begin, and this makes it a prime target for developing microbe-based interventions. When a female mosquito ingest thousands of gametes when acquiring blood mea, and these fuses to form zygotes in the midgut of infected host, less than 10% of these develop to ookinetes, and few of themproceed to invade the epithelium forming sessile oocysts (Drexler *et al*., 2008). However, each oocyst can release thousands of sporozoites leading to a dramatic amplification of infectious sporozoitesthat proceed to the salivary glands (**Figure 2.3**). This demonstrates that in spite of a severe bottleneck to the parasite development in the midgut, oocyst-to-sporozoite phase act as a compensatory mechanism to increase infectious stages(Wang & Jacobs-Lorena, 2013). Therefore, the midgut has been hypothesized as the prime target for testing microbe-based strategies that aim to ascertain whether microbes provide refractory or modulatory effects on the development and transmission of malaria.



Wang & Jacobs-Lorena (2013)

Figure 2.3: Development of malaria parasite in mosquito. a) Shows*Plasmodium* stages in the midgut.b) Shows severe bottleneck experienced by the parasite in the midgut.

Previously it was shown that fungi confer refractoriness to pathogens such as dengue virus or *Plasmodium* parasite when , adult *An. gambiae* were fed on *Penicillium chrysogenum* and found to have enhanced susceptibility to malaria parasite indicating that the fungi made it easy for the vector to acquire the pathogens (Angleró-rodríguez *et al*., 2016). Interestingly, another form of fungi termed as *Wickerhamomyces* sp formed a stable association with *An. stephensi* (Ricci *et al*., 2011), and produced killer toxin that could be exploited for preventing midgut development of malaria parasites (Cappelli *et*

al., 2014). Recently, it was shown that an obligate intracellular fungus named *Microsporidia MB* blocks the transmission of malaria in *Anopheles* mosquitoes (Herren *et al*., 2020). While this *Microsporidia MB* is naturally found in populations of *Anopheles* mosquitoes in Kenya, a possible reason why Kenya still report *Plasmodium* infection is that its prevalence in the field range from 0 to 25% (Herren *et al*., 2020). To have the impact of this fungus felt in terms of reduced malaria burden, field prevalence would need to range between 40-60%. It is hypothesized that a dissemination strategy to increase field prevalence could reduce the occurrence of malaria disease. Taken together, these studies form the basis for studying fungal symbionts since they demonstrate the prospects of utilizing fungal symbionts. However, there are gaps on whether these isolates were true symbionts of mosquitoes (with the exception of *Wickerhamomyces*) since limited information is available on their persistence across generations or developmental stages.

2.5.2The Un-cultured *Microsporidian-Anopheles* symbiont is a classical example of a novel fungal symbiont

Microsporidia are obligate, intracellular organisms that infect several hosts, including vertebrates and invertebrates and have been shown to inhabit both terrestrial and aquatic environments(Vossbrinck & Debrunner-Vossbrinck, 2005).*Microsporidia* undergo two main phases of development namely meront phase that ensures proliferation and the spore stage, which ensures long-term persistence to harsh environmental conditions. Characterizations of *Microsporidia* in arthropods indicate that

they exploit vertical and horizontal transmission (Stentiford *et al*. 2013). To undergo vertical transmission, *Microsporidia* spores lodge onto the insect ovaries to colonize developing eggs. This mode of transmission ensures that the symbiont attains greater host specificity by lessening the virulence and burden of infection(Vávra & Lukeš, 2013). It has been hypothesized that *Microsporidia* spores ingested orally germinate to sporoplasm that invades the host intestinal cells through a polar filament (Han and Weiess, 2017). Collectively, *Microsporidia* sp relying on oral horizontal transmission pose high virulence and lower levels of host specificity because upon death, it is likely that more spores will be available to infect more hosts (Han and Weiess, 2017). Sexual transmission has been shown to occur in microsporidian species*Nosema plodiae*that infect *Plodia interpunctella* (Indian meal moth) and achieves these features by invading the host reproductive tissues and is passed from male to female during insemination (Kellen & Lindegren, 1971). It is possible that *Microsporidia MB*, which is vertically transmitted in *An. gambiae* and inhibit the transmission of *P.falciparum* could be exploiting mixed modes of transmission namely sexual and vertical transmission. To utilise *Microsporidia* symbiont to lower the burden of malaria transmission in the wild, it is essential to increase the prevalence of symbiont above ~40% from the current 0-25%. This can be achieved by establishing an alternative strateg that could spread *Microsporidia MB*in *Anopheles* mosquito populations. The understanding of whether male-mediated transmission of *Microsporidia MB* in *An. gambiae*could possibly offer ways of manipulating its population in several ways of importance.

A possible way of manipulating *Anopheles* field population in a beneficial way involves exploiting the rear-and-release strategy. In this approach, males harbouring symbionts are reared in the laboratory and released in the wild with the hope that they have equal mating chances with wild females because the physiology of such males have not been intereferred with like what happens with those irradiated under sterile insect technique (Crotti *et al*., 2012). Males do not require a blood meal and therefore humans would not need to worry about increased biting rates from the released males. Conversely, female *Anopheles* only mate once in their lifetime to undergo postmating switch that ensures refractoriness to further mating in what is termed as enforced monogamy (Gabrieli et al., 2014). This phenomenon is not common among insects and is thought to be induced by steroid hormone 20-hydroxyecdysone transferred to females during insemination (Gabrieli et al., 2014). Ostensibly, one male could mate with several females establishing infected progeny of several matrilines and increasing chances of persistent infection. It is hypothesized that sexual transmission could offer an efficient strategy for increasing the prevalence of *Microsporidia MB* into the wild populations (>40%).

2.5.3 Mycosymbionts enhance host's inducible and constitutive immunity

Insects mount potent and highly effective humoral and cellular innate immune responses when invaded by foreign cells such as parasites, fungi or bacteria. These defensive mechanisms are characterized by a transient release of antimicrobial peptides with anti-parasitic/anti-bacteria/anti-fungal properties some of which are listed (**Figure**

2.3), and previously reviewed by Wang and Jacobs-Lorena, (2013). Upon encountering a foreign cell, a number of pattern recognition receptors (PRRs) are involved in the recognition of non-self through binding to specific surfaces of microbial cell structures, and this is believed to activate a proteolytic cascade (Pan *et al*., 2018;Dimopoulos, *et al*., 1997; Waterhouse *et al*., 2007). Two components of basal immunity (Immune deficiency and Toll pathways) are well described in *Anopheles* as discussed below.

When Grams positive bacteria and fungi infect insects, NF-$_k\beta$ proteins are activated to induce Toll pathway. These proteins namely Dorsal and Dorsal related immune factors have been characterized in *Drosophila*(Bian*et al*., 2005), where they are associated with the production of antimicrobial peptides (AMPs) and antifungal molecules such as drosomycin. Other than AMPs, microarray studies augment these findings by revealing significant up-regulation of genes when Toll or immune deficiency (IMD) pathways)are active (De Gregorio*et al*., 2002). Activation of IMD by gram negative bacteria requires Relish protein, which is a different kind of NF-$_k\beta$ protein with genome sequence of *An. gambiae* showing that NF-$_k\beta$ protein contains Gambf1 and Relish genes(De Gregorio *et al*., 2002). This signifies that fungal infection could trigger antifungal immune responses in a similar or related fashion to observations made in *Drosophila*.

Inducible immunity of *An. gambiae* has been shown to play a crucial role in anti-plasmodialresponse. Inducible immunity is one that occurs when insects are invaded by foreign materials while basal (or constitutive) immunity is the surveillance system

monitoring cells prior to invasion. It was found that a decrease in the immunity through silencing of immune related genes such as thioester-containing protein (TEP1) and leucine-rich repeat proteins (LRIM1) resulted in 200% increase in *Plasmodium* parasites developing in the midgut while boosting resulted in complete blockage of parasite development (Frolet, *et al*., 2006). Microbes influence mosquito immunity in a way that microbe-based strategies are believed to exploit this mechanism to influence development of parasites. Commensal microbes co-existing with mosquito influence their basal immunity in a manner that when eliminated the host cell develops increased susceptibility to malaria or dengue virus (Dong *et al*., 2009; Lu, *et al*., 2012). Reports indicate that gram-negative bacteria and *Plasmodium*parasites invading *Anopheles* activate immune deficiency pathway (IMD) (Meister *et al*., 2005), while invasion by gram-positive bacteria, dengue, fungi, and *P. bergei* elicits Toll pathway of basal immunity in *Anopheles* (Bian *et al*., 2013; Frolet *et al*., 2006; Pan *et al*., 2018).

Understanding the role of eukaryotic symbionts on the hosts' parasitesis important. Studies on fungal symbionts that co-exists with *Anopheles* have linked novel isolates to the production of killer toxins that could directly interfere with development of malaria in the midgut (Cappelli *et al*., 2014; Valzano *et al*., 2016). Others found fungi to modulate midgut trypsin activity in a way that it enhances host susceptibility to dengue (Angleró-Rodríguez *et al*., 2017). Other fungi were found to recruit ornithine decarboxylase gene in sequestering arginine needed for the synthesis of polyamine thereby providing protection against *Plasmodium*(Angleró-Rodríguez *et al*., 2016).

Whether native fungi of *Anopheles* directly activate Toll pathway or the aftermath thereof remains unknown. It is hypothesized that immune responses mounted by *Anopheles* when invaded by fungal infection does not impose developmental fitness but could have a negative feedback mechanism in blocking the development of malaria parasites.

2.5.4 Melanotic pathology as a feature of symbiont infection enhances its propagation in host

Insects respond to pathogen infections by relying on a number of defencestrategies. Inducible and constitutive defence mechanisms are common among most arthropods. While most insects lack adaptive immunity, innate immune response is well developed and is the main strategy for fighting infections (Kumar *et al*., 2018). There is a striking similarity between insect innate and mammalian innate immune responses, with components of cellular and humoral responses cutting across between them(Kumar *et al*., 2018; Shaw & Catteruccia, 2019). When infected with microbes, their first line of defense is structural, which consists of barriers in the tracheal lining, gut peritrophic matrix, and the external barrier enhanced by the cuticle. However, when invading pathogen breaches these barriers, a wide range of reactions are induced. It has been observed that proteolytic cascades are rapidly activated when cuticles are perforated by microbial infections or any injury, and this leads to melanization and blood coagulation (Gulley *et al.,* 2013; Zou, *et al*., 2010). At this stage, persistentinfection by microbes lead to activation of cellular immune response to

enhance the clearing of invading microbe through encapsulation or phagocytosis (Shaw & Catteruccia, 2019). Systemic infections lead to secretion of inducible effector molecules into the hemolymph, examples of these molecules are proteins involved in stress response, antimicrobial peptides, molecules required for iron sequestration and opsonization(De Gregorio *et al*., 2002). These responses are critical for the arthropods such as mosquitoes to absorb the shock induced by invading microbes or eliminate these microbes from their system.

While antimicrobial peptide (AMP) regulation has received immense attention in response to microbes and pathogens, there is limited knowledge on other defence mechanisms utilized by insects to combat infections particularly in *Anopheles*. Apart from AMP regulation, other mechanisms that confer defense are poorly understood especially in *Anopheles*. When microbes invade insects, the latter's immediate immune response is the melanization process, which takes place at the point of cuticle injury leading to blackening of the wounded segment (De Gregorio *et al*., 2002). The blackening occurs from the intense deposition of melanin derived from *de novo* synthesis (Akhouayri, Habtewold, & Christophides, 2013; De Gregorio *et al*., 2002). In *Anopheles*, there is paucity of information about the occurrence of melanization especially from artificial introduction of fungal symbionts and the role played by this defence strategy to evade invasion.

In other arthropods such as *Drosophila melanogaster*, *Tenebrio molitor*, and *Manduca sexta*, melanization reaction, a humoral response to invasion, has been poised

to play a role in killing microbes invading insects by combined production of toxic intermediates, sequestering, and encapsulating microbes thereby helping in healing the wound (An, *et al*., 2011; Chu, *et al*., 2017; Gulley *et al*., 2013; Zhang*et al*., 2016). For melanin to be produced and deposited, phenoloxidase enzyme involved in the production of orthoquinones is activated to oxidize mono-phenols and di-phenols (Akhouayri *et al*., 2013). These orthoquinones are polymerized to release melanin which is deposited at the site of infection. Insect cuticle and hemolymph plasma have been reported to harbour phenoloxidase (PO), which exists as pro-phenoloxidase (PPO), the inactive form (Zhang *et al*., 2016). Serine proteases cleave PPO to its active form PO through a process termed as prophenoloxidase activation. When microbes invade insects, their cell wall components such as proteins, lipopolysaccharides, β-1, 3 glucan, peptidoglycans are recognized at the site of injury (De Gregorio *et al*., 2002), and this triggers activation of melanization cascade. The involvement of serine protease cascade in melanization is tightly regulated to avoid instances of fatal events that may occur from systemic activation. It is likely that fungal symbiont induces a similar melanotic response in anopheline mosquitoes.

2.6 Transmission of symbionts is essential for co-existence and exploitation

A number of symbionts associating with insects such as mosquitoes establish a stable and intimate relationship with them. These associations are what support the investigation of symbiont-based methods in controlling vector-borne diseases. A stable relationship could signify beneficial relations between the host and symbiont

(Cirimotich *et al*., 2011; Ott *et al*., 2014). A predominant view is that for colonization and persistence of a symbiont in a host vector to occur, a transmission mechanism that utilizes and/or relies on the host reproductive biology is useful(Ott *et al*., 2014).. Several strategies namely vertical, horizontal, and mixed mechanism (vertical and horizontal) have been deduced to drive the association between *Anopheles* and known symbionts as described below.

2.6.1 Vertical and horizontal transmission of symbionts

A predominant relationship exists between hosts and symbionts that colonize reproductive tissues such as ovaries and testis. Microbial symbionts have evolved features that compliment host biology such as nutrient acquisition, and these could explain their minimal fitness cost on the host, which increases their chance of retention along insects' development phases (Bright & Bulgheresi, 2010). Some of these symbionts achieve transmission by tapping onto features of the host reproductive biology to enhance their persistence to subsequent progeny through vertical transmission. Evolution of microbial genome within the host gut leads to elimination of loci (i.e. genome reduction) that could confer extraneous physiological functions thereby acting as a trade-off that reduces their response to environmental changes and rendering symbionts less free-living in a manner that enhances their co-existence (reviewed in Moran *et al.*, 2008). Classical examples of vertically transmitted *Anopheles* symbionts include those in the genus *Asaia*(Favia *et al*., 2007), and *Elizabethkingia* that leads to melanosis in *An. gambiae*(Akhouayri *et al*., 2013). Besides, it has been proposed that

*Wolbachia*sp exploits host immunity to enhance its transmission across generations (Pan *et al*., 2018). Previously, fungi of order Blattaria in cockroach and wasps were found to utilize vertical transmission to establish an intimate relationship (Gibson & Hunter, 2009). In *Anopheles*, yeast *Wickerhamomycesanomalus*was found to localize reproductive tissues suggesting prospects of vertical transmission (Ricci *et al*., 2011). A *Microsporidia MB*fungal symbiont of *Anopheles* was shown to undergo vertical transmission in these hosts at a rate ~40%(Herren et al., 2020). However, this is an obligate symbiont that relies on natural acquisition from the field infection and has not been cultured in the laboratory. Perhaps the best strategy of developing this symbiont into a tool is to study other modes of horizontal transmission to complement vertical transmission. While these studies demonstrate the importance of vertical transmission and the prospects of fungal symbionts assuming a similar trend, there is limited knowledge on whether re-introduction of cultivable fungi previously isolated from *Anopheles* could achieve similar features and whether uncultured *Microsporidia MB*could be exploiting an additional mode of transmission in *Anopheles* hosts(Herren et al., 2020).

Horizontal transmission happens more often when the male is infected and transmit symbionts to the female during insemination or through direct contact (paternal transmission). It utilises both direct and indirect contacts between host insect and symbionts, and is believed to be the primary route utilized by most insects during pre-adult and adult stages to acquire significant portions of new microbes from their

environment as they develop (Favia *et al*., 2007; Moran & Dunbar, 2006). Classical way is when vectors obtain symbionts through oral route, usually from the diet or through direct microinjection (Ott *et al*., 2014). However, it is suggested that horizontal transmission is less evolutionarily stable compared to vertical transmission (Ebert, 2013). While vertical transmission ensures a long-term persistence between symbiont and host because it favours evolution of benign infections, horizontal transmission favours overt expression of the symbiont and has been associated with increased prevalence under certain conditions such as intensities and temperature (Ott *et al*., 2014).

2.6.2 Mixed transmission of symbionts

Mixed transmission blends both vertical and horizontal modes of transmission. Mixed strategies are considered transitory and often considered useful if a symbiont is not exclusively transmitted through either modes described(Ott et al., 2014). There is limited information on features that enhance persistence of symbionts through mixed transmission.

Paternal transmission has elements of both modes of transmission and has been shown to influence symbiont population dynamics in a way that imparts evolutional effects on the host as well. For instance, if a male vector possess a different strain from the female, mating leads to coinfection and recombination, which results in exchange of genes that impact on the interaction between the host and symbiont(Moran & Dunbar, 2006). This transmission leads to elimination of sex bias because it acts as a tradeoff among symbionts that are maternally inherited to favor daughters at the expense of sons

(Moran & Dunbar, 2006). It is also believed that horizontal transmission is plausible in increasing symbiont densities and population since a male could mate with several females and this could facilitate a prolonged widespread sweep of novel isolate in a host population (Moran & Dunbar, 2006).In *Wolbachia*-based strategy, cytoplasmic incompatibility has been utilized as the most effective way of driving its spread in the mosquito populations. However, symbionts such as *Microsporidia MB* do not posses such mechanisms. Therefore, identification of alternative transmission routes that could be utilised to increase the spread of *Microsporidia* is critical in driving its spread through *Anopheles* population. In *Anopheles* there is hardly any information on mixed modes of maternallyand horizontally transmitted fungal symbionts and their effect on the biology of host as well as the spread in a given population. This signifies the need to explore the existence of other routes of transmission if any, and elucidate their role on vector biology.

2.7 Emerging methods adopted for studying mycobiomes of *Anopheles*

Eukaryotic yeast and fungi have been isolated using midgut-depended culture isolation and amplification of the 18s or 26s rRNA (Ricci, *et al*., 2011; Urubschurov & Janczyk, 2011). For instance, a study identified isolates of 18 non-pathogenic yeasts belonging to the genera *Cryptococcus*, *Rhodotorulasp*, *Yarrowiasp*, and *Candidasp* from the adult and larval stages of the three main mosquitoes namely the *Anopheles*, *Culex*, and *Aedes*(Jupatanakul *et al.*, 2014). Others isolated *Pichia* yeast and *Candida* from the midgut of *Ae.aegypti* through culturing of the midgut and amplifying 18s

rRNA segments of the cultured cells (Gusmao *et al.*, 2010). Besides, a yeast *Wickerhamomycesanomalus*was isolated from the reproductive organs and the midgut of *An.stephensi* species (Ricci *et al.*, 2011a; b), signified the existence of a complicated interaction between eukaryotic mycobiome with the host tissues.

There is an agreement that most of the methods reported in literature depend on culturing the midgut homogenate and amplifying either 18s or 26s rRNA, and these methods provide a narrow spectrum of species of yeast endosymbionts that are difficult to cultivate yet inhabiting the vector's midgut (Gusmao *et al.*, 2010; Ricci *et al.*, 2011a; b). A comprehensive approach exploiting high throughput methods that give a GPS surveillance of all symbionts irrespective of bacteria, viral, spirochete, or fungal origin as shown previously is considered as useful in identifying detailed microbes in these hosts (Chandler *et al*., 2015). Besides, combining both culture dependent and culture-independent methods has been proposed as an alternative to the high throughput method (Gusmão *et al*., 2010). It is important to note that isolates of yeast and fungi were reported from the midgut and cuticles of sandfly(Akhoundi *et al.* 2012), and recently in the midguts of *Aedes* and *Anopheles* (Angleró-rodríguez *et al*., 2016; Angleró-Rodríguez *et al*., 2017; da Costa & de Oliveira, 1998; Pereira*et al*., 2009; Ricci,*et al*., 2011). While these findings demonstrate that mosquitoes co-exist with eukaryotic symbionts, it is not clear whether they confer any fitness cost to the vector, get transmitted across generation or to offspring (true symbionts), or whether they augment these traits with beneficial interaction with the malaria parasite.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Isolation of fungi from *Anopheles* mosquito developmental stages obtained from semi-field environment.

3.1.1 Study site and design

Mosquitoes used for fungi isolation were obtained from the *Anopheles gambiae* complex reared in the screen houses that mimics natural environmental setting. These screened greenhouses are located at the icipe- Thomas Odhiambo Campus (*i*-TOC Mbita) along the shows of lake Victoria in Homabay Western Kenya (0°26′06.19″ S, 34°12′53.13″ E; altitude 1,137 m). Field samples screened for the isolated symbionts were obtained from Mwea (central Kenya–37.3538W, –0.6577N) and Ahero (Western Kenya–34.9190W, –0.1661N) as shown in the map below (**Figure 3.1**). These areas have been shown to have high densities of *Anopheles gambiae*complex mosquitoes(Muturi *et al.*, 2008; Mwangangi *et al.*, 2006). Additional mosquito samples were obtained from old discarded tire wheels near Pwani University in the Kenyan coast.

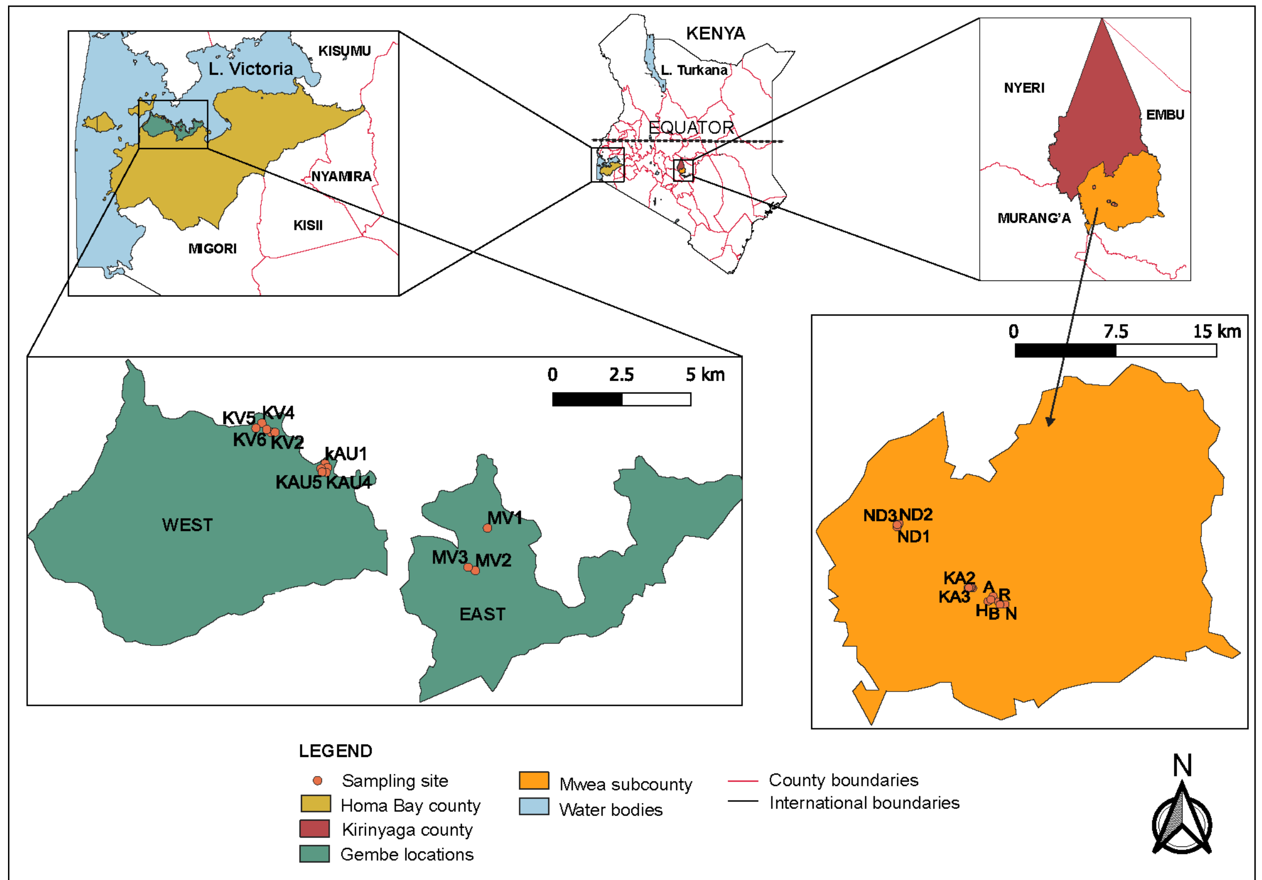


Figure 3.1: Map showing locations where mosquito samples were collected

3.1.2 Sampling of semi-field *Anopheles* mosquitoes

Semi field mosquitoes namely *An. gambiae*s.s and *An. gambiae*s.l (*An. arabiensis*)were maintained in screen-houses at *i*ToC-Mbita campusand used for fungi isolation. The immature insects maintained in large greenhouses that mimic natural environment while adults supplied with 6% glucose solution in a laboratory confinement maintained at room temperature (27.5 ±1.5^{o}C; 68-80% relative humidity) were collected

at larvae, pupae and adult stages. Equal numbers of *An. gambiae*s.s and *An. gambiae*s.l (*An. arabiensis*) were either placed in troughs or aspirated in small cups awaiting downstream experiments (total n=348).

3.1.3 Dissections of mosquitoes

Pre-adult immature (L3 instar larvae, pupae placed in troughs) and adult stages of *An. gambiae*s.s and *An. arabiensis*placed in small cups were prepared for culture work. Briefly, adults were sexed by macroscopic examination of antennae to separate females from males (method given at www.mr4.org). Males were not utilised hence were discarded. Females were washed in three series of 70% ethanol to remove surface contaminating microbiome before rinsing in 1× PBS. These specimens were dissected under the microscope using sterile dissection pins to separate the midgut, ovary and gonads, which were segregated into pools of three and pulverized using sterilized pestle. Carcasses of whole individual mosquitoes were also included, and these were washed as described and homogenized, these procedures were undertaken as previously decribed (Dickson *et al*., 2017; Dong *et al*., 2009; Ramirez *et al*., 2012).

3.1.4 Cultivation of mycobiome from *Anopheles sp*tissues

The homogenate from dissections were serially diluted to 10^3 (10^{-1}, 10^{-2}, 10^{-3}) and each dilution of the aliquot (100uL) in Potato Dextrose Agar [PDA] (20 g of sucrose, 10 g of peptone, and 20 g of agar in 1 L of double distilled water) while the

other half was plated in Yeast Peptone Dextrose Agar (Yeast extract (1%), glucose (2%), malt extract (1%), peptone (2%), and agar (2%)) (Oxoid Ltd., Basingstoke, Hamp- shire, England). These agar plates were prepared with a cocktail of antibiotics consisting of Tetracycline (50 µg/mL) and Chloramphenicol (50 µg/mL) to selectively inhibit bacterial contaminants. The plates were incubated aerobically at room temperature (27.5± 1.5°C) and checked daily for fungal growth. Fungal colonies obtained were labelled and sub-cultured in new PDA or YPDA plates as previously described(Gusmão *et al*., 2010). These fungal isolates have been maintained in glycerol and water stocks at the Martin Luscher Emerging and Infectious Diseases Laboratory (ML-EID) facility at *icipe*.

3.1.5 Morphological characterization of fungal isolates

Fungal isolates were sub-cultured in PDA and/or YPD containing chloramphenicol (50 µg/mL) and incubated at room temperature for 3-5 days. Microscopic and morphological examination of features such as spores, conidia, coloration, and mycelium were done under a microscope after staining with lactophenol cotton blue (0.01% w/v) as previously described (Pereira *et al*., 2009). Growth rate, based on radial growth of the isolate plated at the centre of culture plate was also frequently observed as previously described on fungus isolated from *Aedes* mosquitoes (Darbro *et al*., 2011).

3.1.6 DNA extractions from fungal isolates and amplification of 18S rRNA gene

The pure cultured isolates were prepared by culturing fresh mycelium in YPD broth for 2-3 days on a rotor shaking at 180rpm as previously described (Gibson & Hunter, 2009). The resulting broth was centrifuged to pellet the cells for DNA extraction. Genomic DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions and stored at -20°C awaiting downstream experiments. Briefly, samples placed in 1.5 mL tubes, 200µL of lysis buffer added and samples homogenized with pestle. This was followed by addition of 20µL of Proteinase K and before mixing by inverting tubes several times. The mixture was incubated at 56°C for 2 hrs and 200 µL of absolute ethanol added. They were shaken vigorously by vortexing and centriguged at 13200 rpm for 5 minutes. These were passed through a spin column and washed before eluting with 200µL of distilled water.The rRNA internal transcribed spacer (ITS) was amplified using ITS1 (5'-TCCGTAGGTGAACCTTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3')gene primers as previously described (White *et al.*, 1990). Briefly, the ITS-PCR amplification was achieved in a 30 µL reaction mixture containing 19.5 µL PCR H_2O, 6 µL of Solis BioDyne - 5× FIREPol® Blend Master Mix - with 7.5 mM $MgCl_2$, 0.75 µL of each primer, and 3 µLof DNA template to achieve a final concentration of 1× FIREPol® Blend mixture (Solis Biodyne, Estonia). The amplification of DNA was undertaken in a SympliAmp PCR machine using the following PCR conditions: initial denaturation at 95°C for 15min, followed by 35 cycles of denaturation at 95°C for 30 s,

60°C for the annealing of primers at 30 s, 45 s of extension at 72°C, and a final chain elongation step of 72°C for 10 min. Aliquots of PCR amplicons (5µL) were resolved on a 2% agarose gel stained with ethidium bromide (0.35 µg/mL) and allowed to resolve for 30 min under voltage 90V and current 300 Amps. Resulting DNA was visualized under VWR® Genoplex gel documentation system fitted with a GenoCapture and Genosoft v.4.0 software.

3.1.7 Sequencing

All the PCR products were cleaned with ExoSap-IT PCR Product Cleanup Reagent (USB Corporation, Cleveland, Ohio, USA) according to the manufacturer's instructions in preparation for sequencing (Macrogen Co., Ltd., South Korea). Briefly, 5 µL of the PCR product was mixed with 2 µL of ExoSAP-IT and mixed by spinning. The mixture was incubated at 37°C for 15 mins and inactivated at 80°C for 15 minutes. Sequencing was undertaken on automated dye termination kit and sequenced on a 16-capillary 3100 Genetic Analyzer from Applied Biosystems. The Big Dye terminator kit was used according to the manufacturer's instructions. Sequencing of the fungal DNAsampleswas undertaken using ITS1 gene primers.All sequencing was undertaken by Macrogen in South Korea. Sequences obtained were visualized and edited in Geneious Software (version R11.1)before carrying out similarity search in NCBI.

3.1.8 Phylogenetic identification of close relatives

Sequences were aligned in Geneious Software v8.1.9 and trimmed before undertaking a search of similar and related sequences fromBLASTn-NCBI search engine(http://www.ncbi.nlm.nih.gov) on the Geneious Software platform. Obtained sequences similarities were saved in fasta format andMUSCLE algorithm used for multiple sequence alignment of the isolate sequences and their related sequences obtained from GenBank as previously described (Edgar, 2004). Phylogeny was constructed using maximum-likelihood method on a Genious Software v8.1.9 by treating alignment gaps as missing data and all characters treated as unordered and equally weighted. Statistical support of the tree was evaluated by performing 1000 replicas of bootstrapping. Gene sequences reported in this work were deposited in GenBank under accession number (MT437213-MT437220; MT433814; MT435649).

3.2 Investigation of the effects of selected isolates on the *Anopheles* host fitness.

3.2.1 Ethical statement

All procedures involving use of animals were handled in strict accordance with good animal practices defined by the *icipe*Animal Use and Care regulatory body. Mice were used solely as source of blood meal for rearing and maintaining insectary mosquitoes.

3.2.2 Revival of isolates

To ensure maximum viabilityfungi that were either isolated from midgut and or reproductive tissues (*Penicillium*, *Periconia*, *Epicoccum*, *Leptosphaerulina*, *Alternaria*, *Lichtheimia*, *Cladosporium*, and *Hasegawazyma* sp) were revived by transferring them from -80°C, to -20°C, and 4°C, at 3 hrs intervals before taking them to room temperature and inoculating PDA/YPD media to maximize growth. Pure isolates were maintained on solid agar and in liquid broth medium. The solid agar (PDA) consisted of yeast extract (1%), glucose (2%), malt extract (1%), peptone (2%), and agar (2%), while the liquid medium (YPD) consisted of yeast extract (0.5%), glucose (1%), malt extract (0.5%), and peptone (1%) pH 5.7 (Oxoid Ltd., Basingstoke, Hamp- shire, England). These cultures were maintained at room temperature (27.5±1.5^{o}C) and monitored for growth. Confirmatory tests were based on colony morphology and HRM-PCR profiles using ITS1/ITS4 primer sets.

3.2.3 Preparing fungal mycelium for inoculating mosquitoes

Fungal mycelia were obtained from the individual plates using 2mL double distilled water with sterile forceps into a pre-weighed 15mL centrifuge tube. The isolates were suspended in distilled water, transferred to the centrifuge tubes and washed by centrifugation at 16500 rpm for 15 mins to remove traces of media. This was done three times to ensure complete drainage of media from the isolates. The pellets were tapped to break the compactness at the bottom of the tube. They were dried at 30°C in the oven, cooled down to room temperature before taking the final weight to estimate amount of fungalspores per mililitres. One portion was stored at 4°C, while the remaining were mounted on hemocytomer and stained with lactophenol blue for easy enumeration of spores.

3.2.4 Curing mosquito eggs and infecting larvae/adults with fungal isolates

*Anopheles gambiae*eggs from a colony maintained in the insectary were cleaned from fungi by washing with 1% HCl and rinsed with double distilled water. This was done by adding 2mL of 1% HCl to approximately 500 eggs placed in 100mL double distilled water swirled and diluted after 20 mins by topping up laying water to 500mL. The immature larvae (L1) and adult stages obtained from cleaned colonies were tested for the presence of fungi by DNA extraction and undertaking PCR using ITS1/ITS4 primers as previously described (White *et al*., 1990). These were deemed fungal-free if amplification with fungi-specific ITS primers turned negative. The colony from

thistreatmentwas taken as negative control and maintained on Tetramin™ baby fish food previously tested with ITS1/ITS4 primers toavoid cross contamination.

3.2.5 Infecting larvae and adults with fungal mycelium through oral feeding

Mosquito colonies that previously turned negative (upon treatmentwith 1% HCl) were infected by inoculating with a one-off dose of 60mg (3.9×10^3 - 5.5×10^5 spores/mL) of ground mycelium and spores of *Penicillium*, *Periconia*, *Epicoccum*, *Leptosphaerulina*, *Alternaria*, *Lichtheimia*, *Cladosporium*, and *Hasegawazyma* sp. These were done in triplicates of 60 larvae per standard trough. Controls not exposed to fungi were maintained on Tetramin™ baby fish food previously tested with ITS1/ITS4 primers toavoid cross contamination. Double distilled water used for rearing was changed 18 hrs post inoculation and 36 hrs interval subsequently. To collect samples, they were allowed to develop for 6 – 8 days (larvae sample), 10-13 days (pupae) and 12-15 days (adults). These samples were dissected to obtain midgut and reproductive organs or homogenized (whole mosquito). The homogenate was divided into two portions with one meant for plating and the other half for extraction of DNA. Samples from culture were deemed positive when the number of larvae or pupae andadults for which at least one fungal colony was detected. For adult infection, newly emerging adults from clean lines were allowed to feed on fine whole fungus mixed with 6% glucose solution soaked in cotton wool and a wick system to maximize infection.Fine fungi were obtained by grinding whole cultures using a bead beater.Metal cage**s** used for rearing adults were

dismantled, autoclaved, and wiped with wet cotton wool containing antifungal (ketaconazole –50mg/mL) and antibiotics (tetracycline –50mg/mL). These adults were kept in a room with temperature maintained at 27.5±1.5°C and 68-80% relative humidity with a cycle of 12 hrs day (light) and 12hrs night (darkness) (Gonzalez-ceron *et al*., 2003). After 18 hrs of feeding exposure, new cotton wool free of fungi soaked in 6% glucose was availed to maintain the colony and allow development of infection (Lindh *et al*., 2008)

3.2.6 Transmission of fungi and effects on survival, fecundity and longevity

Fungal isolates were evaluated for their influence on the developmental stages of the vector. The study of fitness involved determining mortality rate (survival/ longevity) – by assaying the number of surviving larvae and adults over time compared with their controls not exposed to fungi. Prevalence of survival was obtained from the number of larvae that acquired the isolates and pupated, while reproductive capacity – the ability of infected male or female to mate and produce offspring as measured by the number of eggs (fecundity), viability of eggs over time, duration to oviposit, development to adult – F1, were assessed. Mating combination aimed to ascertain whether infection was sex biased or whether one sex was better in transmitting the isolate was undertaken by co-housing either infected males or uninfected females or infected females and uninfected males that were sexed at pupae stage to ensure they remained virgins. To test paternal transmission, adult offspring obtained from a mating combination of male infected at larval stage co-housed with clean females (confirmed fungal negative *post hoc*) was

tested with HRM-qPCR ($^{+}$♂×$^{-}$♀ -> offspring). To test vertical transmission, offspring obtained when virgin infected female that mated with virgin males from clean colonies was tested with HRM-qPCR ($^{-}$♂×$^{+}$♀). Other combination included mating both genders previously exposed to fungi ($^{+}$♂×$^{+}$♀), and controls that were free of fungi($^{-}$♂×$^{-}$♀). Females from these experiments were supplied with blood meal using restrained mice. Well engorged mosquitoeswere transferred into individual cups with wet filter papers for egg laying and monitored for three days. Eggs laid per mosquito were counted to ascertain fecundity, which was considered as standard measure of fitness. Females that laid eggs were screened *post-hoc* to ascertain infection status. Fecundity was also analyzed for the offspring (F1 generation). Adult offsprings were sexed and female/males counted to obtain sex ratio. These samples were washed in PBS and dissected to obtain midgut, which were tested for persistence and dissemination of fungal candidates using culture and DNA extracted for HRM-qPCR experiments. Transstadial transfer aimed to assess whether there was retention of the isolate from larvae to adult and/or from adult to larvaas previously described (Lindh *et al*., 2008). A total of 4 rounds of experiments were undertaken in replicates for each treatment and variability between experiments established by comparing the range of data obtained.

3.2.7 DNA extraction and re-culturing infected samples

Sample homogenates from mosquito dissections and carcasses previously exposed to fungal isolates or their offspring were divided into two portions, one for DNA extraction whilst the other was used for plating. DNA was extracted using a

modified Qiagen protocol. Briefly, protein precipitation technique was used for extracting high quality DNA. This involved homogenizing each sample in 1.5-mL Eppendorf®tube containing 4 sterile 0.5 mm beads with 300 µL of cell lysis buffer (5 mM EDTA, 0.5% SDS and 10mM Tris (pH 8.0). A BioSpec Mini Beadbeater -16 was used to homogenize samples for 25 sec. Crushed samples in cell lysis buffer were incubated at 65°C for 1 hr before adding 100µL of protein precipitation solution (8 M Ammonium acetate and 1 mM EDTA) and vortexed in 20 second pulse before placing them in ice for 5 mins. These were centrifuged at 16400 rpm for 5 mins at 4°C and supernatant transferred to a new 1.5mLEppendorf® tube with 300 µL molecular grade isopropanol (99.8%). These were mixed by inverting 100 times and centrifuged at 16400 rpm for 60 mins at 4°C. Supernatant was carefully pipetted off retaining the pellets that were washed twice in 300 µL of ice-cold 70% ethanol by centrifugation at 16400 rpm for 30 mins at 4°C. Following the removal of washing solution, tubes were inverted on paper towel to air-dry and eluted with 50 µL nuclease free water and allowed to elute at 4°C overnight before determining DNA quality and quantity using Thermo Scientific™NanoDrop™ 2000c Spectrophotometer. Samples were serially diluted and used for checking efficiency of qPCR machine before running stock samples. Mosquito samples from G_0 as well as F1 or F2 were homogenized and divided into two portions. One part was used for genomic extraction whilst the other half was plated to ascertain persistence and inheritance of the isolates. These experiments were repeated for 4 rounds in replicates.

3.2.8 HRM RT-PCR to estimate prevalence and densities persistence or inheritance

Relative densities of fungal isolates were estimated using primers previously generated from whole genome sequences relative to the host ribosomal S7 protein (**Appendix 1**). PCR was done using Rotor-Gene Q HRM real time thermocycler machine (Qiagen). To verify PCR amplification efficiency, DNA samples from pure culture of nine fungal isolates were used as positive control while those from clean mosquitoes deemed free of fungi by previous cleaning of eggs with 1% HCl acid were used as negative controls. Samples were either used as whole mosquitoes or dissections of midgut and reproductive tissues. Whole mosquito carcasses were also included to ascertain systemic infection with the fungus. To undertake qPCR, a mastermix was prepared by adding 2 µL of HOT FIREPol®EvaGreen® HRM (without ROX), whose components included 5× EvaGreen® HRM buffer, HOT FIREPol® DNA polymerase, dNTPs, BSA, EvaGreen® dye, 12.5 mM Magnesium chloride), 0.5 µL of 10 pmol/µLforward and reverse primers (**Appendix 1**), and topped by adding 5µL UltraPure nuclease free water (Invitrogen, UK). The amount of template used was 2 µL. The PCR amplification program included: initial denaturation at 95°C for 15 mins, 35 cycles of denaturation at 95°C for 30 secs, annealing at 60°C for 30 secs, and extension at 72°C for 45 secs. A final elongation at 72°C for 10 mins was included before melting the products from 65°C to 95°C at a rise in 1°C interval.

3.2.9 Prevalence of *Leptosphaerulina* sp. on diverse mosquitoes from field collection

Adult mosquito samples collectedby indoor aspiration (manually using mouth aspirators and torches)from homesteads in Mwea and Mbita, or larvae collected by dippers from Nairobi (*Anopheles*) and Kilifi and Malindi (*Aedes*), were screened to determine theprevalence and densities offungal infection to ascertain retention and transmission at these developmental stages. This involved using whole mosquitoes from these sites to undertake HRM-RT- PCR analysis alongside positive controls and clean insectary samples as negative controls. For all samples that turned positive for fungi infection, DNA samples were prepared for sequencing by cleaning the PCR products with ExoSap-IT reagent as described in the manufacturers manual (USB Corporation, Cleveland, Ohio, USA) and shipped for Sanger sequencing (Macrogen Co., Ltd., South Korea).

3.3 Investigating mechanisms utilised by *Leptosphaerulina* sp. to establish within the *Anopheles* populations.

3.3.1 Ethical review and acquisition of blood from donors

Approval was obtained (KEMRI/RES/7/3/1) to collect blood samples from parasitemic adult patients or children whose parents/guardians accepted to donate gametocytemic blood by signing the study consent form before being enrolled as volunteers (**Appendix 2**). Parasitemia survey to identify blood donors was undertaken in primary schools adjacent to Mbita along the shores of Lake Victoria. A consent form

translated in local language was used as the instrument for enrolment (**Appendix 3-5**). A qualified and registered laboratory technician assisted with blood collection (500mL from each participant). The collected blood samples wereimmediately fed to starved femalemosquitoes from experiments with fungi experiments.

3.3.2 Microscopic examination of infected larvae and adults

Fungi-infected larvae (at stage L3), pupae and adult were placed on a wet microscope slide and observed for evidence of microscopic fungal infection previously described as melanotic pathology(Akhouayri *et al*., 2013). These melanotic pathologies were compared with non-infected pre-mature and mature stages of mosquitoes. Melanotic lesions were categorized as either dispersed or localized to specific segments as previously described (Akhouayri *et al*., 2013). Melanised samples were separated, counted and allowed to develop separately to estimate survival rate by counting larvae that pupated or pupae that eclosedas well as adults that survived to acquire blood meal and laid eggs. Besides, fungal densities of infections were determined for the G_0 and their F1 offspring.

3.3.3 Total RNA extraction from compartments and whole infected mosquitoes

Female adult stages of *Anopheles* from fungal infection experiments were dissected and three individuals pooled for extraction of total RNA using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, this involved placing 200 µLof sterile glass beads (500 µm diameter) in the samples to homogenize the midgut in 750 µLof TRIzol reagent. This was followed by adding

200µLchloroform (Sigma Aldrich) and incubating samples at room temperature for 5mins. The homogenates were vortexed, centrifuged at 13600rpm for 15mins at 4°C. The upper aqueous phase was transferred to a new 1.5mL eppendorf tube. To the precipitate RNA samples, 300 µLof chilled isopropanol were separatelyadded and centrifuged at 13600rpm for 10 mins at 4°C and followed by two rounds of centrifugation at 13600rpm for 5 mins at 4°C with 200 µLof chilled 75% molecular grade ethanol. The RNA pellets were re-solubilised by adding 50 µLRNase-free water. Quality and purity were assessed using Nanodrop spectrophotometer (Thermo Scientific, USA) based on the $A_{260/280}$ ratio. The RNA was either used immediately for cDNA synthesis or stored at -80°C.

3.3.4 cDNA synthesis and RT-PCR on immune genes to ascertain fungal effect on immunity

To determine the expression levels of stress (CYP6M2 and hsp70)genes, Toll (Tep1, Lrm1, Fbn9 and PGRPLC)genes, and IMD pathway genes (cecropin1, defensin and relish2 gene) (sequences shown in **Appendix 1**) fungus infected and uninfected controls were used to extract RNA, which was treated with DNasae I (Ambion) to degrade any residual DNA that could have contaminated the samples.FIREScript RT cDNA synthesis kit (Solis BioDyne) was used for cDNA synthesis using supplied oligo (dT) primers following the manufacturer's instructions (Solis BioDyne). Preparing a 20µLreaction mastermix involved adding 2 µL 10× RT buffer with DTT, 0.5µL dNTP mix (20mM), 1µL random primers (100 µM), 0.5µL oligo dTT8 primers, 1µLFireScript

RT, 0.5 RiboGrip RNase, and topping up to 10μL with nuclease free water. Master mix and RNA templates were added aliquoted in equal volumes (i.e. 10μL master mix and 10μL RNA sample) to make cDNA of 20 μL. Measurement of gene expression levels involved using the cDNA in duplicates to perform real-time RT-HRM-PCR using 5× HOT FIREPol®EvaGreen qPCR Mix Plus (no ROX) as described in the manufacturer's instructions. Delta ct and efficiency were obtained from the machine and used to estimate relative densities of gene expression. Gene expression was calculated as the ratio between target gene ct and ribosomal host gene ct. All cDNA reagents were procured from Solis BioDyne (Estonia). All the primer sequences used for qPCR are shown (**Appendix 1**).

3.4 Determining the effects of selected isolate on *Plasmodium falciparum* and fungus genomic features

3.4.1 Membrane feeding assay

Gametocytemic blood collected from voluntary donors during a parasitological survey carried out in local primary schools around Mbita in Western Kenya from April 2017 – August 2018 was used for the membrane feedings. Briefly, *P. falciparum* gametocyte carriers were identified by certified medical technologists; consent sought from guardians, and offered transportation to *i*-TOC for blood donation and subsequent provision of antimalaria drugs. Each willing volunteer was requested to donate 500 mL of blood, which was drawn by venipuncture, centrifuged, and filled in a membrane feeder's pre-warmed mini glass (Chemglass Life Sciences) covered with stretched parafilm and maintained at 37°C as previously described (Boissière *et al*., 2012). To

avoid variability between infection rates, each donor blood was treated as a single experiment. Plastic cups containing100 females previously sugar-starved for 5 hours (*Anopheles gambiae*, *An. arabiensis*from semi-field and *Anopheles gambiae* complex field mosquitoes initially collected as pupae (all 3-4 day old)) were bloodfed through a parafilm membrane for 30-40 minutes. Sorting of engorged females was done and placed in standard cages maintained at 27.5 ± 1.5°C, 70-80% relative humidity and supplied with 6% glucose solution *ad libitum*. These mosquitoes were dissected into two (head/thorax) for ELISA sporozoite quantitation and abdomen for fungal screening after two weeks post infection with parasite.

3.4.2 Sporozoite quantification by ELISA

Sections of head and thorax of female mosquitoes previously supplied with gametocytic blood were used for sporozoite ELISA as previously described by Wirtz(Wirtz *et al*., 1989). Briefly, individual mosquitoes sections were homogenized in 50µL blocking buffer: NP40 (1:1) and incubated for 60 mins before transferring to -20°C overnight. A 96 well plate was coated with 50µL MAb capture antibody containing 0.5 mg/mLPf2A10-CDC. Following overnight incubation at room temperature, Mab was washed and dried on paper towel before adding blocking buffer (200µL). This was followed by incubation at room temperature for 60 mins. Samples from the -20°C incubation were thawed and positive controls serially diluted with blocking buffer. A sample aliquot of50µL was added to each well excluding the first and second rows preserved for positive and negative controls. A negative control consisted of male

mosquito from clean colonies of insectary mosquitoes prepared in solution of blocking buffer and NP40. *Plasmodium* antigen was allowed to bind to the capture antibody by incubating for 2hr at room temperature. Content of each well was discarded and washed four times with BioTek ELx50® ELISA washer using 200 µL PBS–Tween. To avoid cross contamination between wells, distilled water was used for rinsing aspirators. Plates were dried by inverting on fresh paper towels several times followed by addition of freshly prepared 100µL MAb peroxidase conjugate 0.5mg/mLperoxidase labelled Mouse Ab Pf2A10-CDC. The conjugate had been pretested and confirmed to develop colour when mixed with substrate previously prepared separately. Plates were shielded from direct light by covering with aluminium foil, re-incubated at room temperature for one hour followed by washing with PBS-Tween solution, and dried on paper towels. ABTS® Substrate was added (50µL) and plates wrapped in aluminium foil and incubated for 30 mins at room temperature. Using a Gen 5® software, optical densities were read at 405 nm on BioTek® ELx808® ELISA reader. To determine quantitative cut-offs, mean of negatives were obtained for each plate, and pooled to obtain corrected absorbance by subtracting pooled means from the mean negative of each plate. Corrected values were substracted/added to all optical distance reading to normalize values in all plates. Positive controls were serially diluted to obtain absorbance for generating standard curves. Sporozoites were quantified using the equation obtained from the standard curve, each donor had three replicates.

3.4.3 DNA extractions and PCR of abdomens of *Plasmodium* exposed samples

Abdomen sections were subjected to DNA extraction using protein precipitation method (modified Qiagen protocol) described previously (section 3.2.7). DNA samples were used for estimating therelative densities of fungi against the mosquito host ribosomal S7 protein(**Appendix 1**). HRM RT-PCRwas undertaken as previously described (section 3.2.8) and relative densities calculated based on efficiency and ct values generated by the machine for the individual samples.

3.4.4. Preparing fungus samples for draft whole genome sequencing

Whole genome sequencing was undertaken to unravel possible role of *Leptosphaerulina* sp on their host*Anopheles gambiae* complex. A total of 5 pure isolates of *Leptosphaerulina* sp was used for extraction of quality DNA using hot phenol extraction protocol based on a modified CTAB method. Briefly, 100µL of beads was added to samples in 2mL tubes to homogenize on a Bead-beat 1x45sec, 6000 rpm. Working on ice, they were mixed with 640µLof CTAB and 2µLof β-Merc-EtOH and mixed by inverting for 30sec. Tubes were spun down at 2000rpm for 30sec before adding 860µL of phenol. The resulting mixture was mixed by vortexing for 10sec and incubated in water bath at 65°C for 6 mins. During the incubation, tubes were mixed by shaking. Samples were chilled on ice for 5mins and divided in two portions (2 ×700µL), each portion was transferred to a fresh tube containing 400µL chloroform taking care not to plug the pipette tip with beads. In a centrifuge set at room temperature, these samples were spun at 13000 rpm for 10mins. The resultant aqueous phases (~500µL)

wereseparately transferred into a new tube containing 500µL Phenol: Chloroform: Isoamyl alcohol (25:24:1) and mixed by inverting the tubes 100 times. Samples were centrifuged at 13000rpm for 3mins at room temperature. The aqueous phases were carefully transferred into new tubes and 500µL of chloroform added and mixed by inverting for 30secs. These tubes were spun at 13000 rpm for 3 mins at RT before transferring the aqueous phase (~300µL) to new tubes. DNA was precipitated by adding 900µL 100% EtOH previously chilled at –20°C. These tube contents were mixed by inverting and incubated at -80°C overnight. The following day, the outsides of tubes were cleaned with RNase away and allowed to equilibrate on ice for 5mins and spun in a centrifuge set at 4°C at 13000rpm for 30mins. These DNA samples were washed with 1mL 70% EtOH at 13’000 rpm for 15 mins at 4°C. Ethanol was pipetted off the tubes and DNA pellets dried at 37°C for 5mins. Subsequently, the DNA samples were solubilizedby adding 100µL RNase-free water and flicked (or quick vortex) and incubated on ice while flicking to enhance elution. These resultant DNA samples were incubated at 64°C for 5 mins and incubated on ice before storing in -80°C awaiting quality assessment.

3.4.5 Quality and quantity check of genomic DNA

All samples were resolved on 2% agarose gel stained with 3% ethidium bromide by loading 2µL of whole genomic DNA and visualizing them under UV-transilluminator. Using Thermo Scientific™NanoDrop™ 2000c Spectrophotometer, the quality of same gDNA samples (1µl) was assessed. Samples with an OD ratio $_{260/280}$

>1.8<2.2 and a distinct band upon resolution on 2% gel were selected for shipment to South Korea ed for Illumina sequencing (Macrogen Co., Ltd., South Korea).

3.4.6 Illumina sequencing with TruSeq Nano DNA Kit

Before samples were used for Illumina sequencing, quality control was undertakento check the quality of the 5 DNA samples by resolving them on a 1% agarose gel and undertaking NanoDropspectrophotometry analysis to estimate the concentration. Samples that qualified based on concentration >200ng/uL, and formed a sharp band proceeded to the library preparation step. Library construction begins with random fragmentation of the genomic DNA (gDNA)and ligation of 5' and 3' terminal ends of the fragments with TruSeq adapters. These modified DNA samples are then subjected to PCR and gel purification. To undertake sequencing using a TruSeq Nano DNA kit, the DNA was loaded into the flow cell, shreading samples, adapters enriched by PCR according to the manufacturer's instructions. Final stages involved pooling groups of eight per lane and running them with paired-ends generating reads of approximately 150bp. The entire process was undertaken on a HiSeq 2000 platform at Macrogen (South Korea).

3.4.7 Quality assessment of the raw reads (FASTQC)

Raw sequences of high throughput sequencing were received from Illumina platform and downloaded. For quality control checks, a FastQC(http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used. The

program provides an output report [$ ~/FastQC/fastqc*.fastq] that included overrepresented sequences, duplication levels, sequence length, GC content, average read quality, and per-base quality in HTML report. Reads below the blue section (~30) were deemed of poor quality and trimming was necessitated.

3.4.8 Trimming of reads with trimomatic and fastqc

Reads were trimmed to remove adapters and repeated nucleotides that lowered the quality of sequence reads. To undertake trimming a timmomatic program was used as shown below.

```
Module load trimmomatic-0.35

trimmomatic PE Lepto_B_R1.fastq.gz Lepto_B_R2.fastq.gz
Lepto_B_R1_Trimmed6_paired.fq.gz
Lepto_B_R1_Trimmed6_unpaired.fq.gz
Lepto_B_R2_Trimmed6_paired.fq.gz
Lepto_B_R2_Trimmed6_unpaired.fq.gz ILLUMINACLIP:TruSeq3-
PE.fa:2:65:20:2:keepBothReads LEADING:30 TRAILING:30
SLIDINGWINDOW:4:30 MINLEN:45 &

Quality encoding detected as phred33

Input Read Pairs: 84885026 Both Surviving: 73826485 (86.97%) Forward
Only Surviving: 8695241 (10.24%) Reverse Only Surviving: 1083928
(1.28%) Dropped: 1279372 (1.51%)

TrimmomaticPE: Completed successfully
```

These codes instruct the program to remove the sequencing adapters [TruSeq3-PE.fa:2:65:20:2] eliminate the N bases or the leading low quality (below quality 30 – LEADING 30) as well as remove N bases, trailing low quality (TRAILING 30), and scan each read based on 4-base wide sliding window trimming the sequence when there

is a drop in average quality to 30 (***SLIDING WINDOW***: 4:30). It also commands the program to drop reads whose base length is below 45 nucleotides (MINLEN: 45). The preferred average quality was 20

3.4.9*Denovo* assembly with Abyss and Spade

A *de novo* assembly was undertaken by comparing two pipelines namely Abyss and Spade. These are alternative to velvet. Abyss command [abyss-pe] was used for the assembly of trimmed paired-end reads. The command was followed by the number of processors assigned [e.g. np=8 refers to assigning 8 processors], kmer, which signifies the parameter of the size of kmer was selected [k=31], and the name of the output file [name=Abyss_Ass_kmer31_lepto]. Other parameters included the path for the forward/reverse trimmed reads [in], and the path of singles files [se]. Several iterations were tried based on kmer sizes such as k=33, 35, 41. In summary the commands listed below were used to undertake the assembly.

```
module load abyss/2.1.4

abyss-pe np=8 k=31 name=Sample_Kmer31
in='/Godfreyn/Whole_genome/Lepto/Assembly_Abyss/
Lepto_B_R1_Trimmed6_paired.fq.gz / Godfreyn/Whole_genome/Lepto/Assembly_Abyss/
Lepto_B_R2_Trimmed6_paired.fq.gz'
se='/Godfreyn/Whole_genome/Lepto/Assembly_Abyss/Trimmed_Lepto_s.fastq'

# repeated with different kmer sizes
```

A *de novo* assembly with SPAdes v.3..11.1 algorithms, which is unique because it generates a final assembly based on multiple kmers was used. It automatically selects a list of kmers based on the maximum read length of the trimmed reads used as the input file, and the final assembly has components of each kmer contributing to its assembly. To run the program, spades.py command was used alongside option [--careful], in order to minimize mismatches arising from the contigs. The output file is noted by [-o], while path to the forward and reverse reads are noted by [-1] and [-2] respectively. The single reads path is denoted by [-s], while a list of kmers to be considered by the program could be specified by kmer command [-k]. To undertake SPAdes assembly the following procedures were used.

```
module load SPAdes/3.13.0

spades.py --careful -o SPAdes_out -1
/Godfreyn/Whole_genome/Lepto/Assembly_Abyss/
Lepto_B_R1_Trimmed6_paired.fq.gz -2
/Godfreyn/Whole_genome/Lepto/Assembly_Abyss/
Lepto_B_R2_Trimmed6_paired.fq.gz -s
/Godfreyn/Whole_genome/Lepto/Assembly_Abyss/Trimmed_Lepto_s.fastq

Outup file

/Godfreyn/Whole_genome/Lepto/Assembly_Abyss/Trimmed_Lepto/SPAdes.
```

3.4.10 Assembly statistics and quality control with QUAST

Using multiple pipelines to undertake whole genome assembly is paramount to ensure comparison of essential parameters. Of interest are parameters thatinclude number of contigs, N50, and the total length. A good assembly is one that has high N50 value, total length that tallies with the specie requirement, and low number of contigs. Therefore, QUAST is an assessment tool used for analyzing these parameters on iterations of Abyss assembly and the output of SPAdes assembly. The module runs as follows.

```
module load quast/5.0.2

# ABySS statistics

quast.py /Godfreyn/Whole_genome/Lepto/Assembly_Abyss/ABySS/Sample_Kmer*-scaffolds.fa -o ABySS

# SPAdes statistics

quast.py
/Godfreyn/Whole_genome/Lepto/Assembly_Abyss/Assembly/SPAdes/scaffolds.fasta -o SPAdes
```

3.4.11*Ab-initio* gene prediction with Augustus platform

Using emboss, the contig file was converted to a single fasta file and glimmer loaded for gene prediction as shown

```
module load glimmer/3.02
```

g3-iterated.csh joined_file.dnajoined_file

glimmer3totab.pl joined_file.predict>   joined_file.predict.tab

Augustus platform was also used for gene prediction as shown below (Hoff and Stanke 2013). This platform predicted protein coding genes and provided output as shown below to obtain the "output_lepto_prediction.codingseq" that contained CDS DNA fasta sequence of predicted genes.

A BLAST2GO was used to undertake gene ontology annotation with the aim of describing the cellular, molecular, and biological functions associated with the predicted genes (Ashburner et al. 2000). Briefly, enzyme commission number cut off was set at 55; weight of 5; and hit filter 500. To identify conserved domains, protein coding genes were annotated on the InterPro database as described previously (Mulder and Apweiler 2008). Obtained gene systems and categories were represented in tabulated formats.

3.5.Establishment of a self sustained propagation strategy for field dissemination of the novel fungal symbiont of *Anopheles* mosquitoes

3.5.1 Field collection of *Anopheles arabiensis*

To establish mosquitoes harbouring*Microsporidia MB*, resting gravid and engorged female mosquitoes were collected through manual aspiration. Collections were undertaken in Ahero(−34.9190W, − 0.1661N) Kenya between 0630hr and 0930hr from

February 2020 to March 2021 using electric torches/lights and aspirators. Collected females were placed in large cages supplied with 6% glucose and transported to *icipe*-Thomas Odhiambo Campus (*iTOC*) for processing.

3.5.2 Mosquito processing and identification

Wild collected females were placed in individual microcentrifuge tubes supplied with wet 1 cm x 1cm Whatman filter paper for oviposition, the mosquitoes were maintained in an insectary at 27.5 ± 1.5°C, humidity 68-80%, and 12L: 12D light cycle. They were monitored daily for egg laying. After 3 days, females that had laid eggs were prepared for whole mosquito DNA extraction using protein precipitation method (Puregene, Qiagen, Netherlands). PCR was undertaken using the method recently described (Herren *et al*., 2020) to screen for the presence of *Microsporidia MB* in the mosquito as well as mosquito species identification. The abundant species of the *An. gambiae* species complex from the site of collection was confirmed by PCR (Federica Santolamazza, 2008). Eggs from each female were counted under a compound microscope using a paint brush and then dispensed into water tubs for larval development at 30.5 °C and 30-40% humidity. The offspring of *Microsporidia MB* positive field caught female mosquitoes were maintained in medium sized troughs and placed in temperature and humidity regulated incubators. *Microsporidia MB* uninfected controls were obtained from the *An. arabiensis* colonies at *i*-TOCMbita colony. Tetramin^{TM} baby food was used for rearing the larvae.

3.5.3 Cage experiments

To ensure that females and males F1 used were virgin, they were sexed at pupae stage after visual examination of the terminalia and placed in the same sex cage. Emerging insectary virgin females were placed in the same cage with virgin F1 male from the infected lines. A separate control experiment of uninfected virgin F1 or insectary males with virgin insectary female was included. These combinations were maintained under insectary conditions in 30 x 30 x 30 cages by supplying them with 6% glucose for 36-48 hrs before F1 males were screened *post hoc*to confirm *Microsporidia MB* infection status. Virgin females were either allowed to acquire a blood meal before mating or bloodfed after mating. These were placed in individual tubes with wet filter papers to induce ovipositioning. In the experiments where sex could not be used to differentiate male and female mosquitoes, dyes (red and blue) were used to mark mosquito wings and indicate donors and recipients.To investigate the efficiency of horizontal transmission and the importance of *Microsporidia MB* intensity, additional cages with single *Microsporidia MB* infected donor males and 10-50 virgin *Microsporidia MB* uninfected recipient females were established and maintained for 2 days. Serial mating involved reusing males in several cages to increase chance of transferring symbiot. This involved transfering F1 male from one cage of 10 females to a second cage of 15 females. Upon completion of the transmission experiment all *An. arabiensis* mosquitoes were screened by qPCR to determine the infection status and intensity of donor and recipient *An. arabiensis*. To investigate mating rates and the link

between acquiring *Microsporidia MB*and female insemination status, the presence of sperm in *An. arabiensis* females maintained in some of the cages with *Micrsporidia MB* infected males were examined by the dissection of spermathecae and scoring sperm presence.

3.5.4 Determining whether*Microsporidia MB* acquired horizontally undergoes vertical transmission.

In the experiments where a single male was maintained with 10-50 females, all surviving females were supplied with a blood meal and placed in individual tubes with wet 1 cm x 1 cm filter papers to induce oviposition. Upon laying eggs, all females were screened for *Microsporidia MB* by qPCR. The offspring from *Microsporidia MB* infected recipient females were reared until they were 1-2 day old adults and then screened to determine if vertical transmission had occurred.

3.5.5 Determining whether*Microsporidia MB* invades multiple host tissues and proliferates in the reproductive tracts

Microsporidia infected *An. arabiensis* adults were used to establish tissue tropism of the symbiont. This involved conducting dissection on males 3-5 days post emergence. Using sterile pins, the midgut and gonads were separated from the whole body (carcass). These tissues together with the carcass were used for screening the presence and intensity of *Microsporidia MB* using qPCR. Quantification of

Microsporidia MB in the male seminal fluid was carried out on different *An. arabiensis* specimens.Briefly, 10-12 day old males were decapitated and used immediately in forced- mating experiments with virgin females (detaild procedures at www.mr4.org). Upon successful copulation, seminal secretions produced by the male were collected with a pulled capillary tube and transferred to a 10uL 1xPBS and placed under ice. Genomic DNA was collected as previously described prior to *Microsporidia MB* quantification by qPCR.

3.5.6Determining whether*Microsporidia MB*proliferates in the host reproductive tissues

Dissections of male gonads of both F1 *Microsporidia MB*-infected and uninfected *An. arabiensis*was used for microscopy. This involved fixing these tissues in 4% Paraformaldehyde (PFA) solution for 30 minutes before undertaking a series of three quick wash with the PBS-T. Clean samples were stained with Syto-9 constituted in PBS (0.1mM) for duration of 1 hour. These were followed by two steps of washing before the products could be mounted on a slide for visualization using a Leica SP5 confocal microscope (Leica Microsystems, USA). Resulting images were analyzed with the ImageJ 1.50i software package(Schneider et al., 2012).

3.5.7 Molecular detection of*Microsporidia MB*

Microsporidia MB specific primers recently described (Herren *et al*., 2020) were used to detect infections in the *Microsporidia*-infected F1 male mosquitoes that were

used in mating *posthoc*. If a F1 male(s) was found to be positive, its partner insectary females from the same cage were screened for the presence of infection. Briefly, a 10 µL PCR reaction consisted of 2 µL HOTFirepol® Blend Master mix Ready-To-Load (Solis Biodyne, Estonia, mix composition: 7.5 mM Magnesium chloride, 2 mM of each dNTPs, HOT FIREPol® DNA polymerase), 0.5 µL of 5 pmol μL^{-1} of MB18SF/ MB18SR primers, 2 µL of the template and 5 µL nuclease-free PCR water was undertaken. The PCR conditions used were; initial denaturation at 95°C for 15 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 62°C for 90 secs and extension at 72°C for a further 60 secs. Final elongation was done at 72°C for 5 min. The scores and densities of infected F1 males and their partners from insectary acquiring infection were determined by qPCR assay using*Microsporidia* specific (MB18SF: CGCCGGCCGTGAAAAATTTA and MB18SR: CCTTGGACGTGGGAGCTATC) primers. These were normalized against the *Anopheles* ribosomal S7 host gene (primers, S7F: TCCTGGAGCTGGAGATGAAC and S7R GACGGGTCTGTACCTTCTGG)(Dimopoulos et al., 1998), and presented as relative densities of the target against host gene.

3.5.8 Statistical analysis

Statistical analyses of fungi growth parameters, prevalence, densities, fitness parameters, immunity, and *Plasmodium* assays were performed using either GraphPad Prism 7.01 or R statistical software version 3.2.3 (Core Team R, 2017). Where necessary, data were log transformed and logistic models fitted to estimate odds ratio

between parental infections. Prevalence of melanosis was presented as percentages while log transformed relative densities were compared with sporozoite densities. A two-tailed unpaired t-test was used to compare unpaired data values with a normal distribution (*Plasmodium* data). Where data distributions were non-normal, the two-tailed Mann–Whitney U test was used (e.g. immune assay data) or paired spearmans rank test (compare intensities between paired donor and recipient *Microsporidia MB* intensity). To establish if donor *Microsporidia MB* intensity or number of available mates affected the odds of *Microsporidia MB* transmission, a genitive binomial generalized linear model was fitted. Survival curves were produced using GraphPad Prism 7.01 for Windows (GraphPad Software, San Diego, USA). Data are presented as Mean ± SEM of experimental replicates. The *P*-values of $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ and $****p < 0.0001$ were considered to be statistically significant.

CHAPTER FOUR

RESULTS

4.1 Isolation of fungi from *Anopheles* mosquito developmental stages obtained from semi-field environment.

4.1.1 Mycobiomes of *Anopheles* are abundant in the gut and reproductive tissues

The fungal isolates (25) obtained by resolving PCR amplicons were of different sizes indicating they belonged to different gerera (**Figure 4.1**), and on sequencing these amplicons it was observed that themost dominant genera were;*Aspergilus*, *Penicillium*, *Periconia*, *Epicoccum*, *Leptosphaerulina*, *Hyphopichia*, *Alternaria*, *Lichtheimia*, *Cladosporium*, *Hasegawazyma*, *Marasphaerium*, and *Dothideomycetes*sps (**Table 4.1**).Thesefungal isolates were common in the guts and reproductive tissues of adult *An. gambiae*hostswith the distribution in tissues summarized as follows: ovary (11), midguts (11), pupae (5), whole mosquito (4), and larvae (4) as shown (**Figure4.2**). Notably, eight of theisolates namely *Penicillium*, *Periconia*, *Epicoccum*, *Leptosphaerulina*, *Alternaria*, *Lichtheimia*, *Cladosporium*, and *Hasegawazyma*sps resided in both the midguts and ovaries(**Figure4.2**).

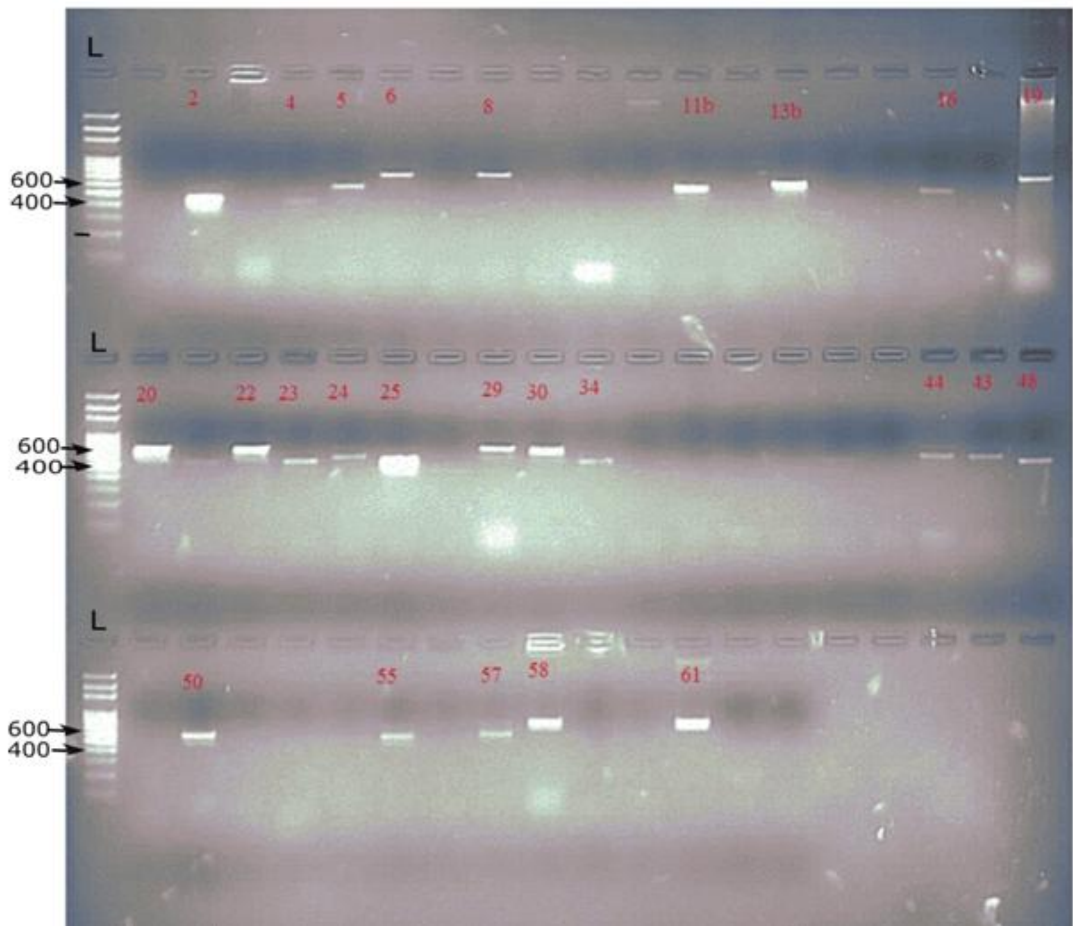


Figure 4.1: PCR amplification of 18s rDNA for fungal isolates.

Pure cultures of the isolates were amplified using rDNA-ITS gene primers (ITS1/ITS4) and resolved in 2% agarose gel. These amplicons were purified and prepared for sequencing. L represents a 1kb ladder while fungal isolates prepared for sequencing are represented by numbers (2, 4, 5, 6, 8, 11b, 13b, 16, 19, 20, 22, 23, 24, 25, 29, 30, 34, 44, 43, 48, 50, 55, 57, 58, and 61). The expected band size was ~600bp.

Table 4.1: Fungal isolates, their related taxas from GenBank, percentage similarity, and taxonomic classification.

Isolate Code (GenBank Accession no.)	Related Taxa (GenBank Accession no.)	BlastN (%)	Taxanomic division Phylum
Isolate 2	*Hyphopichia burtonii*(KY103604)	97.3	Ascomycota
Isolate 4	*Hyphopichia sp.* (KY103604)	93.7	Ascomycota
Isolate 5	*Penicillium georgiense*(NR_121325)	96.9	Ascomycota
Isolate 6	*Marasphaerium gattermannii*(HQ322127)	85.5	Ascomycota
Isolate 8	*Lichtheimia hyalospora*(NR_111440)	97.5	Ascomycota
Isolate 11	*Periconia sp.* (LN813031)	99.4	Ascomycota
Isolate 13B	*Leptosphaerulina chartarum*(KJ863505)	99.1	Ascomycota
Isolate 16	*Cladosporium cladosporioides*(KP689176)	99.2	Ascomycota
Isolate 19	*Marasphaerium gattermannii*(HQ322127)	85.7	Ascomycota
Isolate 20	*Marasphaerium gattermannii*(HQ322127)	85.7	Ascomycota
Isolate 22	*Marasphaerium gattermannii*(HQ322127)	85.6	Ascomycota
Isolate 23	*Cladosporium cladosporioide* (KF159973)	99.6	Ascomycota
Isolate 24	*Aspergillus niger*(KX426976)	99.6	Ascomycota
Isolate 25	*Cladosporium sp. Strain LCZ2* (KY643766)	98.6	Ascomycota
Isolate 29	*Marasphaerium gattermannii*(HQ322127)	85.4	Ascomycota
Isolate 30	*Hasegawazyma lactosa*(FJ515208)	77.1	Basidiomycota

Isolate Code (GenBank Accession no.)	Related Taxa (GenBank Accession no.)	BlastN (%)	Taxanomic division Phylum
Isolate 43	*Aspergillus tamari* (NR_135325)	92.5	Ascomycota
Isolate 44	*Fungal sp.* (KY404943)	93.6	Ascomycota
Isolate 48	*Dothideomycetes sp*(KX909022)	96.3	Ascomycota
Isolate 55	*Epicoccumsp*.(KX965725)	99.4	Ascomycota
Isolate 50	*Alternaria alternata*(MF099865)	99.4	Ascomycota
Isolate 57	*Alternaria brassicae*(KU844330)	99.8	Ascomycota
Isolate 58	*Lichtheimia hyalospora*(NR_111440)	97.5	Ascomycota
Isolate 61	*Marasphaerium gattermannii* (HQ322127)	85.7	Ascomycota

Accession numbers are given in brackets.

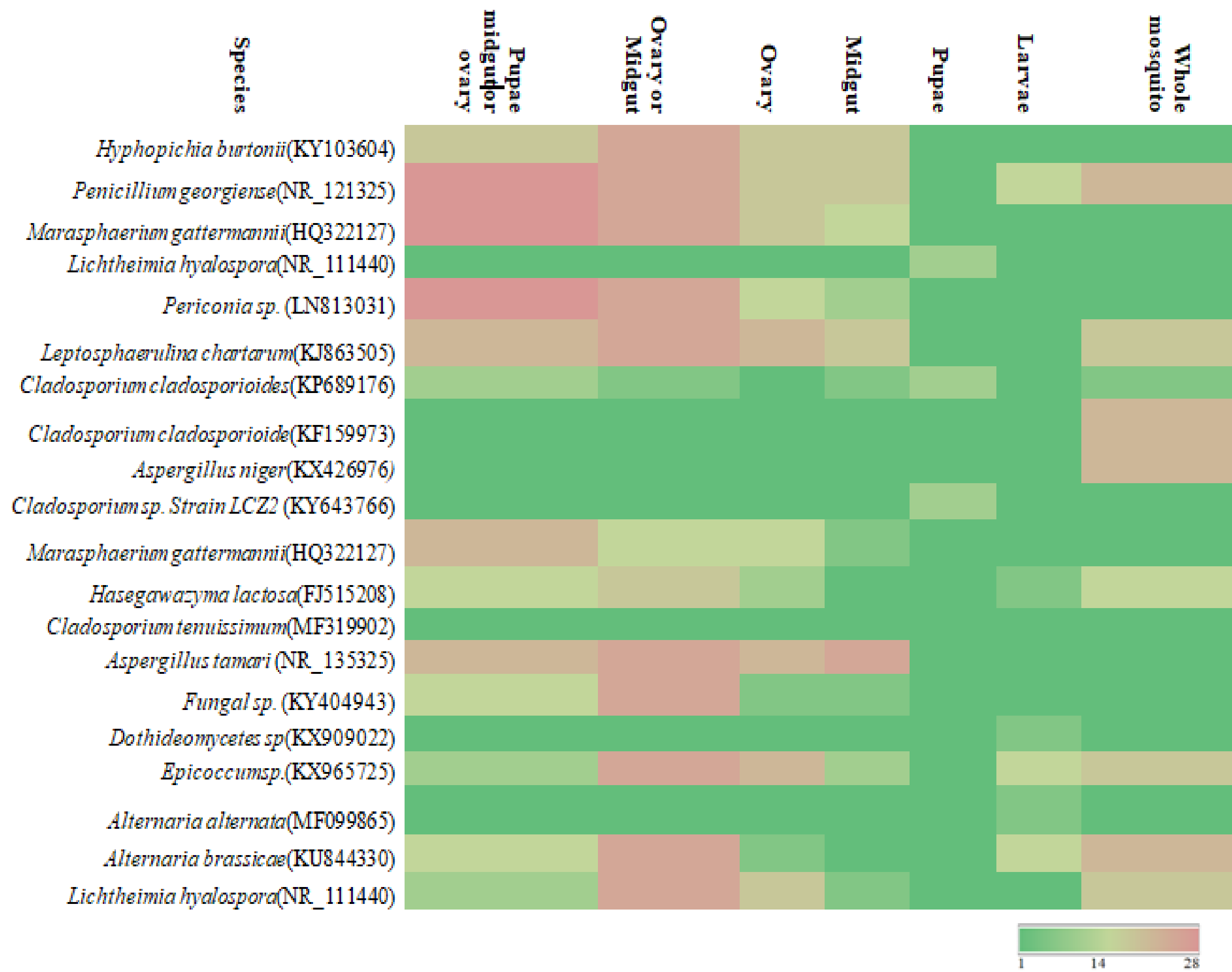


Figure 4.2: A heat map showing the abundance/distribution of fungal isolates from various compartments of adults and immature stages of *An. gambiae*.
The isolated fungi were rich in the guts and reproductive tissues of adults and less rich in the immature stages and whole *An. gambiae* hosts.

4.1.2 Phylogenetic characterization of fungal isolates from *An. gambiae*

Sequences were compared with their closest relatives from NCBI and aligned in Muscle before developing a phylogenetic tree in Geneious software (**Figure 4.3**). Phylogenetic

relationship shows that these isolates were grouped into several clades, where the genus *Hyphopichia* belonged to its clade while genera *Leptosphaerulina*, *Alternaria*, *Periconia*, and *Marasphaerium* belonged to the major clade.

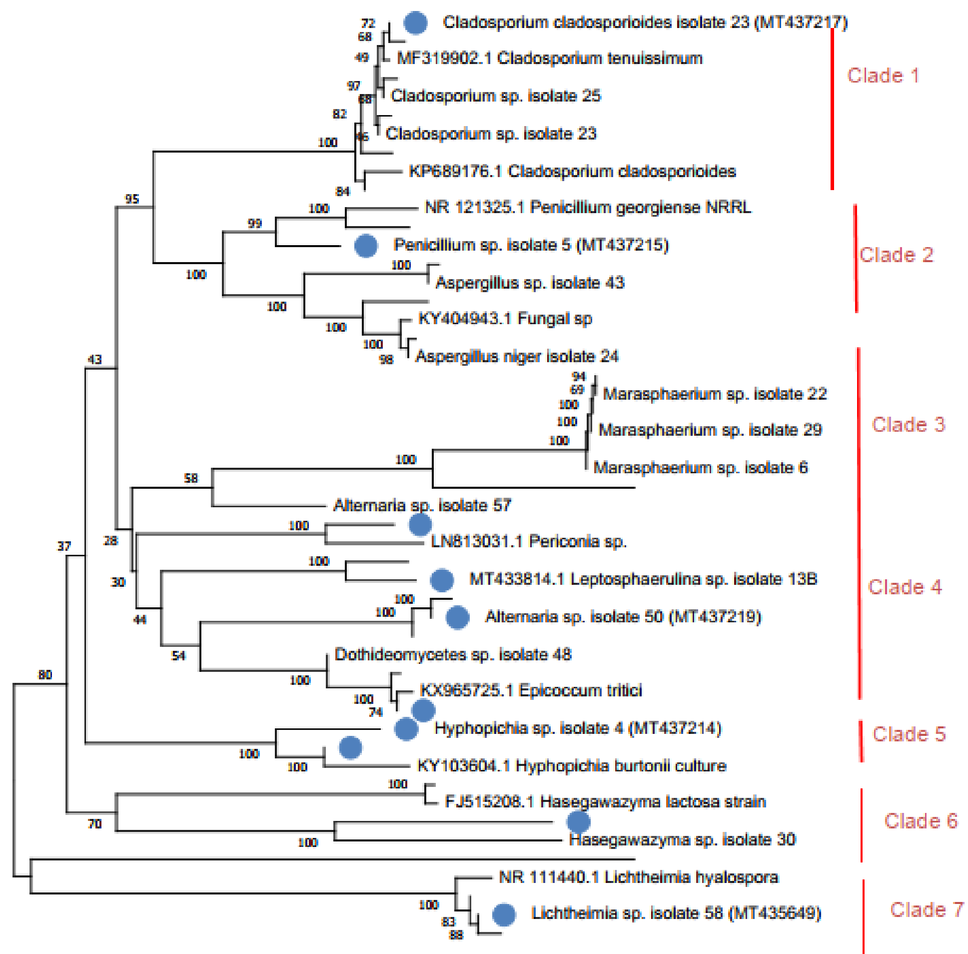


Figure 4.3: Phylogenetic relationship of rDNA-ITS cultured fungal isolates.

The bars represent a scale of 0.02 substitutions per nucleotide. Samples were analysed based on 1000 bootstrapping, and values represented in each branch signify percentage. Sequences of fungal isolates were aligned with their closest neighbors obtained from the NCBI GenBank using Geneious Software. This alignment was used to infer phylogenetic tree using Geneious Software based on maximum-likelihood algorithm. The tree signifies that fungal isolates were in seven major clades as shown.

4.1.3Microscopic evaluation of representative fungal symbionts from *An. gambiae* indicate that they are ascomycetes

Isolates were characterized macroscopically and microscopically and found to form aerial conidiophores that were branched into hyphae forming conidia (**Figure 4.4; Appendix 6**). A representative light microgram of the growth colony and outcome of lactophenol staining of selected fungi are as shown (**Figure 4.4; Appendix 7**).

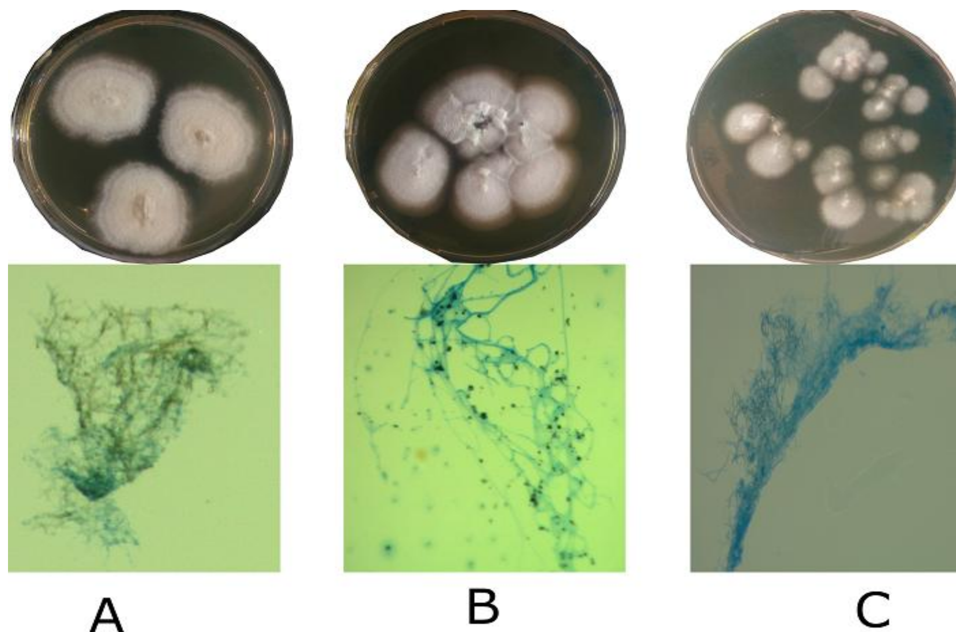


Figure 4.4: Microscopic representation of isolated fungi candidates.

Light micrographs of four-day old fungi on Sabourads dextrose agar and stained with lactophenol blue. **A** represents colony of *Leptosphaerulina* sp front view on SDA plateand stained with lactophenol blue. **B** represents *Lichtheimia* sp. colonies while **C**represents *Hyphopichia* sp. Isolates **A-C** were characterized by the presence of aerial conidiophores and branchedhyphae that form conidia.

4.1.4 Several of *Anopheles* fungal isolates are conserved and recovered by culture and non-culture isolation techniques

A *Wickerhamomycesanomalus* (36%), vittaformacorneae (25%), and uncultured fungus (16%) were identified by high throughput techniques as the core mycobiomes of *An. gambiae* (**Figure 4.5**). Of the identified fungi, 4 species were common in both cultivable and non-cultivable approaches. The most abundant symbiont obtained by both methods were*Lichtheimia* sp., and *Hyphopichia* sp. (**Figure 4.6**).

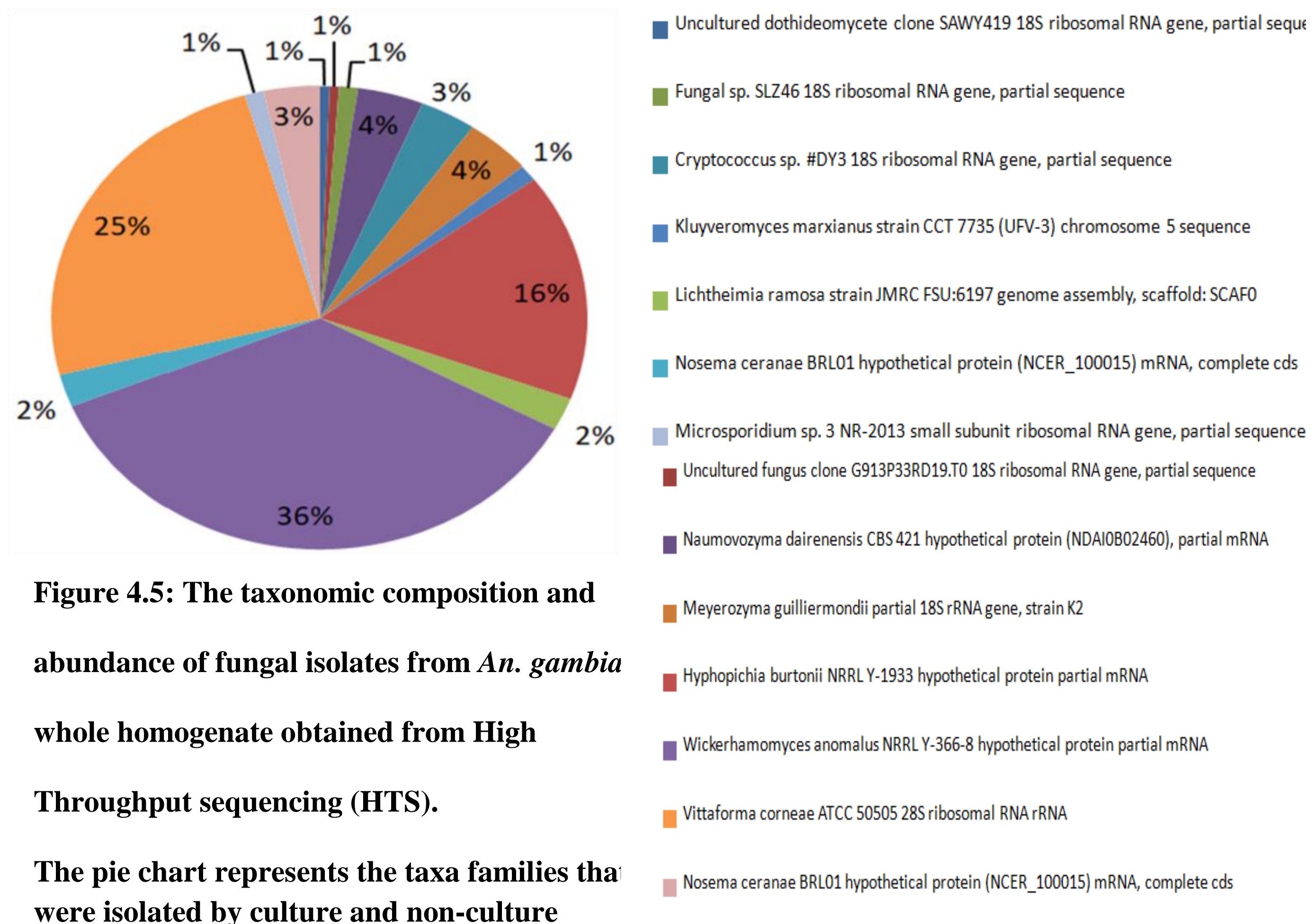


Figure 4.5: The taxonomic composition and abundance of fungal isolates from *An. gambia* whole homogenate obtained from High Throughput sequencing (HTS).

The pie chart represents the taxa families tha were isolated by culture and non-culture

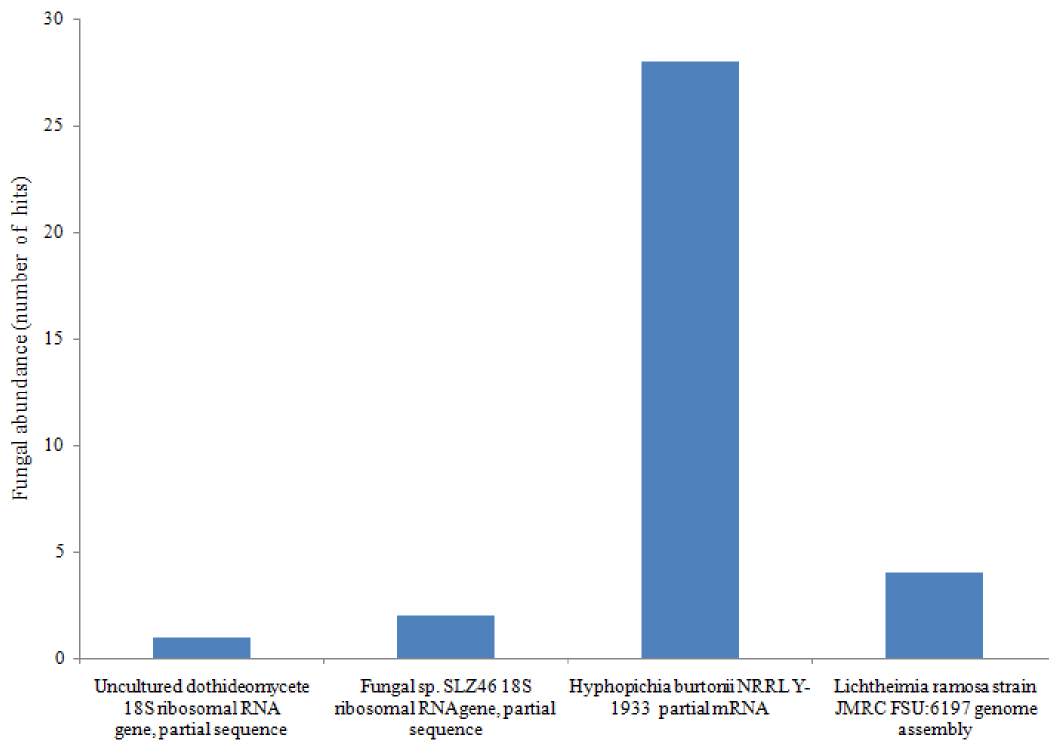


Figure 4.6: Abundance of culture-related symbionts that were obtained by high throughput sequencing.

There were 28 and 4 hits for the genera *Hyphopichia* and *Lichtheimia* fungus respectively. These genera were also obtained through culture method.

4.2 Investigation of the effects of selected isolates on the *Anopheles* host fitness.

4.2.1 The outcome of curing mosquito eggs to establish fungus free *An. gambiae* host colonies

Amplification of rDNA-ITS gene (using ITS1 and ITS4 primers) in the adult and immature stages of *An. gambiae* maintained in the *icipe* insectary showed that the mosquitoharboured diverse fungi (**Figure 4.7A**). Washing of *An. gambiae* eggs with 1% hydrochloric acid in the first generation lowered the burden of fungal infection (**Figure 4.7B**), and cleared the infection in the second generation leading to the establishment of fungus free colonies (**Figure4.7C**) that could be artificially infected by reintroduction of candidate isolates. Per-oral introduction of *Leptosphaerulina*sp to the uninfected laboratory colonies at larval stage (L1) led to the establishment of this fungus in later stages L3/L4 of these larvae (100%), which were retained to pupae (~80%) and adults (>60%) (**Figure 4.7D**).

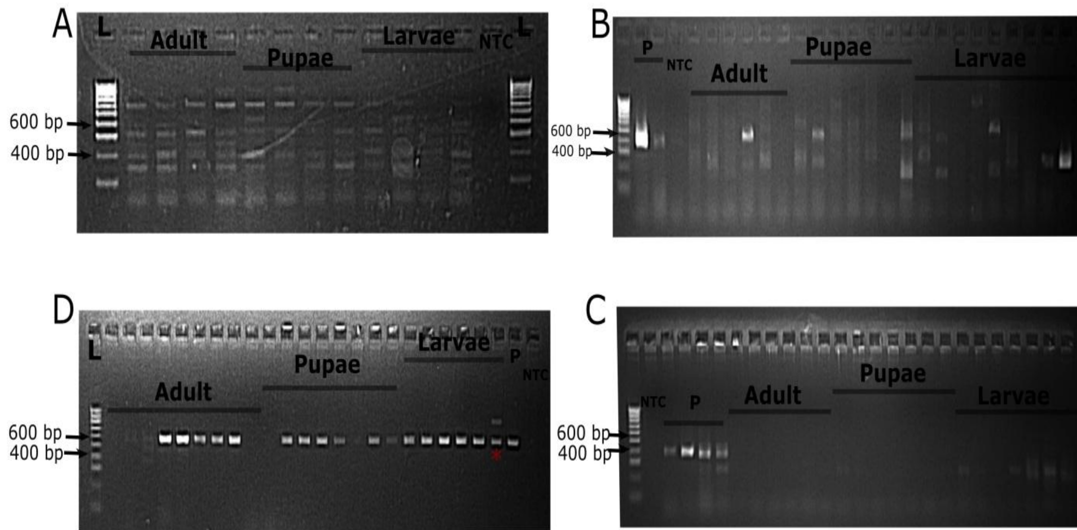


Figure 4.7: Establishment of fungus free colonies of *An. gambiae*.

Fungi in the insectary mosquitoes were screened with ITS1/ITS4 primers for the expected band size ~600bp. These colonies were found to inhabit diverse fungi across all developmental stage (**A**). Cleaning of mosquito eggs with 1% HCl during the first generation was found to lessen the burden of fungi infectionacross larvae, pupae and adult stages. A non-template (**NTC**) and fungus positive controls (**P**) were included in each experiment. (**B**), complete establishment of uninfected colonies were achieved after second generation of clearing with 1%HCl acid (**C**). When these clean (uninfected) mosquito colonies were infected with isolated fungi, 100% of L3/4 larvae carried the infection, which were retained in ~80% of pupae and at least 60% of the adults (**D**).

4.2.2 The occurrence of transstadial transmission of fungi across *An. gambiae* developmental stages

It was observed that fungi introduced at immature stages of *An. gambiae* persisted across the subsequent developmental stages. All the fungus re-introduced in the 1st instars larvae were found to persist to the late stages of larvae (L3/L4) (**Figure 4.8A**). Analysis of relative densities in the 4 days-old adults obtained from the infected colonies indicated that one such isolate named *Leptosphaerulina* sp. persisted to the adult

stage(**Figure 4.8B**). Other fungi such as *Epicoccum and Penicillium, Cladosporium* and*Lichtheimia* which had high infection intensities at L3/L4 were lost at adult stage. Assessment of effective transstadial transmission and monitoring of*Leptosphaerulina* sp. in the *An. gambiae* host indicated that the fungus establishes intimate association with thishost (**Figure4.9A-B**).

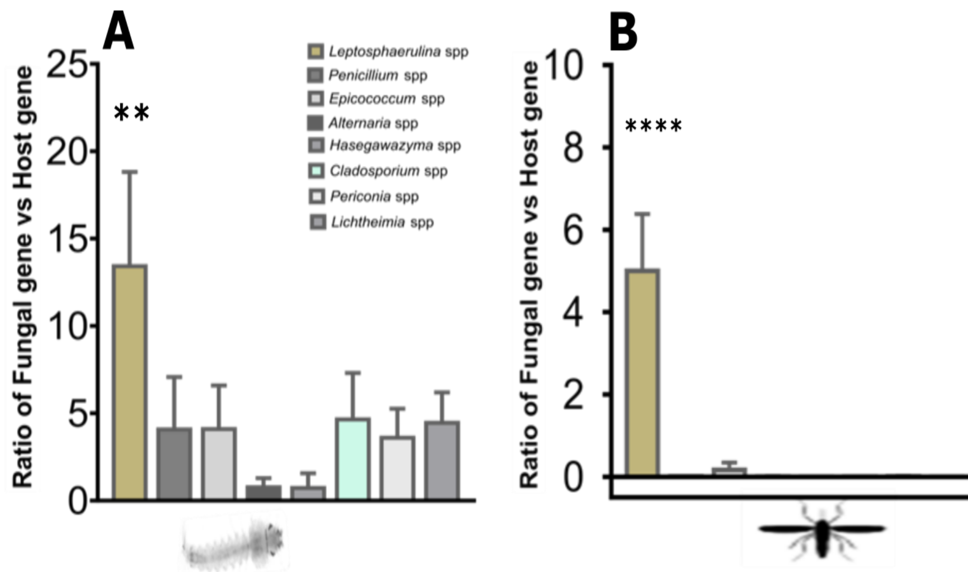


Figure 4.8: Persistence and transmission of *Leptosphaerulina* sp. fungus isolate across developmental stages.

(**A**) Density of *Leptosphaerulina*sp. was found to persist in 90.92% of surviving larvae (N = 128, $p=0.001$) and 66.43% of emerging adult stages (**B**; $p=<0.0001$). These were confirmed by culture re-isolation and re-sequencing methods. The mean bar represents mean±95%CI, while ** represents$p <0.001$, **** represents$p <0.00001$

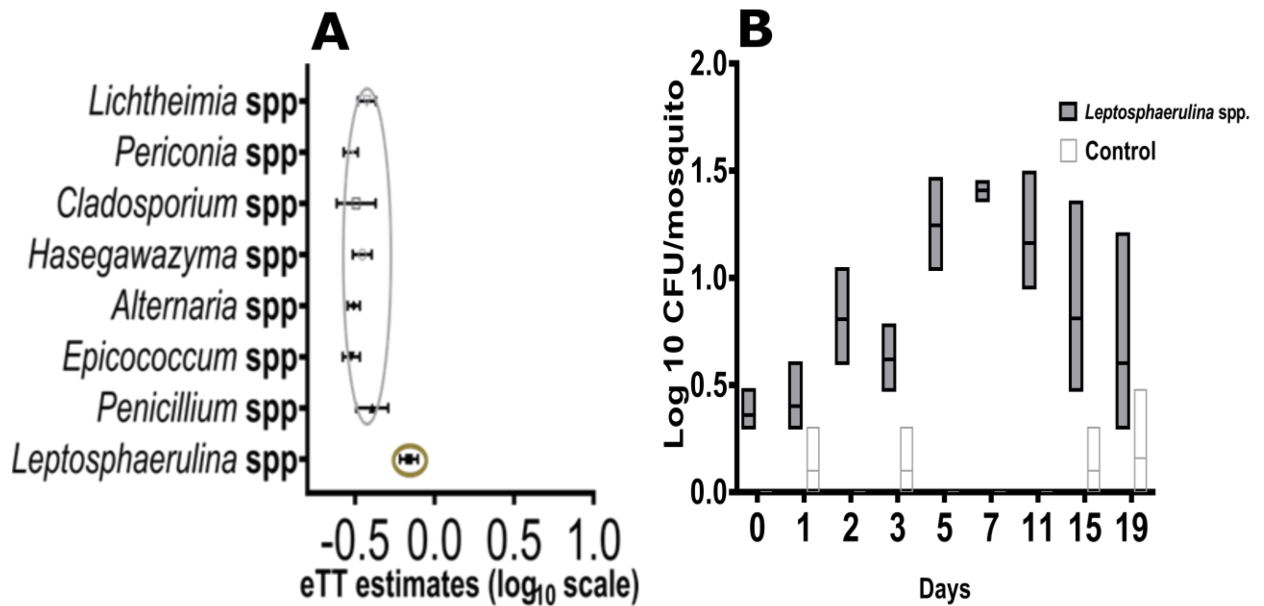


Figure 4.9: Effective transstadial transmission and monitoring infection indicate that *Leptosphaerulina* sp. establishes intimate association with *An. gambiae*.

(**A**) The effective transstadial transmission (eTT estimates) based on transformed log differences between densities of infection between larvae and adult denotes that *Leptosphaerulina*sp. persisted along developmental stages. (**B**) Monitoring *Leptosphaerulina*sp. in female for 19 days by culture and enumerating fungal CFUs on Sabouraud agar laced with 1% HCl and tetracycline indicate the symbiont co-existed with the host.

4.2.3 Transstadial transmission of *Leptosphaerulina* sp. has low virulence on *An. gambiae* host.

To establish whether *Leptosphaerulina* sp. could induce deleterious effects on the host's development, standard fitness costs parameters such as survival of infected larvae and rate of pupation were evaluated. It was observed that *Leptosphaerulina* sp. does not impose deleterious effect on larvae development (**Figure 4.10A**); however, duration

taken by the fungus infected-larvae to pupate was significantly delayed (*p* = 0.0161; **Figure 4.10B**). *Leptosphaerulina* sp. was found to cause a significant reduction in the number of eggs laid by the infected females (**Figure 4.11A**), however, survival of the infected adults did not differ significantly (**Figure 4.11B**).

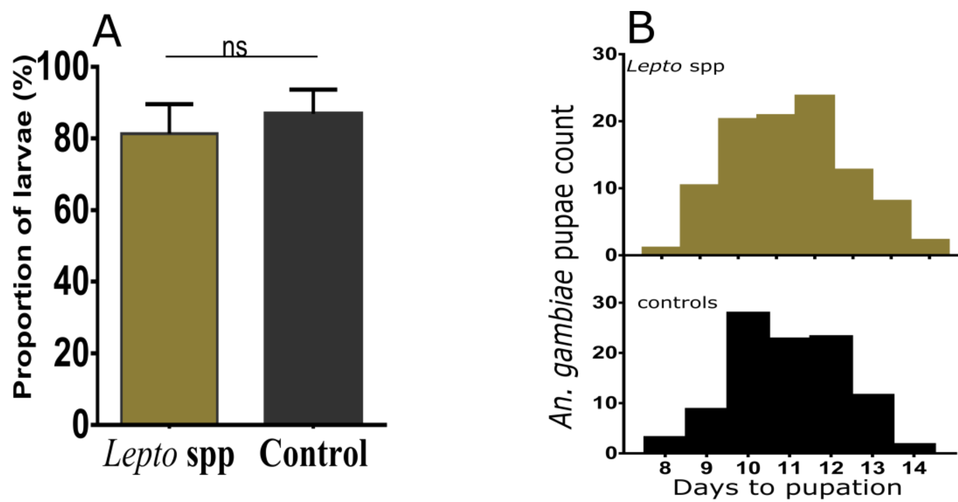


Figure 4.10: *Leptosphaerulina*sp (Lepto sp.) does not affect development of *An. gambiae*.

A) The proportion of larvae pupating upon fungus infection indicate that the isolate did not impose deleterious effect on their development (Mann-Whitney U = 24.5, *p* values = 0.1675). **B**) The duration taken by fungus infected larvae to pupate was significantly delayed (Mann-Whitney U = 15837, *p* = 0.0161). Infections were confirmed *post hoc* using qPCR-HRM assay. The mean bar represents mean±S.E.M, while ns = not significant (*p*>0.05).

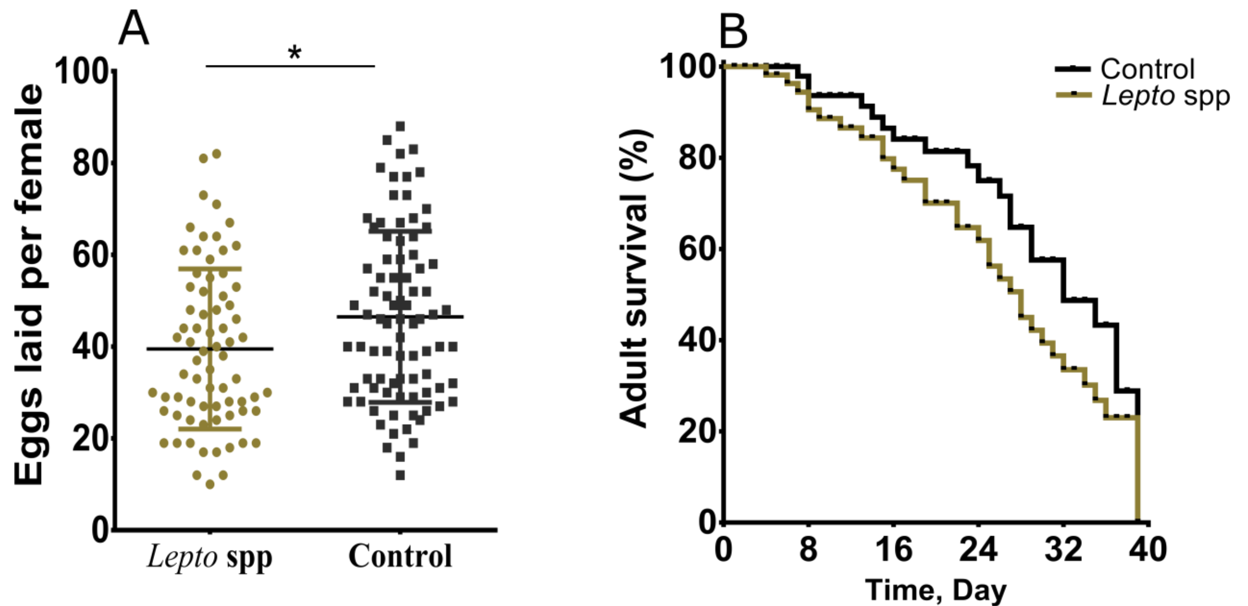


Figure 4.11: Effects of *Leptosphaerulina*sp on fecundity and survival of *An. gambiae.*

A) A reduction in the number of eggs laid by individuals females infected with fungus was noted (Mann-Whitney U = 2758, *p*=0.047). **B**) Adult survival did not differ significantly between fungus infected and uninfected mosquitoes lines (two-sided log-rank Mantel–Cox, $\chi 2$ = 0.6855, df = 1, *p*=0.4077). Infection statuses were confirmed *post hoc* using qPCR-HRM assay. The mean bar represents mean±S.E.M, while * represent p <0.05.

4.2.4 The outcome of establishing whether *Leptosphaerulina* sp. is found in field collected *Anopheles* and *Aedes*mosquito samples

To ascertain whether *Leptosphaerulina* sp. co-existed with field collected *An. gambiae* hosts, larvae and adults collected from Mwea and Huruma (central Kenya), Nyawira, Kirindo, and Ahero (Western Kenya), while Aedes from Kilifi and Malindi(Coastal Kenya) were found to harbour *Leptosphaerulina* sp. with prevalence ranging from 12-

27% (**Table 4.2**). The presence of *Leptosphaerulina* sp in the mosquito hosts from the sampled areas of Mwea and Huruma (central Kenya), Nyawira, Kirindo, and Ahero (Western Kenya), and Kilifi and Malindi (Coastal Kenya) gives an indication that this symbiont is naturally found in the wild mosquitoes with a prevalence range of ~18% (**Table 4.2**). Assessment of the dosages of wild infection indicates that this fungus harboured increased infection status in both the immature and adult stages(**Figure 4.12**) and this finding corroborates persistence properties observed in the laboratory experiments (**Figures 4.8A, B**).

Table 4.2: The prevalence of candidate fungal species in *Anopheles gambiae* sl. mosquito vectors across geographically dispersed regions of Kenya.

	Central Kenya		**Western Kenya**			**Coastal Kenya**	
	Mwea (n=305)	Huruma (n=70)	Nyawira (n=149)	Kirindo (n=93)	Ahero (n=429)	Kilifi (n=97)	Malindi (n=102)
Prevalence of Lepto (%)	22.62	27.14	18.12	12.9	16.55	18.56	11.76

Lepto species naturally co-exist with major malaria vector are moderately prevalent

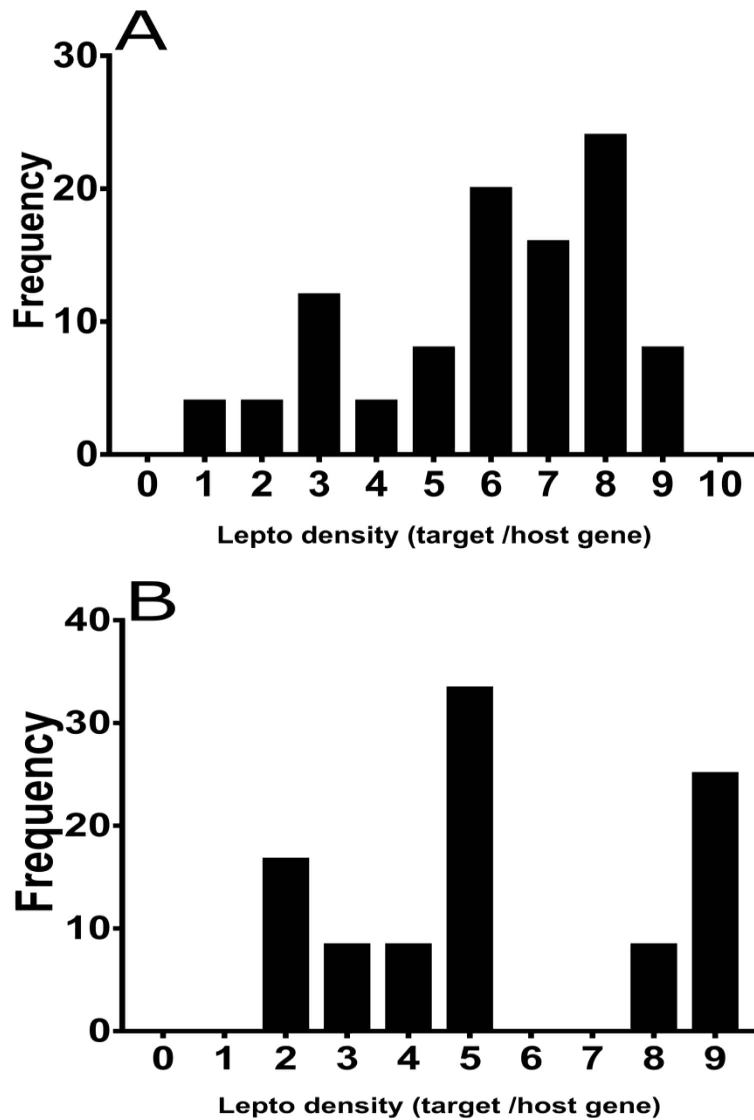


Figure 4.12: Variation of *Leptosphaerulinasp* densities in field caught mosquito samples from Mwea location.

Relative densities of *Leptosphaerulinasp* in field Mwea samples indicate thatlarvae were more likely to harbour fungus infection (**A**, consisting of 25 pools of five) and these persisted to adult stages (**B**, made of 12 pools of 5). These were assayed using qPCR-HRM and expressed as the ration of Lepto521f/896r fungal gene against host ribosomal gene.

4.3 The outcome of the mechanisms utilised by the *Leptosphaerulina* to establish within the *Anopheles* populations.

4.3.1 *Leptosphaerulina*sp induces melanotic pathology that is maintained throughout the *Anopheles gambiae* development stages

Striking phenotypic lesions were observed in live and diseased mosquitoes. These melanotic aggregates were either diffused or localized in distinct compartments of larvae, pupae, and adult stages (**Figure 4.13A-C**). Notably, variations in lesions were also observed in the thorax of the infected larvae and pupae (**Figure 4.13D-E & G-H**). The presence of these aggregates in partially dissected adults (**Figure 4.13F**), and re-isolation of the fungus through culture method suggest the establishment of persisting infection in *An. gambiae* (**Figure 4.13 X1-X2**). The prevalence of melanotic pathology was high in the larvae abdomen (53.75% ±4.11), thorax of pupae (42.75±4.211) and the abdomen of adults (59.75%±7.273) (**Figure 4.13Y**). This prevalence remained relatively constant irrespective of the number of mosquitoes exposed to the fungus. The severity of infection varied between individuals; however, we could spot trails of melanotic aggregates in most individuals exhibited at different body parts (**Figure 4.13A-H**). Monitoring of the fungus in the infected adults reveals that *Leptosphaerulina*sp. had preference for the hosts' midgut (**Figure 4.14A – B**), and re-infection of adults with melanized tissues lessened midgut colonization (**Figure 4.14C**). Notably, comparison of direct and indirect inoculation indicates that increased fungus colonization of the midgut was observed when funguses were inoculated directly at larval stage (**Figure 4.14C**).

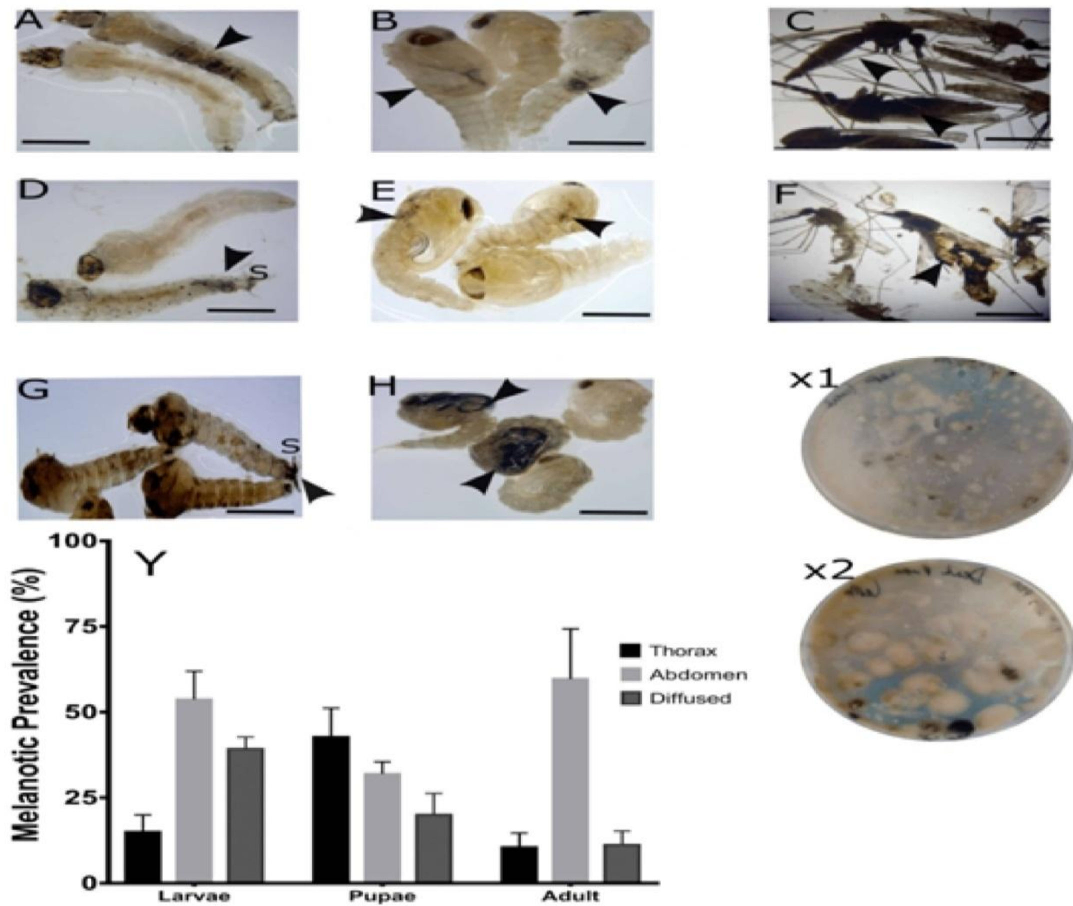


Figure 4.13: *Leptosphaerulina* fungus causes distinct melanotic phenotypes throughout developmental stages of *An. gambiae*.

Fungal infection persisted across developmental stages manifesting as melanotic phenotypes. Phenotypes were grouped according to infection status based on sections the fungus invaded to include thorax, abdomen, and diffused as shown with an arrow on infected tissues. (**A**) Light micrographs of lesions developed from the 3rd to 5th segment of third instars larvae, these were later observed on the abdominal segments of the pupae (**B**) and adults, which were prominent between the 3rd and 6th abdominal segment (**C**). (**D**)Variations in lesions were noted with some larvae acquiring diffused infection leading to thoracic manifestation in larvae and pupae (**E, H**). Partial dissections of the infected adults' abdominal sections reveal these melanotic aggregates (**F**). Fungus induced systemic infection of mosquitoes with pronounced prevalence in the abdominal sections of the adults as indicated by bars corresponding to mean +/- SEM of three experiments (**Y**). Fungal aggregates were isolated on Potato Dextrose Agar from melanised tissues at pupae and adults (x1 and x2 respectively). Scale bar ~1mm.

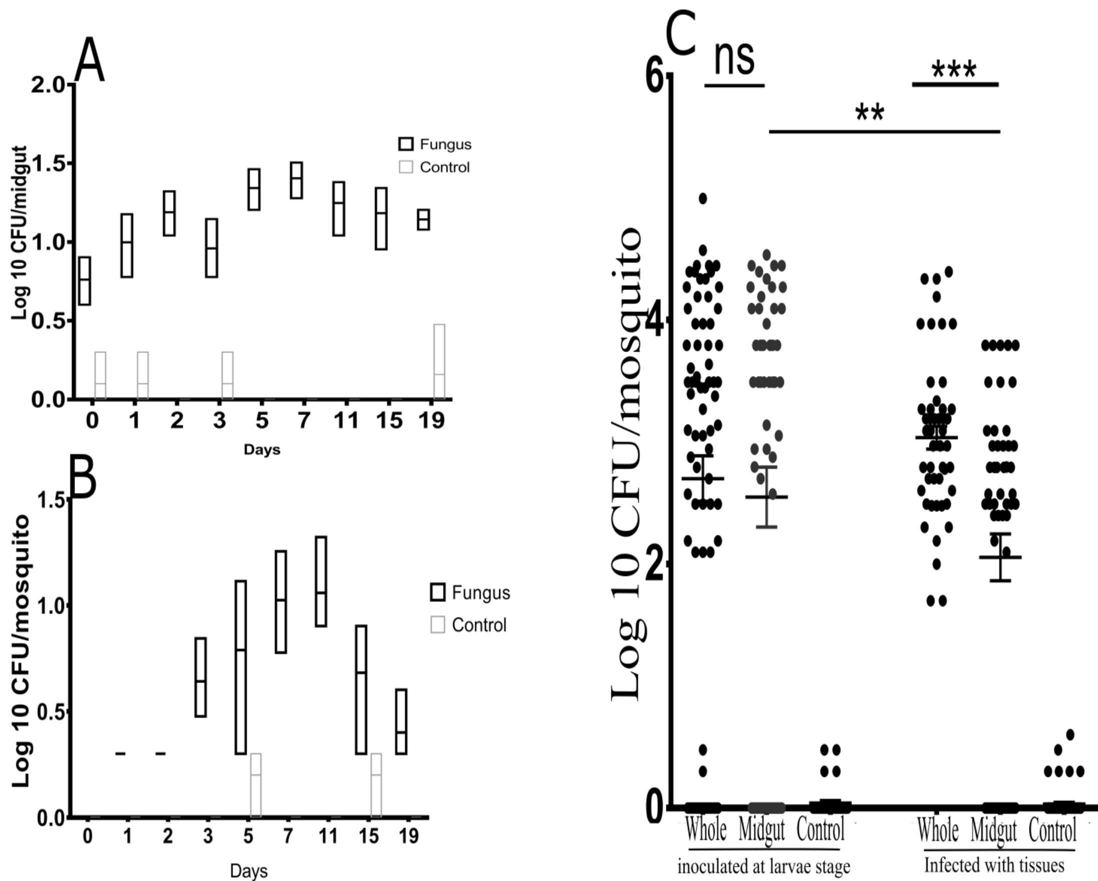


Figure 4.14: Melanosis by *Leptosphaerulina* sp. causes midgut colonization.

The presence of melanisation induced by fungus in the infected mosquitoes was monitored by enumerating midgut fungal CFUs on PDA laced with a cocktail of antibiotics. **A**) Using Bonferroni-Dunn method assuming all rows were from the same population with similar scatter, the $p<0.05$ for all days assayed in samples infected at larval stage. **B**) Based on the same approach, significant infection was observed on infected individuals from the third to 15th day when they acquired infection through tissues. **C**) Using Kruskal-wallis test, individuals infected directly at larval stage and those inoculated with infected tissues acquired fungus infection through melanin formation ($P<0.05$) were compared 7 dpi by enumerating fungal CFUs on midgut and whole mosquito. Error bars represent the standard error of mean, **$p<0.001$, ***$p<0.0001$. Re-infection of *Anopheles gambiae* with melanized tissues lessens the development of lesions.

4.3.2 The outcome on vertical transmission of *Leptosphaerulina*sp. indicates that this fungus does not induce fitness cost on the offspring of *An. gambiae*.

Using mating combinationassay that involved either uninfected partner of the opposite sex mating with infected individual (**Figure 4.15A**), high prevalence of infected offspring (~73%) was observed when both parents were infected with *Leptosphaerulina* sp. at L1 (**Figure 4.15B)**.In contrast, 61% of the offspring acquired infection when mothers were infected relative to 49% when male mosquitoes had acquired the fungus and transferred it to females before ovipositing (p=0.606, Pearson's chi-squared test, n=12). Fewer offspring developed lesions when melanized males were mated with clean females, interestingly, a higher prevalence of offspring developed lesions when their mothers were melanized, suggesting the possibility for sex biased inheritance (**Figure 4.16A**). These mothers were found to harbour significant amounts of fungus in the midgut, which suggested occurrence of vertical inheritance(**Figure 4.16B& C**). It was also observed that female progeny were more likely to acquire and retain infection based on higher infection levels in female whole mosquito and dissected tissues compared to their male counterpart (**Figure 4.16B-C)**. Notably, the presence of *Leptosphaerulina* sp. did not affect fecundity because larvae hatching from the F1 infected females from all mating combinations confirmed *post hoc* did not vary significantly (**Figure 4.17A**). Fungus was likely to invade the midut (**Figure 4.17B**). Comparison of midgut densities between Go and their offspring indicate that maternal infection influences the amount of fungus acquired by the offspring (r^2 = 0.1008, p<0.05; **Figure 4.18A)**. Maternal

transmission occurred with greater efficiency than paternal transmission, as determined by comparing densities of *Leptosphaerulina* sp. in offspring infection from maternal and paternal (**Figure 4.18B).**

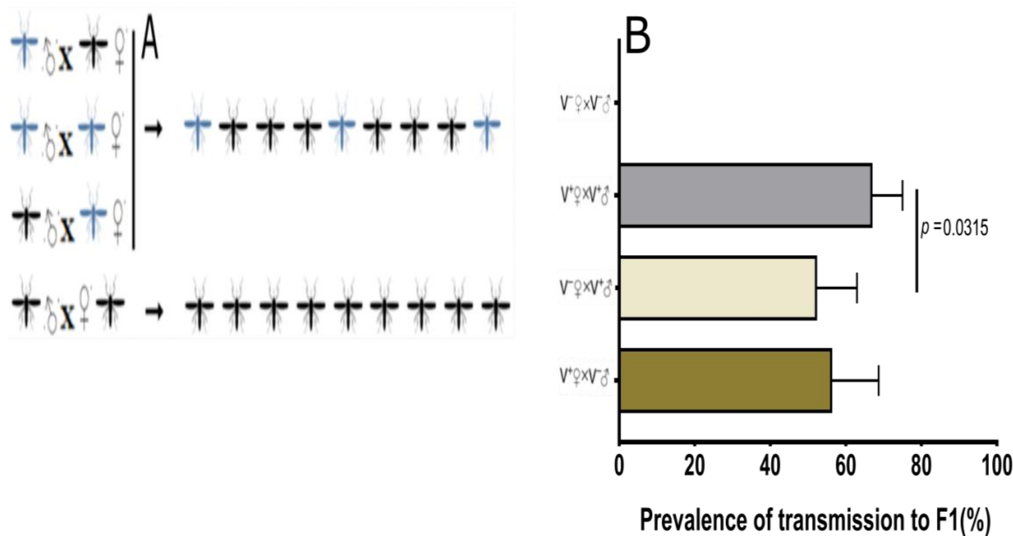


Figure 4.15: Generational transmission of *Leptosphaerulina* sp and effects on offspring of *An. gambiae*.

The graphics illustrate reproductive manipulation utilized where black *Anopheles* signify wild type adults (uninfected) that were mated with adults exposed to fungi through co-feeding at 1st instars larvae stage (in blue) with their corresponding offspring indicated by an arrow(**A**). Mating infected female with clean males (V^+♀×V^-♂) or mating combination when both sexes have infection (V^+♀×V^+♂) resulted in 61.2% and 72.6% of offspring acquiring infectionrespectively. The offspring arising from mating combinations when both sexes were infected acquired higher infections than their counterparts from uninfected females and infected males (*p*=0.0315) (**B**).

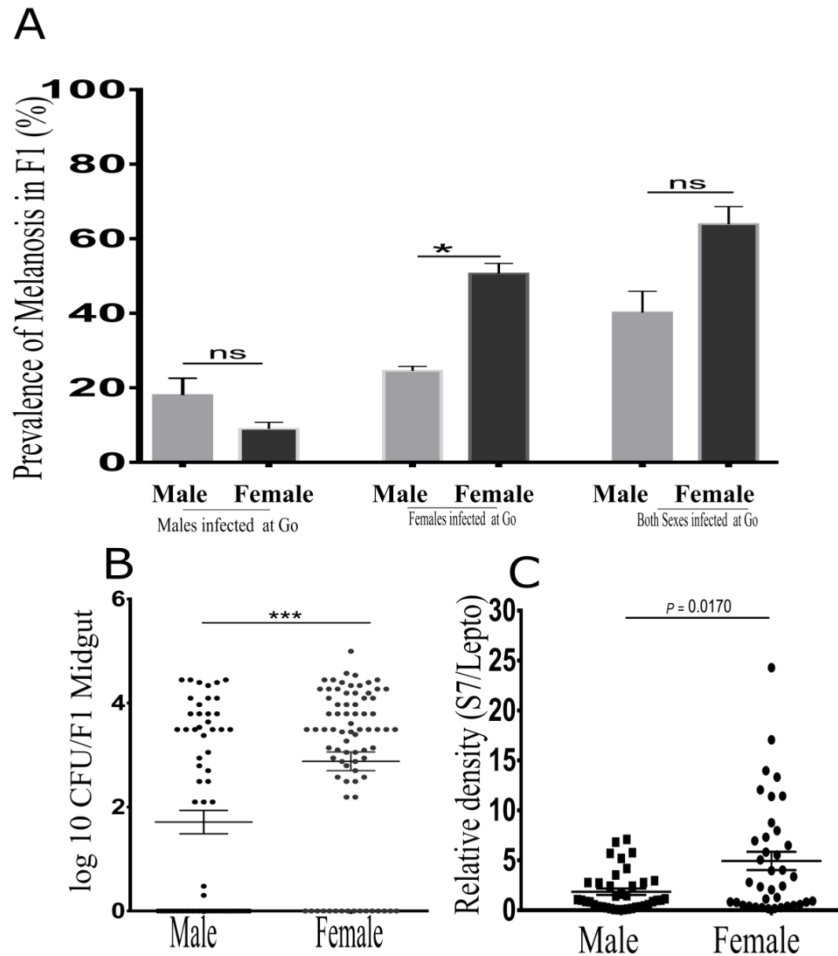


Figure 4.16: Maternal spread of fungus infection increases the development of melanosis to subsequent generation.

(**A**) Mating clean females with melanized males lowered the prevalence of tumor formation in subsequent offspring but this did not differ between the sexes. A mating combination of melanized females with clean males favoured the emergence of melanized female offspring (Mann Whitney U test, p=0.0286). **B**) Enumeration of fungus in these offspring indicates a biased infection of the female offspring midgut (Mann Whitney U test, p=0.0005). **C**) Using qPCR to estimate infection density between sexes of the offspring indicate that females inherited enriched fungus (unpaired t-test, t(60)=4.944, *p*=0.017). Densities were evaluated 7 days post emergence, three independent experiments were considered in each case. Error bars represent the mean±standard error of mean, ***p<0.0001; *p<0.01; ns represent not significant

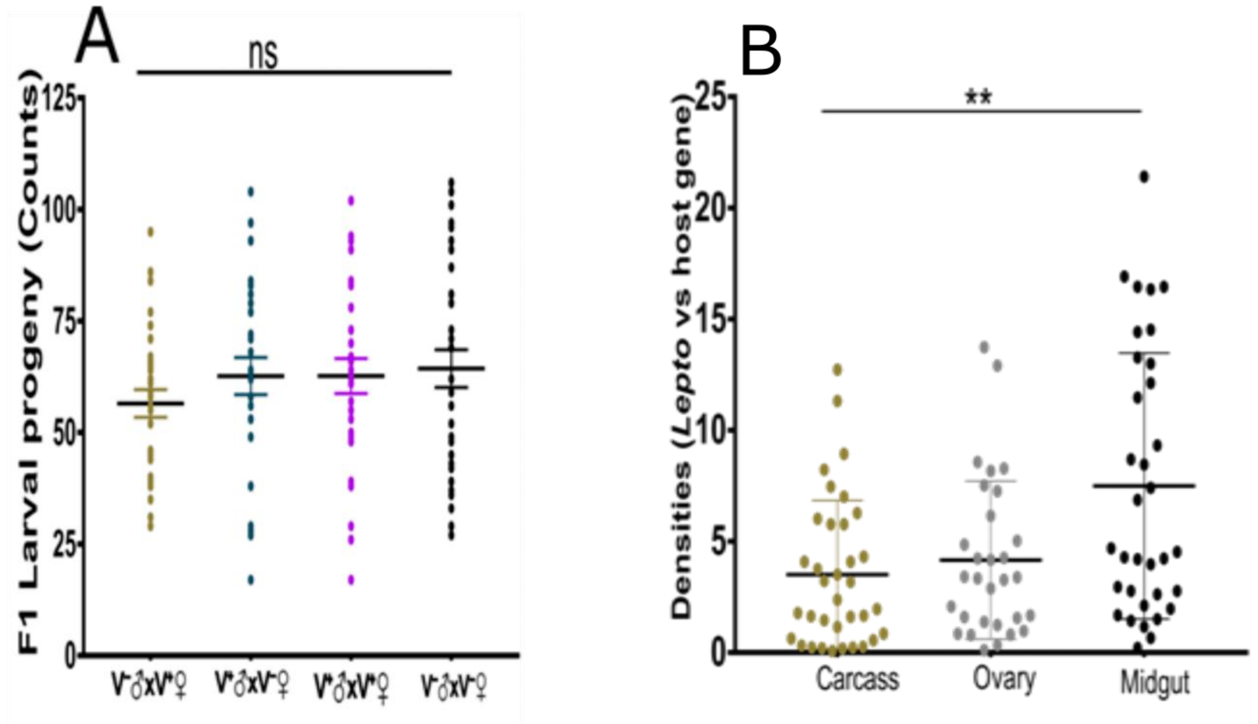


Figure 4.17: ***Leptosphaerulina*****sp infection on** ***An. gambiae*** **F1 progeny.**

(**A**) Persistence of the isolate in F1 females did not lower their fertility (Kruska-Wallis test: p=0.291). (**B**) These densities of infection were distributed in major tissues with increased burden in the midgut (Kruskal-Wallis test, p = 0.0056). The mean bar represents mean±S.E.M, while ** represent p value of <0.01, ns = not significant (p>0.05).

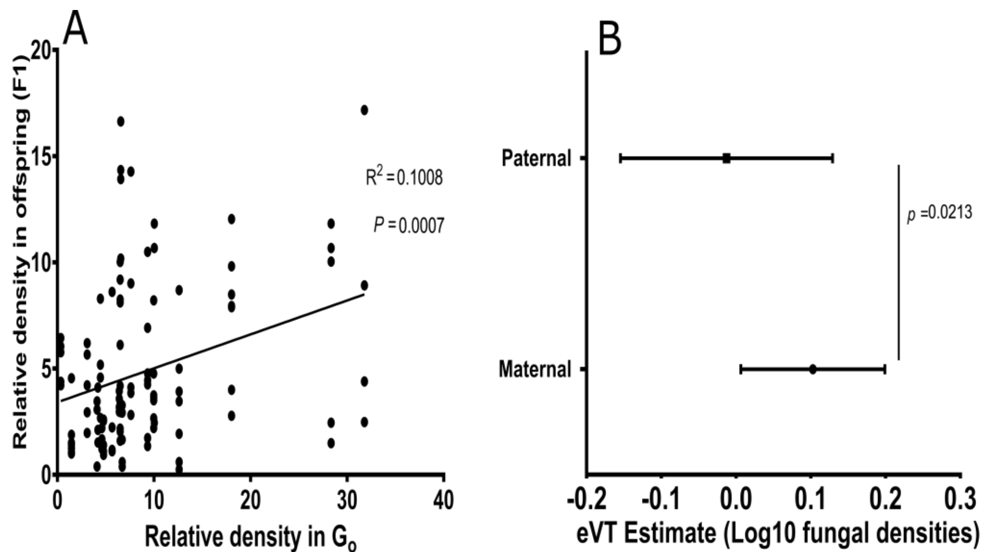


Figure 4.18: Generational transmission of *Leptosphaerulina*sp is density dependent.

A) In a linear correlation, the densities of fungal infections in parents influenced transmitted densities in offspring (r^2 = 0.1008, p<0.05). **B**) Comparison of transmission efficiency between infected parents denotes that offspring are more likely to inherit fungus when mothers were infected (Mann – Whitney test: p =0.0213)

4.3.3 *Leptosphaerulina*sp. infection induces stress and activates host innate immunity contributing to its transmission

*Leptosphaerulina*infection coincided with widespread infection dynamics manifested by melanotic tissues persisting across developmental stages and that this could also elicit immune boosting. In response to fungus infection, a dynamic immune response in *An. gambiae* was observed. Stress gene *Hsp70* previously shown to influence ability of pathogen propagation in *Anopheles* (Sim *et al*., 2007) was moderately elevated while a gene involved in xenobiotic metabolism (*Cyp6m2*) was significantly expressed

(**Figure4.19A**). Genes of IMD pathway namely *Tep1* and *Fbn9* were significantly expressed (**Figure 4.19B**). Changes in the luciferase gene expression measured by assessing activity of *cecropin 1* promoter, a gene previously linked to drive its expression (Ramirez *et al*., 2014), *defensin 1* and *Rel2* were also significantly expressed in fungus-infected tissues (**Figure 4.19C**).

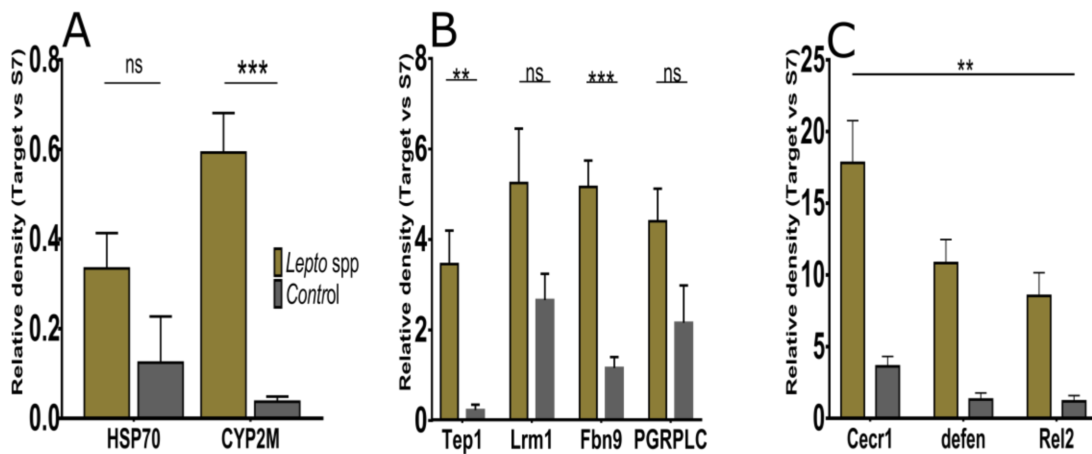


Figure 4.19: *Leptosphaerulina*sp infection induces expression of stress and immune genes in *An. gambiae* female.
A) Genes involved in xenobiotic metabolism were significantly expressed (*Cyp6m2*) (Mann-Whitney test, $p<0.05$). **B**) Two of the Toll genes Thioester-containing protein (*Tep1*) and a fibrinogen-related protein (*FBN9*) were significantly expressed while leucine-rich repeat immune protein (*LRM1*) and peptidoglycan recognition protein (PGRP-LC) did not indicate any statistical differences. **C**). IMD pathway genes cecropin (*Cecr1*), defensin 1 (*defen*), and Relish ortholog (*Rel2*) were significantly upregulated (Mann-Whitney test, p<0.05) Error bars signify mean±SEM, while * represent p <0.05, ** represents p<0.001, ns = not significant ($p>0.05$). Samples were obtained by pooling 5 dissected guts of confirmed positive.

4.4 The outcome on the effects of selected isolate on *Plasmodium falciparum* and fungus genomic features

4.4.1 *Leptosphaerulina sp* does not protect *Anophles* hosts against *Plasmodiumfalciparum*

The investigation of whether *Leptosphaerulinasp* have implications for *P.falciparum* transmission indicate that the presence of *Leptosphaerulinasp*did not reveal a direct blocking of *Plasmodium*infection when mosquitoes acquired fungus through artificial or natural means. These were assayed in *An. arabiensis* (**Figure 4.20A**), *An. gambiae* (**Figure 4.20B)**, and field collected *An. gambiae* complex (**Figure 4.20C**) that were exposed to infected blood and sporozoites quantified using ELISA on head and thorax samples sections while abdomen section used to confirm fungus density by qPCR *post hoc*. However, females that acquired higher fungal densities had moderate to low levels of *Plasmodium*, but these were not sufficient to suggest a negative correlation (**Figure 4.21A-C**; linear correlation were significantly non-zero).

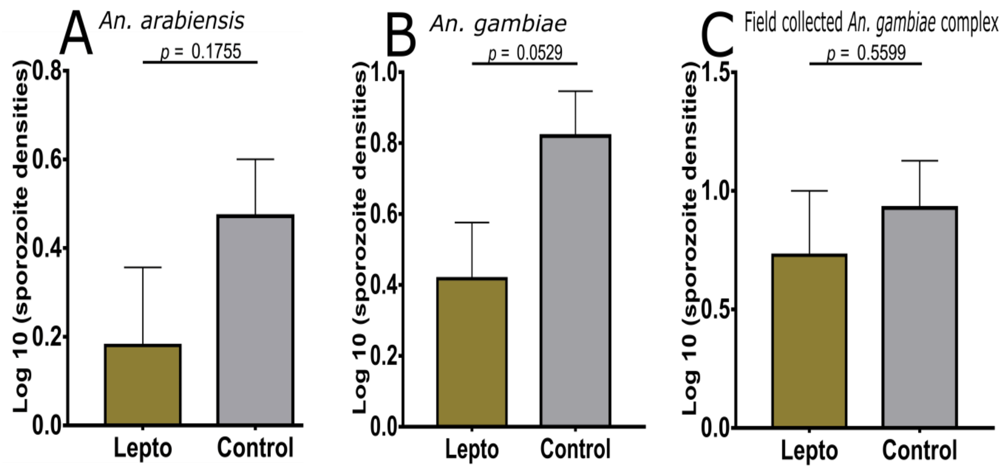


Figure 4.20: Effects of *Leptosphaerulina* sp. on *Plasmodiumfalciparum* on laboratory-reared and field collected *Anopheles*.

Bar graphs (**A-C**) display transformed densities of sporozoites per salivary gland determined from individual malaria vectors including *An. arabiensis*, *An. gambiae*, and field collected *An. gambiae*s.l complex. Using unpaired t-test, comparison of sporozoite densities indicate that fungus infected and uninfected mosquitoes were equally likely to acquire parasites and develop sporozoites (**A:**t(98)=1.365, *p*=0.1755; **B:** t(121)=1.955, *p*=0.0529; **C:** t(36)=0.5885, *p*=0.5599). Error bars represent the standard error of mean

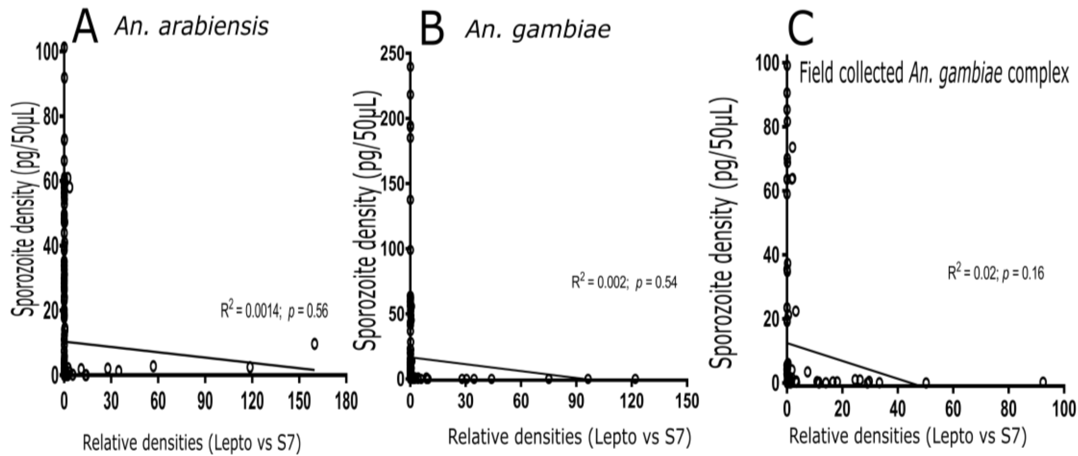


Figure4.21: A lack of correlation between *Leptosphaerulina* sp. infection and the burden of *Plasmodiumfalciparum* on laboratory-reared and field collected *Anopheles*.

Using linear correlation, a non-significant negative correlation was observed from the slope of sporozoite density plotted against fungus density for the laboratory reared mosquitoes (**A:** $F_{1,243}$=0.335; *p*=0.564; r^2=0.0014; **B:** $F_{1,248}$=0.384; *p*=0.536; r^2=0.002) and field collected *An. gambiae* complex (**C:** $F_{1,99}$=2.016; *p*=0.1588;r^2=0.02).

4.4.2*Leptosphaerulina* sp genomic features does not indicate evolutionary tendencies

The high quality paired-end reads were assembled with both Abyss and Spades platforms to establish the best outcome for gene predictions. The total reads were as shown (**Table 4.3**). Quast report of the draft genome indicate that the number of contigs ranged from 574 to 853 while the largest contigs varied from 2087884bp to 2092350bp and GC contents from 53.09 to 53.12 (**Table4.4).**

Table 4.3: The number of reads in *Leptosphaerulina* sp genome

Total read bases (bp)	Total read	GC(%)	Q20(%)
22780229950	150862450	54.68	93.8

Table 4.4: Comparison of Quast report on genomic features between the two methods of assembly

	Abyss v.2.0.2	Spades
N50	407983	724084
GC content	53.12	53.09
Number of contigs	853	574
Number of contigs (>1000bp)	570	438
Largest contig	2087884	2092350
Total length	47227476	47259800

4.4.3 Outcome of gene prediction

A total of 1592 genes identified to code for proteins occupied 86.91% of the genome assembled with Abyss platform (**Table 4.5**). Using AUGUSTUS to predict protein coding gene by BLAST search against the NCBI nr database indicates that sub-system features such as cofactors, vitamins and prosthetic groups, ion acquisition and metabolism, protein metabolism, carbohydrates and lipids were the most dominant groups suggesting that this symbiont could be playing an important role in metabolism. A Blast search against *Anopheles* host features in the *Anopheles* Vectorbase database did not yield similarities suggesting a lack of shared genes, which could have indicated evolutionary association.

Table 4.5: Summary of gene annotation statistics

Total number of genes	1592
Number of CDSs	1531
Pseudogenes	87
rRNA genes	25
tRNA genes	104
Protein-coding genes with function prediction	1124
Protein-coding genes without function prediction	407
Protein-coding genes encoding enzymes	629
Protein coding genes connected to KEGG pathways	701
Protein coding genes connected to KEGG Orthology(KO)	920

4.5. The outcome on the establishment of a self sustained propagation strategy for field dissemination of the novel fungal symbiont of *Anopheles* mosquitoes

4.5.1 Horizontal transmission of *Microsporidia MB*in *An. arabiensis*does not occur between the immature stages or through inoculation with infected tissues

Horizontal transmission was determined by assaying whether infected individuals could contaminate uninfected hosts and cause transfer of *Microsporidia*. To evaluate whether *Microsporidia* in *An. arabiensis* is transmitted horizontally between infected and uninfected larvae, two setups shown (**Figure 4.22A, B**) were evaluated for 48 hrs and using qPCR the absence of infection transmission was confirmed *post hoc*. A lack of transmission was observed in larvae when midguts of infected adults were ground and tissues used for larval infection (**Figure 4.22C**). The contaminated homogenate used for contamination adult sugar sources did not cause infection in the uninfected adults (**Figure 4.22D**).

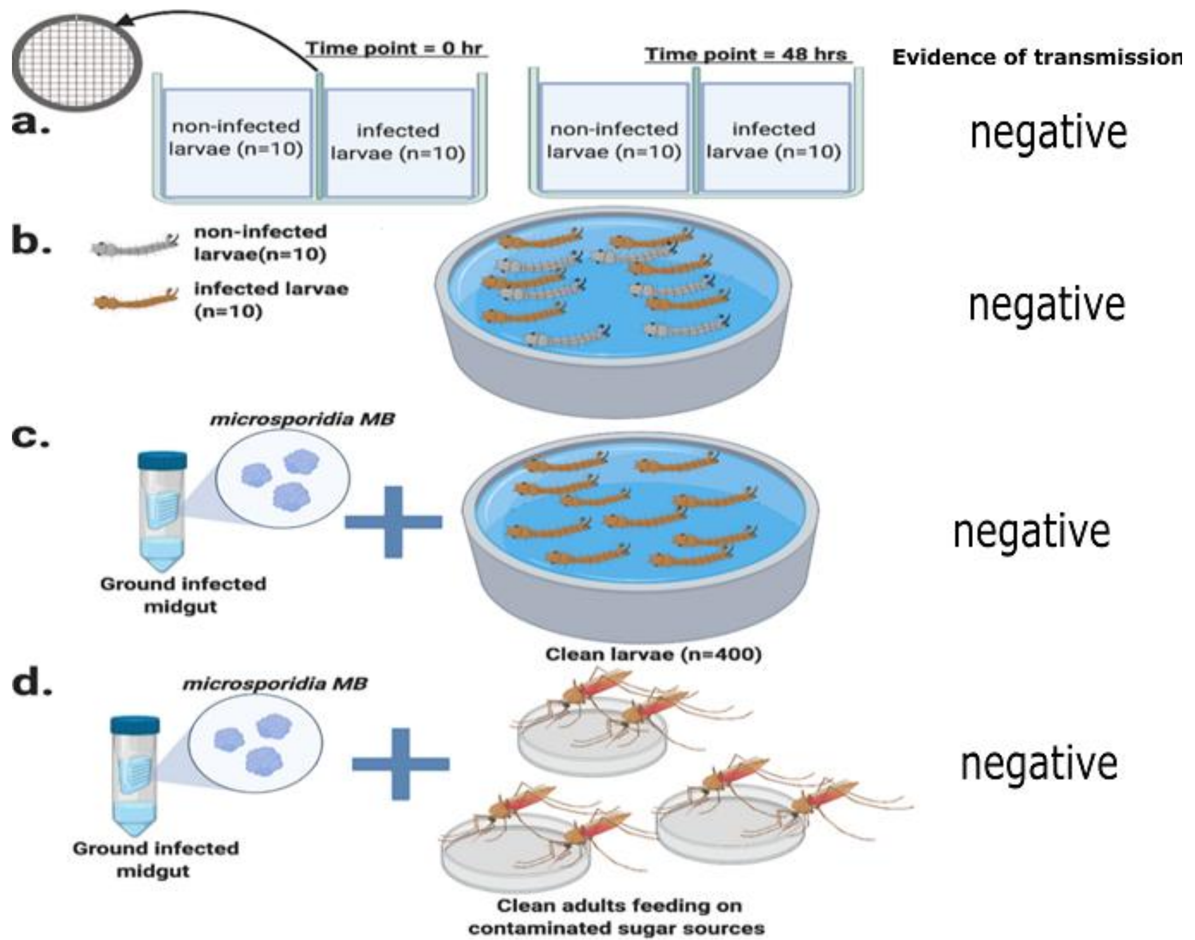


Figure 4.22: *Microsporidia* is not transmitted horizontally in larvae or adult anopheline mosquitoes.

a) qPCR assay of uninfected larvae reared beside infected larvae in a demarcated rearing container turned negative after 48 hours. b) The uninfected larvae did not acquire infection when they were reared in the same container with infected larvae. c-d) When midguts of infected individuals were ground and used to infect uninfected larvae or adults, infection were not transferred to the uninfected larvae and/or adults.

4.5.2 Horizontal transmission of *Microsporidia* occurs through venereal transfer

Venereal and horizontal transmission between infected and uninfected adults was evaluated in a caged mating combination that tested the potential of an infected individual transferring symbiont to the uninfected host. Uninfected males reared in the same cage with infected females were confirmed positive using qPCR assay (**Figure 4.23;** Cross A). Uninfected females maintained in the same cage with the infected females and infection status determined *posthoc*, were found negative (**Figure4.23;** Cross B). Infected males transferred infection to uninfected females (**Figure 4.23;** CrossC), but these males could not transfer infection to the uninfected male counterparts through direct contact (**Figure 4.23;** CrossD). Notably, some of the females acquiring infection from infected males gave rise to infected offspring. However, there was no direct link between male intensity of *Microsporidia MB* infection and successfully infecting uninfected female (exp(b) = 0.982, P = 0.715 df = 31). Besides, the number of females in each cage did influence the odds of transferring *Microsporidia MB* to the females (exp(b) = 0.968, P = 0.421 df = 31). Females acquiring *Microsporidia* gave rise to a generation of infected offspring signifying occurrence of both horizontal and vertical transmission of the symbiont. (**Figure 4.24A**).The correlation between the infection intensities in donor males and recipient females was not statistically significant (R 2 = 0, P = 0.34 df = 31; **Figure 4.24B**). A striking observation was that the relative density of infected females (10.33)was twice the intensities in donor males (5.01).

Establishment of a potent mating strategy did not reveal any apparent preference for either serial, multiple or single mating approach (**Figure 4.25**).

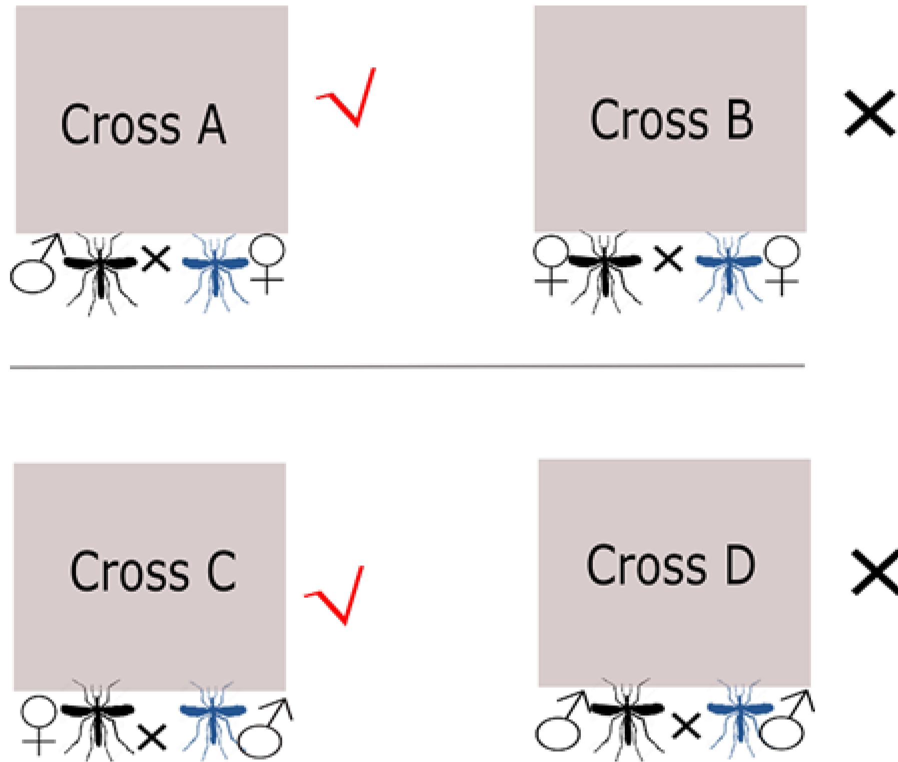


Figure 4.23: ***Microsporidia*** **is sexually transmitted by infected adult mosquitoes**

A cage consisted of one infected and 10-15 uninfected individuals or three infected and 30-45 uninfected individuals. *Anopheles* in black color represent uninfected individual while blue color signify infected mosquito.In cage A (n=12), a mating combination of infected females and uninfected males did not lead to the transfer of symbiont in males. Transfer of infection was not observed in uninfected females maintained in the same cage with infected female (Cage B, n=10). In cage C (n=12), involving a mating combination of infected males and uninfected females, transfer of infection ranging from an individual female to 4 were observed in 25 of 60 cages (n=60). Uninfected males maintained in the same cage with infected males did not acquire infection (Cage D, n=10).

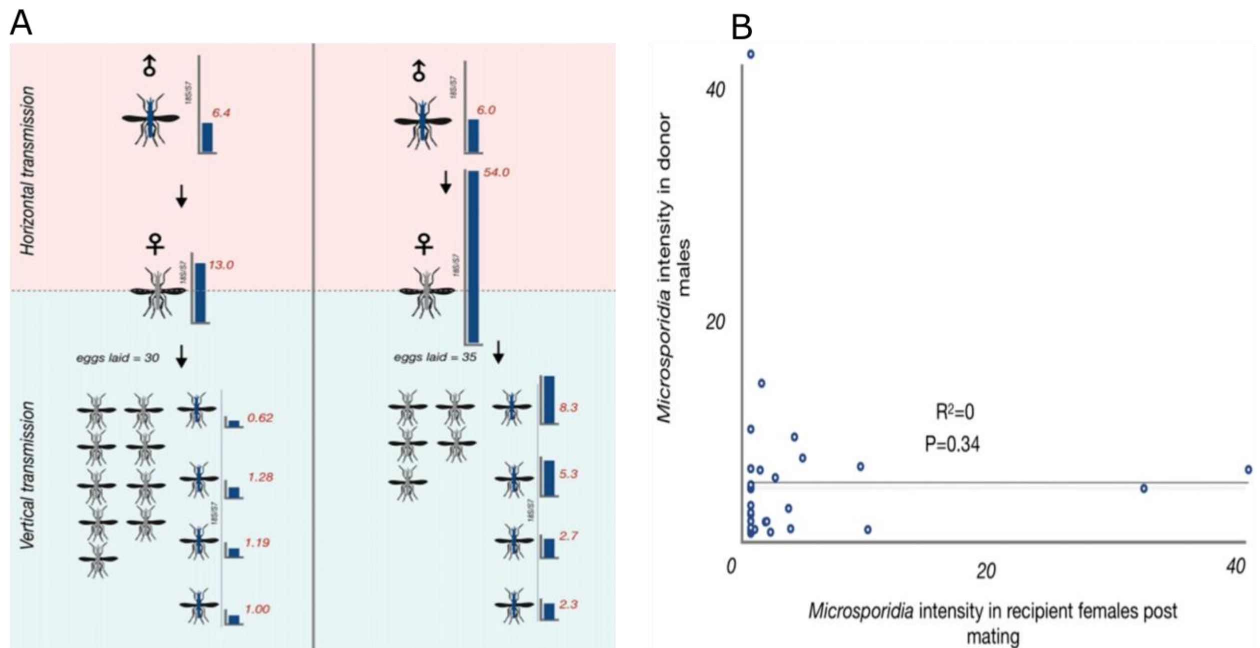


Figure 4.24: Vertical transmission of horizontally acquired *Microsporidia MB* infections in *An. arabiensis*.

A) Vertical transmission of *Microsporidia MB*was observed in the offspring of recipient females that had acquired *Microsporidia MB*through insemination.Numbers coloured in red indicate *Microsporidia MB* infection intensity in individual *An. arabiensis* adultsas determined by qPCR. *Microsporidia MB* infection intensity decreased across each transmission cycle (vertical and horizontal).**B**) The intensity of *Microsporidia MB* in recipient females is not correlated to donor male intensity, with a linear regression of the slope that does not significantly differfrom zero ($P = 0.26$, $r = 0.202$ and n = 33).

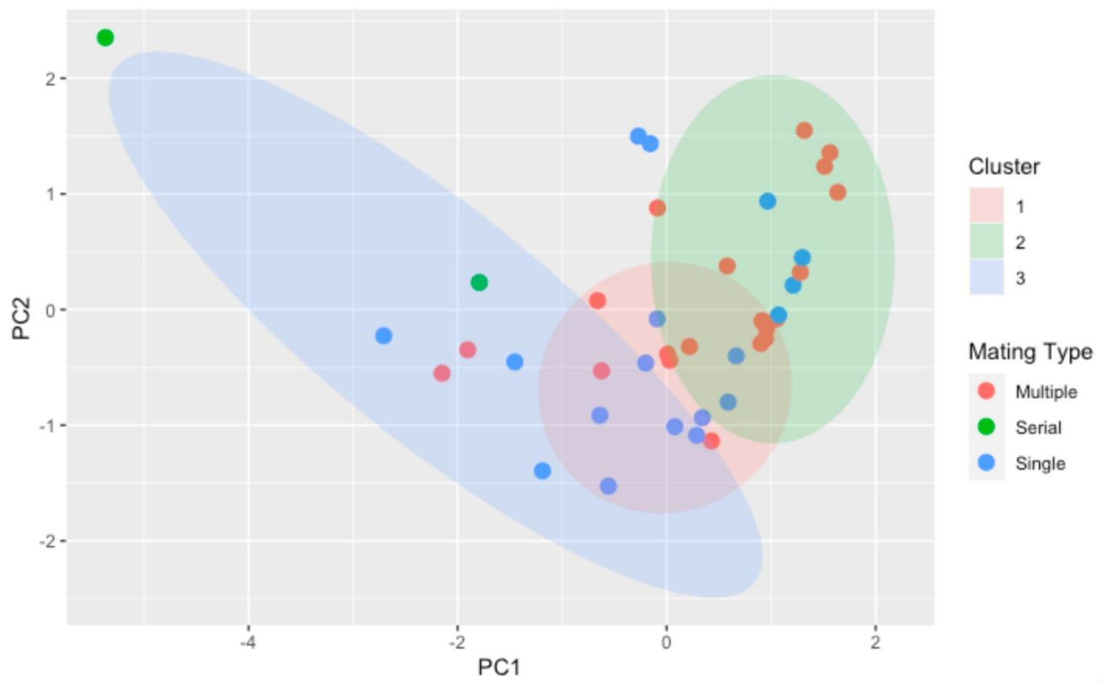


Figure 4.25: Exploiting different mating approaches for optimal dissemination of *Microsporidia* in anopheline mosquitoes.
Mating was grouped in three categories. Single mating involved placing one F1 male in a cage with 3-20 virgin females while batch (multiple) mating involved placing 2-5 F1 males in the same cage with 15-35 virgin females. Serial mating involved transferring an F1 male from one cage of females to a second cage of females and maintained in the insectary conditions for 4 days. The numbers of offspring in each mating combination were compared in a PCA to establish appropriate strategy of disseminating *Microsporidia* in *An. arabiensis*.

4.5.2 The presence of *Microsporidia MB*in male *An. arabiensis* midgut and reproductive system indicate venereal transfer

To determine if *Microsporidia* was distributed in the organs of *An. arabiensis*, tissues such as midgut, gonads and carcasses were dissected and used for qPCR assay. It was observed that *Microsporidia MB* was prevalent in the male midgut (11/22) and gonads (7/22) (**Figure 4.26A**). Systemic infection of more than one tissue was also observed. Notably, the intensities of *Microsporidia MB*werefound to be high in the midguts and gonads of male *An. arabiensis*, but lower in carcasses (**Figure 4.26B**). Seminal fluids collected from infected males were found to harbour high intensities of *Microsporidia MB* (**Figures 4.26C, D**). Besides, *Microsporidia MB* cells were observed in the male ejaculatory duct (**Figures 4.27A, B**). Females acquiring *Microsporidia MB* were found to have sperms (**Table 4.6**) indicating that the the transfer of *Microsporidia MB* occurrs through successful insemination.

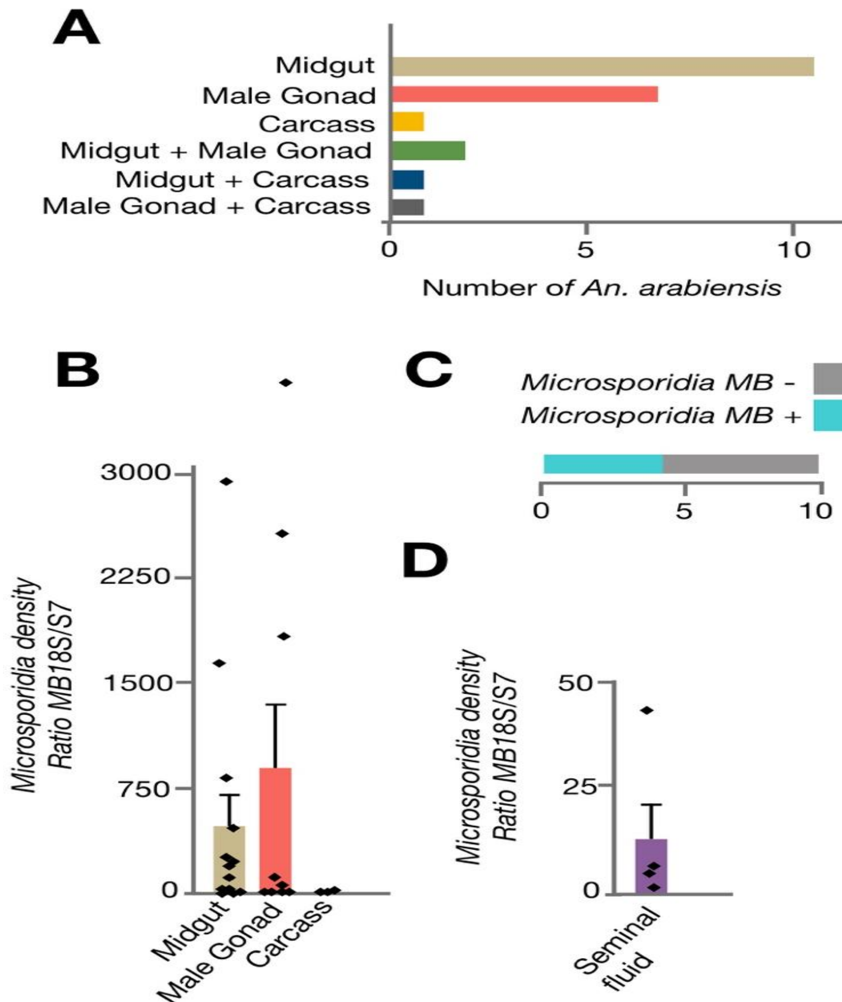


Figure 4.26: Distribution of *Microsporidia MB* in the organs of male *An. arabiensis*.

A) Screening of dissected organs from 22 male *An. arabiensis*specimens, reveals that *Microsporidia MB* is detected primarily in the midguts and male gonads. **B)**The intensity of *Microsporidia MB* infection is highest in the midgut and male gonads. **C)** Screening male *An. arabiensis*seminal fluid revealed that *Microsporidia MB* was detected in 4/10 specimens. **D)** The intensity of *Microsporidia MB* infection in *An. arabiensis* ranges from a ratio of0.87 to 41.8 MB18S/ S7. Error bars reflect SEM.

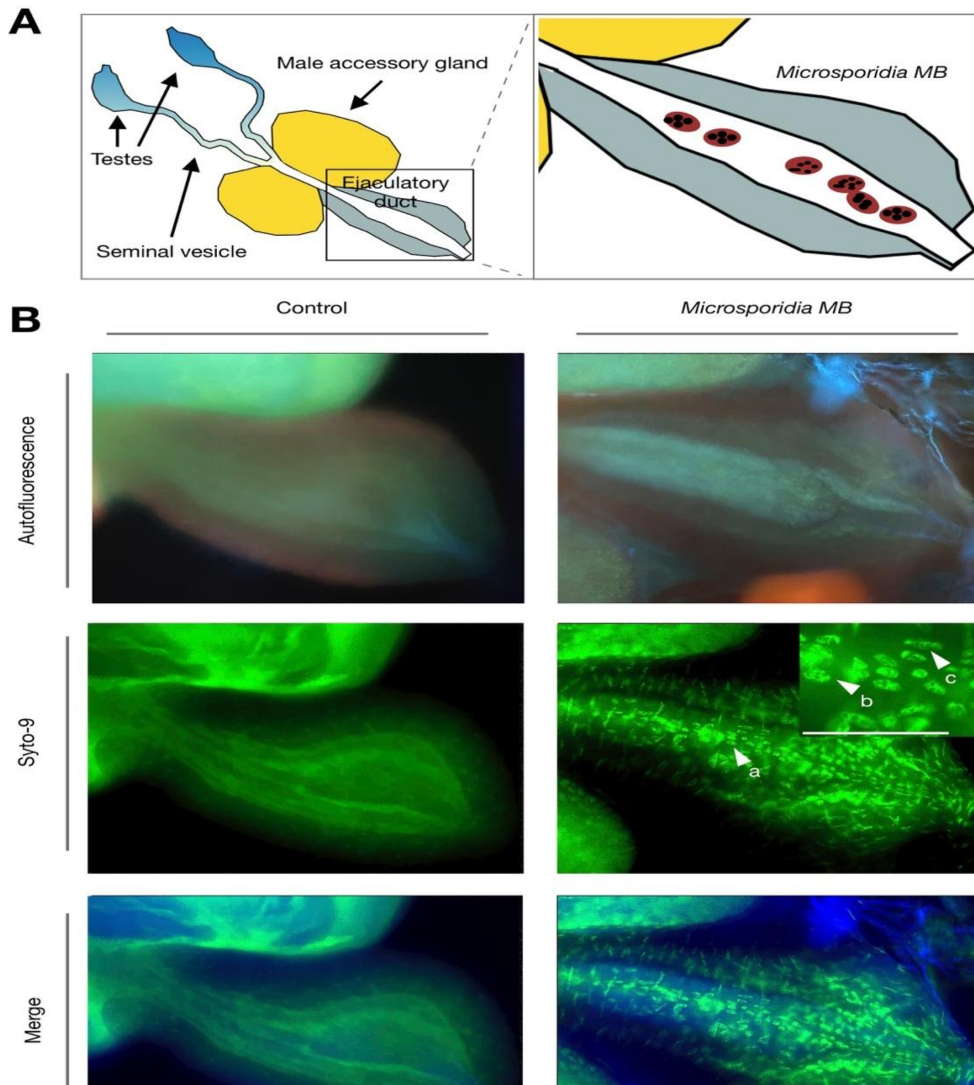


Figure 4.27: Flourescence microscopy of*Microsporidia MB* in *An. arabiensis* male ejaculatory ducts.

A) Schematic diagram of the male *Anopheles* gonad shows the position of the ejaculatory duct in relation to seminal vesicle, male accessory gland and testes. **B)**Flourescence microscopy images indicate that *Microsporidia MB* meronts (a) are found in the male *An. arabiensis* ejaculatory duct. Multinucleate *Microsporidia MB* cells can be observed containing 4 and 8 distinct nuclei (b) and (c), which likely corresponds to the progression on of 4-nuclei sporogonial plasmodia into the 8-nuclei sporogonial plasmodia and ultimately into sporophorous vesicles. Scale bar = 50 µm.

Table 4.6: Transmission of *Microsporidia MB* to *An. arabiensis* females in linked to the presence of sperms in the female spermatheca.

	# of male donors	# of male donors MB +	# of female recipients	# of female recipients with sperm in spermatheca	# of female recipients sperm + and MB +	# of female recipients sperm- and MB +
SPC12	3	1	25	2	1	0
SPC09	1	1	40	0	0	0
SPC15	3	2	59	2	1	0

CHAPTER FIVE

DISCUSSION

5.1 Isolation of fungi from *Anopheles* mosquito developmental stages obtained from semi-field environment.

Diverse ascomycetes fungi isolated from semi-field *An. gambiae*were found enriched in the midgut and reproductive tissues of the host. Semi-field *Anopheles* are maintained in a laboratory setting that mimics natural environment, and this condition is likely to facilitate the establishment of core fungi that associate with these hosts. Field-collected mosquitoes have highly diverse microbial taxa that differ from one location to another,suggesting that the environment shapes microbial composition (Boissière *et al*., 2012; Coon *et al*., 2014; Osei-Poku *et al*., 2012). Most anopheline vectors lose microbes at pupal stage as they shed off their contents while emerging to adults and subsequently acquire additional symbionts from their immediate environment, a phenomenon that was described as microbial reduction (Lindh *et al*., 2008). It is not known to what extent fungal symbionts can evade this microbial reduction to persist in these vectors and influence the hosts' biological fitness across the developmental life stages. Whether adult stages acquire fungal microbes exclusively from their environment or through transstadial transmission from immature stages remains unresolved as previously observed(Coon *et al*., 2016, 2014; Pumpuni *et al*., 1996).

Existing studies on mosquito symbionts focus on bacteria, whereas the midgut

harbors a consortium of microbes ranging from unicellular bacteria to lower eukaryotes such as fungi (Chandler *et al*., 2015; Osei-Poku *et al*., 2012). There are reports of naturally occurring fungi isolated from field-caught mosquitoes (Angleró-Rodríguez *et al*., 2016, 2017; da Costa & de Oliveira, 1998; Pereira *et al*., 2009). Besides, fungi have been isolated from the midgut and cuticles of other vectors such as phlebotomine sand flies (Akhoundi *et al*., 2012). It is not clear what role these fungi play on the host biology, and whether they are true symbionts or were acquired from their immediate environments.

The presence of fungal symbionts (mycobiomes) in the mosquitoes is not striking because these hosts are exposed to diverse environmental conditions such as aquatic ecosystem during immature stage to terrestrial conditions when they become adults, and these ecologies put them in close proximity to different kinds of microbes (Gimonneau *et al*., 2014). Some fungi have been evaluated for their potential to either enhance or inhibit pathogen infection, it was found that *Talaromyces* (*Tsp_PR*), isolated from female *Aedes* and *Penicilliumchrysogenum* from *Anopheles* rendered mosquito more susceptible to DENV and *Plasmodium* infection respectively (Angleró-Rodríguez *et al*., 2016, 2017). While these observations are fundamental,establishing whether these isolates co-exist with the host is fundamental in planning to scale up such findings. In this study it was observed that *Anopheles gambiae* is inhabited by diverse fungi with enriched colonization of midguts and reproductive tissues. Expectedly, high throughput analysis indicates that some of the cultivable fungi could be isolated from these hosts.

5.2 Investigation of the effects of *Leptosphaerulina*isolates on the *Anopheles* host indicate that the fungus has minimal fitness cost

Biological impact of fungal symbionts in *Anopheles* mosquito is relatively unknown. Fungi have been isolated from mosquito species (Cappelli *et al*., 2014; Chandler *et al*., 2015; Gueganet *al.,* 2020; Guégan *et al*., 2018; Lindh *et al*., 2008; Ricci, Damiani, *et al*., 2011; Valzania *et al*., 2018; Valzano *et al*., 2016), and linked to promoting larvae growth (Valzania *et al*., 2018), or pathogen killing (Valzano *et al*., 2016) but studies on germ-free mosquitoes to ascertain host-symbiont interaction especially on development, persistence and emerging phenotypic responses from their association remains limited.

The finding that immature *Anopheles gambiae* acquires *Leptosphaerulina*sp. from larval breeding water and undergoes transstadial transmission to pupae and subsequently from pupae to adult with minimal effect on the host is important. Preliminary assessments showed that surface sterilization of eggs with 1% 1MHCl cleared resident fungi and did not slow larval development compared to conventional rearing as previously depicted when antibiotics were used to produce axaenic mosquitoes (Coon *et al*., 2014; Correa et al., 2018). To prevent adults from imbibing pupal water, pupae from infected lines were surface-sterilized in a series of sterile water laced with 1% 1MHCl and ketaconazole. The treated water was also used previously to establish fungus free colonies, besides this strategy prevented the occurrence of cross contamination. Emerging pupae were immediately aspirated from cages and transferred

to rearing cages. This approach minimized the opportunity for surface contamination as previously observed (Lindh *et al*., 2008). A model proposed by Moll*et al*., (2001) suggests that bacterial reduction takes place in the pupae during eclosion leadingto transfer of fewer microbes from either the habitat water or sugar sources. It was reasoned that pupae could release fungus into their habitat(pupal-water) and when newly emerging adults were not aspirated immediately they imbibed fungus contaminated water. This informed the use of anti-fungus to kill the isolate thereby adults that did not imbibe the contaminated water remained fungus positive. This strategycould support the observation on reduced prevalence and densities of fungus in adult compared to pupae and larvae.

Understanding the role of mycobiomes in mosquito development and how such microbes evolve through the vector's life stages could inform selection of novel strains to be exploited in controlling vector-borne diseases (Ricci *et al*., 2012). Notably, in this study *Leptosphaerulinasp* was found to possess minimal to moderate virulence on the host. Although it slowed the duration of larval development, it did not affect the proportion of surviving larvae. This could be attributed to elimination of other non-fungi microbes since studies have demonstrated the importance of microbes in the development of anopheline hosts (Coon *et al*., 2014; Valzania *et al*., 2018). These observations were consistent with those showing that axenic *Aedes* mosquitoes exhibited delayed time of development (Correa *et al*., 2018), coupled with increased duration of adult life history, which could be explained by a lack of differences between the fungus

infected and uninfected adult longevity in this study. This pattern suggests that elimination of host microbes and re-introduction of resident microbes influence host biology differently thus expanding our understanding on the effects of symbiont on host biology. Fungus infected mosquitoes had decreased fecundity. A possible explanation for this observation is that female hosts colonized by a specific microbiome exhibit reduced capacity to reproduce due to the absence of other symbiont taxa as suggested (Gulia-Nuss, *et al*., 2015; Telang*et al*., 2006). Besides, microbe turnover in the gut has been linked to a steady supply of nutrients to the host; therefore elimination of core members using hydrochloric acid could have influenced nutritional capacity thereby affecting production of eggs.

Once the ability to undergo transstadial transmission in *An. gambiae* was verified, it washypothesized that *Leptosphaerulinasp* infection could be acquired by mosquito offspring. From this finding, it wasobserved that the abundance remained relatively constant among the artificial-laboratory reared offspring with infected Go female playing a role in increased propagation of transmission to progeny. Transmissions from infected males suggest a possible occurrence of contact and/or copulation/venereal transmission. Copulation transmission takes place when infected males release ejaculate molecules contaminated withfungus during insemination (Gonella *et al*., 2012). Whenever infected Go females were mated with uninfected Go males, uneven transmission of ~7% occurred in these Go males. However these males could not infect a new batch of uninfected females upon re-mating suggesting unstable

acquisition of fungus through contact, but this should be investigated further. Notably, male mosquitoes from 1st instar larvae infected lines transferred fungus infection to previously uninfected female, whose offspring also tested positive suggesting the occurrence of both transstadial, and/or vertical transmission. Previously, mosquitoes were shown to acquire *Asaia*bacteria per-oral (Favia *et al*., 2007; Ricci *et al*., 2011), and some through venereal routes before undergoing vertical transmission (Damiani *et al*., 2008), observations that supported these findings. The studydemonstrated that native fungal symbiont co-exists with *An. gambiae*by colonizing the midgut and reproductive tissues suggest the likelihood for occurrence of horizontal and most preferably vertical transmission.

5.3 The outcome of the mechanisms utilised by the selected fungi to establish within the *Anopheles* populations

In *Anopheles*, there is paucity of information about the occurrence of melanization especially those arising from fungal symbionts that were artificially re-introduction and the role played thereof. A dominant bacterial symbiont *Elizabethkingiameningoseptica*was artificially introduced and found to cause melanotic pathology resulting in significant mortality of *Anopheles gambiae*, and presumably enhanced its vertical transmission from eggs to larvae (Akhouayri *et al*., 2013). This study investigated the effects of chronic melanosis on laboratory reared *An. gambiae* and demonstrates that it was linked to a native gut fungus *Leptosphaerulina*sp that was previously isolated from semi-field screen-houses. It was shown that increased

melanosis induced by this fungus was highly virulent but was retained throughout development in a fashion similar to what was observed in *Elizabethkingia meningoseptica*(Akhouayri *et al*., 2013). Interestingly, it was enriched in the midgut and reproductive tissues. This could explain its effects on fitness; however, it is possible that melanosis plays an important role in the increased infections that enhance transmission of *Leptosphaerulina* symbiont. These properties make it a suitable for targeted delivery of anti-plasmodial molecules in these hosts. Functional studies indicate that melanization occurring when bacteria infects *An. gambiae*does not influence host fitness (Schnitger, Kafatos, & Osta, 2007). Field-caught and laboratory strains of *Anopheles gambiae* mosquitoes rarely utilize melanosis derived from bacterial infection to eliminate *Plasmodium* parasites(Michel *et al*., 2006; Schnitger *et al*., 2007).

To understand propagation of melanin in the host and whether this had overt influence on host development, infection were classified according to compartments infected and melanised tissues used to propagate infection. It was found that *Leptosphaerulina*fungus induced the development of chronic melanotic tissues that were either localized in the thorax, abdomen or diffused in tissues persist across life history. It is reported that direct inoculation of this fungus to larval or indirect inoculation with melanized tissues triggered the formation of these phenotypes thereby representing a classical example of reproducing disease in individuals that were previously non-infected. While melanosis by bacterial symbiont has been reported in these hosts, the absence of these phenotypes in the *Leptosphaerulina*-uninfected controlsis a suggestion

that infection by this fungus induces the development of melanotic lesions in mosquitoes. Development of melanin in arthropods contributes to host immune responses to the invading pathogens. The deposition of melanin on the infected surfaces helps in the encapsulation of pathogen, wound healing, and clearing of the pathogens as previously noted (Akhouayri *et al*., 2013). The occurrence of these traits are associated with pathogen recognition that activate prophenoloxidase leading to its*denovo* synthesis and subsequent deposition of melanin (Yuan *et al*., 2017), or through a cellular mediated process that involves activation of immune cells in response to tissue injury. It is postulated that melanosis is triggered when mosquito responds against virulent pathogens leading to a direct formation of melanin at the site of injury(Yuan *et al*., 2017). Formation of melanosis and transmission of *Leptosphaerulina*sp across host developmental phases corroborates previous observations that reintroduction of native bacteria symbionts such as *E. meningoseptica* caused persistent melanotic phenotypes (Akhouayri *et al*., 2013). Notably, microbial rearrangements occurringduringpupal stage lead to loss of microbes and a reduction in diversity (Moll *et al*., 2001). Besides, during blood feeding, a similar trend has been reported but this was not observed in *Leptosphaerulina*sp suggesting that this symbiont may occur as a core gut mycobiome of *An. gambiae*.

A classical symbiont establishes an intimate association with host insect by colonizing either the midgut or/and the reproductive tissues. Localization in the midgut is important for immediate interaction with pathogens such as *Plasmodium*(Romoli

&Gendrin, 2018). Symbionts are known to elicit immune responses that could have a direct effect on the parasite or stimulate immune signals that restrains parasite infection (Meister *et al*., 2005; Meister *et al*., 2009; Romoli & Gendrin, 2018). This study found that fungal infection led to development of melanotic phenotypes that was characterized with striking induction of immune genes in the host *Anopheles*. It was reasoned that fungal proliferation may result in changes to the basal immunity, accounting in part to the mycobiome establishment along the developmental stage and melanotic encapsulation. Monitoring of *Leptosphaerulina*sp in the melanized individuals showed that the fungal isolate was enriched in the midgut irrespective of whether a direct inoculation or tissue infection was undertaken. These observations suggest that melanization in *An. gambiae* could be facilitating colonization in the midgut and reproductive tissues, and this helps with the establishment and persistence. Symbionts that establish in the midgut are suited for paratransgenicbiocontrol strategy which involves the delivery of effectorsthat silence either host transcripts or parasites thereby lowering the transmission of pathogen (Mancini *et al*., 2016).

The expression levels of immune genesassociated with classical pathways namely IMD, Toll, and stress were evaluated. It wasreasoned that fungal proliferation may result in changes to the basal immunity, accounting in part to the mycobiome establishment and persistence along the developmental stages. Genes such as *TEP1* and *LRM1*, whose products are involved in opsonization and melanization response, have been associated with antagonistic effects on the development of the parasite (Kambris *et*

al., 2010; Volz*et al*., 2006). In this study it was found that fungus infection induces a major up-regulation of immune and stress genes in the infected *Anopheles* compared to uninfected colonies. With these observations, the degrees of expression differed among genes tested, a phenomenon that could be explained by varying expression levels in different cell type/organs of the vectors. Previously, it was shown that commensal microbiota influence the basal immunity in a way that it could curb the development of the pathogen in the midgut (Clayton *et al*., 2013; Frolet *et al*., 2006). Changes in the expression profile of effector genes *Defensin* 1, *Cecropin 1*, *FBN9*, *Rel2*, *LRM1*, and *TEP1* suggest the possibility for *Leptosphaerulina*sp infection inducing basal immunity. Mosquitoes exploit melanization/opsonization and activation of immune effector molecules by the action of Relish ortholog, *Rel2*, in *An. gambiae* to curb microbial invasion (Zou *et al*., 2011). During the invasion, *Anopheles* mosquitoes respond by mounting antibacterial defence system through elicitation of peptidoglycan recognition protein LC (PGRPLC) signaling, and these responses were attributed to modulations of parasite infection (Meister *et al*., 2009). Taken together, these observations suggest that fungal infection enhances mosquito immunity and elicits reactive oxygen species, which might have enhanced *Leptosphaerulina*sp to establish in the vector and persist in subsequent developmental stages. It was found that the isolate colonizes the midgut and reproductive tissues, and this in part could have enhanced its establishment and persistence.

5.4 The outcome on the effects of *Leptosphaerulina* isolate on *Plasmodium falciparum* and its genomic features

It was found that the presence of *Leptosphaerulina*sp fungi in the two species of *Anopheles* mosquito –*An. gambiae* and *An. arabiensis*did not render these vectors less permissive to *Plasmodium* infection. Interestingly, in some samples, as the densities of the fungal isolate increased, parasite infection declined, but these were not sufficient to suggest a negative correlation. This could be attributed to the fungus occupying space in the midgut that would otherwise have been occupied by the parasite or fungus infection making these hosts prone to parasite infection. Another possible explanation is that the development of melanotic tissues were only sufficient to enhance the establishment of the microbial infection as previously shown(Lu *et al*., 2012; Pan *et al*., 2018). While development of melanosis have previously been linked to impairment of *Plasmodium* development(Kambris *et al*., 2010; Volz *et al*., 2006). Previously it was shown that increased melanosis did not results in the impairment of *Plasmodium* development (Michel et al., 2006), it is possible that in the case of increased *Leptosphaerulina* infection, the hosts either become susceptible to parsite infection or the fungus occupying the midgut hindered development of parasite. A direct link could not be established between melanosis and the impairment of parasite development, it is possible that this phenotype was only sufficient to confer fungus establishment as shown by others (Akhouayri *et al*., 2013).Besides, it is possible that *Leptosphaerulina*-induced melanosis could have led to an increase in susceptibility due to hyper activation of

immunity and increased melanosis which was shown previously to have limited influence on parasite impairment (Michel *et al*., 2006). The occupation of the midgut by this fungus could have inhibited the establishment of parasites in some individuals hence the observed subtle association. It is also possible that the fungus is less likely to limit resources needed for parasite development hence a lack of protection against *Plasmodium*.

Genome studies of *Anopheles* symbionts suggest that they could be playing nutritional functions (Alonso *et al.,* 2019; Minard *et al.,* 2013). Analysis of *Leptosphaerulina* genome sequence indicates that the symbiont could be enhancing metabolic functions of the host through breakdown of nutrients and toxins. Given that sequences from this analysis wan compared with other symbionts, this evaluation is tentative and requires indepth analysis to link specific nutritional roles to the host. Besides, analysis of other fungal genomes could indicate whether this is a common phenomenon for persistent symbionts.

5.5 The outcome on the establishment of a self sustained propagation strategy for field dissemination of the novel fungal symbiont of *Anopheles* mosquitoes

Vertical transmission of *Microsporidia* in *Anopheles* mosquitoes through transovarial route was shown to play a significant role in establishing host-symbiosis association (Herren *et al*., 2020). The finding that *Microsporidia MB* colonizes*An. arabiensis* tissues such as midgut and reproductive tissues could point to the occurrence of both vertical and horizontal modes of transmission. In this study, horizontal

transmission routes of *Microsporidia MB* in *Anopheles* mosquitoes were investigated. To gain an understanding of various modes of horizontal transmission, larvae fed onhomogenate from infected mosquito tissues and/or allowed to exist in the same environment with infected larvae did not result in the transfer of *Microsporidia MB* to the uninfected cohorts. It was observed that horizontal transmission of *Microsporidia MB*occurred when adult mosquitoes were maintained together in cages. Notably, transmission was only observed in cages that had opposite sexes of *Microsporidia MB* infected and uninfected adult *An.arabiensis* suggesting that *Microsporidia MB*is transmitted sexually. The reproductive organ of *Anopheles gambiae s.l.* males has accessory glands that store seminal fluid, which is transferred to females upon insemination. The components are digested in the female atrium several days after insemination (Giglioli & Mason, 1966). Interestingly, these females only mate once in their lifetime after which they undergo postmating switch that ensures refractoriness to further mating in what is termed as enforced monogamy (Gabrieli *et al*., 2014). This phenomenon is not common among insects and is linked to steroid hormone 20-hydroxyecdysone transferred to females during insemination (Gabrieli *et al.*, 2014). The sperms acquired by females are stored in a dedicated organ called the spermatheca (Tripet *et al.*, 2003).

Findings from this study reveal that intensities of *Microsporidia MB* were much higher in the male gonads and their midgut tissues with fewer infections in the carcasses. This indicates that *Microsporidia MB* has high affinity for midgut tissues which could be

acting as a reservoir or is likely to undergo proliferation and migrates to these tissues. Interestingly, multinucleate *Microsporidia* cells were observed in the male ejaculatory ducts and these were detected by qPCR in the seminal fluid. It is possible that *Microsporidia MB* in the male gonad undergoes a series of developmental stages in the ejaculatory duct and is secreted in the seminal fluid. These observations coupled with the visualization of sperms in females that acquired *Microsporidia MB*is indicative that it could be transmitted through sexual route. In summary, it is likely that the sporogenesis of *Microsporidia MB*in the male ejaculatory ductproduces infectious spores that are released with seminal secretions and therefore transferred to females upon mating. Transmission from female to male *An. arabiensis* was also observed, but further investigation will be required to establish the basis of this transmission route.

Notably, transmission was observed from infected male *Anopheles* to female *Anopheles* mosquitoes. The uninfected males also acquired infection from infected females which indicate that *Microsporidia MB* is likely to exploit bi-directional transfer. This is a strong indication of sexual transmission of *Microsporidia MB* from male to female mosquitoes. Interestingly, the absence of *Microsporidia MB* in same sex cohorts and the presence of sperms in the spermatheca of females acquiring *Microsporidia MB*suggest that horizontal transmission could be happening through venereal/sexual route. Venereal transfers of microsporidian symbionts have been reported in several insects known to harbor these symbionts (Knell & Webberley, 2004). The *Anopheles gambiae s.l.* are monandrous because their females undergo enforced monogamy

(Gabrieli *et al.,*2014)suggesting that *Microsporidia MB* could be exploiting other forms of transmission. Symbionts that are sexually and vertically transmitted are believed to attain high levels of prevalence than when only one route is relied upon (Knell & Webberley, 2004).

Female *Anopheles* mosquitoes that became infected were able to subsequently transmit *Microsporidia MB* vertically to their offspring suggesting the occurrence of both venereal/horizontal and vertical modes of transmission. This corroaborates previous observations that sexual transmission of *Serratia* AS1 bacteria in *An. stephensi* occurred when infected males were allowed to mate with the uninfected females(Favia *et al*., 2007). *Serratia*was found to exploit multiple modes of transmission including vertical, horizontal, and transstadial routes, and did not impair host fitness (Wang *et al*., 2017). Another symbiont *Asaia* that is dominant in male and female reproductive tissues of *An. stephensi*(Favia *et al*., 2007), was also shown to undergo venereal transmission from the infected males to uninfected females and subsequent vertical inheritance by 27-68% of the offspring(Damiani *et al*., 2008). The observation that a *Microsporidia MB*infected male transferred infection to females that gave rise to infected adult offsprings (7.69-33.33%) is indicative of persistent transmission that exploits venereal, vertical, and transstadial routes of transmission.

The efficiency of sexually transmitted symbionts varied from 0% to 100%, and this is attributed to factors such as time of mating and infection status (Magalon *et al*., 2010; Moran & Dunbar, 2006). It is possible that the low rate of transmission could have

been influenced by unknown male-to-female mating ratios and the maturity of mating partners. Besides, some larvae of the offspring died before pupation or during hatching process, and this made it difficult to obtain representative data, but it is suspected that the infection statuses could be higher. Importantly, the number of infected females or the densities of *Microsporidia MB* infections in females was not correlated with the densities of *Microsporidia MB* in donor males. Possibly, this observation is linked to the localization of symbiont to the male gonad, which is a pre-requisite for sexual transmission and that only the intensity of *Microsporidia MB* infection in gonads is correlated with transmission capacity. It is also possible that the midgut acts as a *Microsporidia MB* reservoir, and functions in maintaining *Microsporidia MB* infection in *An. arabiensis*.

Studies on the biology of *Anopheles* males infected with *Microsporidia MB* is useful to underscore whether these males could induce the uninfected females to be non-receptive, and/or whether infected females could prefer mating with uninfected males as opposed to the infected ones. The occurrence of both venereal and vertical transmission favours dissemination of *Microsporidia MB* in the wild and could be helpful when considering ways of increasing the prevalence of infection.These findings suggest that *Microsporidia MB* infected male mosquitoes could be released as a strategy to increase *Microsporidia MB* in wild *Anopheles* populations.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

The study purposed to identifycultivable fungi colonizing and stably associated with the *Anopheles gambiae* species, the main vector for human malaria parasite. The findings indicate that fungi isolated from *An. gambiae*maintained in semi-field setting could be re-introduced in these hosts and found to invade these vectors without causing deleterious developmental effects on these hosts. One of these fungi namely *Leptosphaerulina* sp. was found to establish a stable co-existence across developmental stages of *Anopheles gambiae* and field survey indicate that it occursnaturally in the wild collected species of *An. gambiae* complex.*Leptosphaerulina* sp. is transstadially transmitted and persistsacross generations. Investigation of the effects of *Leptosphaerulina* on the *Anopheles* host fitness indicates that it does not affect larvae development, pupation and adult survival. Experiments to establish a possible mechanism utilised by this fungus to persist in the hosts indicate that *Leptosphaerulina*induced the deposition of melanin in the infected host resulting in the boosting of their basal immunity. It is possible that these phenotypes are useful for the observed transstadial and subsequent establishment in the mosquito host. Besides, the presence of protein coding genes connected to KEGG pathways signify that *Leptosphaerulina* could be playing a nutritional role in these mosquitoes. Experiments with *Plasmodium falciparum* did not reveal an apparent parasite blocking, and two possible reasons could explain this subtle association. First, *Anopheles* hosts with

*Leptosphaerulina*sp infection became susceptible to infection with *Plasmodium*hence the lack of *Plasmodium*protection from the parasite. Second,the growth of fungi in the midgut occupied the space that could otherwise be utilized by the invading pathogen.

A non-culturable fungus in *An. arabiensis*named *Microsporidia MB*was found to exploit horizontal transmission to spread through *Anopheles* population. It was found to exploit sexual/venereal transmission because infected individuals of opposite sex were found to cause transmission through insemination as evident by the presence of sperm in females acquiring *Microsporidia* and the presence of *Microsporidia* in the male seminal fluid. Females acquiring *Microsporidia* from the infected males gave rise to infected offspringsuggesting that infection acquired through horizontal means was followed by vertical and transstadial transmission. These underpinnings are useful in the context of developing a symbiont-based tool for the rapid dissemination of novel symbiont with the potential to managemalaria because male mosquitoes do not bite or carry malariaparasites and could be deployed for the release and dissemination of a *Plasmodium* transmission blocking *Microsporidia MB*.

Recommendations for further studies

1. There is need to undertake deep sequencing targeting uncultured fungi, which could reveal *Anopheles* mycobiomes that were not isolated by tissue culture.
2. Whether these symbionts establish association with the mitochondrial hyplotypes of the host is appreciable and is necessary to validate the occurrence of maternal

transmission. Presence of several mtDNA haplotype could suggest the occurrence of paternal transmission associated with an old infection. A metabolomics study to profilesecondary metabolites secreted by *Leptosphaerulina*sp could further inform the strategies utilized by this fungal symbiont to invade and co-exist with the host vectors. It is possible to extract either peptides or secondary metabolites from this fungus and study their direct effects on these hosts. Besides, transformation of *Leptosphaerulina*sp could also validate its direct utilisation in paratransgenesis. There is need to further screen other species of mosquitoes such as *Culex* and *Aedes* and document whether *Leptosphaerulina*sp is specialists and/or generalist and whether it exerts the same traits in these hosts.

3. Whether bacterial symbionts co-existing with these fungal symbionts could be contributing to host fitness would need to be established.
4. Further, there is need to establish types of serpins involved in fungus melanization and unravel whether these molecules play a role in subtle association with *P.falciparum*.
5. Validation of paternal transmission of *Microsporidia MB* in the semi-field screen houses is needed before undertaking large-scale field releases of infected males to underscore mating competitiveness of the infected males.

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APPENDICES

Appendix 1: List of oligonucleotide primers utilized in the amplification experiments

		Real-Time QRT-PCR primers used to quantify specific transcripts	
Accession no.	Gene acronym	Primer Sequences (5’ → 3’)	References
M27607	ITS1	TCCGTAGGTGAACCTTGCGG	(White et al., 1990)
	ITS4	TCCTCCGCTTATTGATATGC	
MT433814	Lepto379F	CGAGCGTCATCTACACCCTC	This study
	Lepto566R	CCCTACCTGATCCGAGGTCA	
	Lepto521F	CCACTGAGGTCAAGTCCGTC	
MT433814	Lepto896R	CACATGGGCTTGGAGGGAAT	This study
AGAP010592	S7_F	TCCTGGAGCTGGAGATGAAC	(Bahia *et al*., 2014)
	S7_R	GACGGGTCTGTACCTTCTGG	

Accession no.	Gene acronym	Primer Sequences (5' → 3')	References
AGAP005203	PGRP - LCF	AGAATACCACACTAAGGCACAGT	
	PGRP - LCF	AGACTTACGATCCTGGTAAATGT	(Stephan Meister *et al*., 2009)
AGAP000693	Cecropin_1F	CCAGAGACCAACCAACCACCAA	(Bahia *et al*., 2014)
	Cecropin_1R	CGACTGCCAGCACGACAAAGA	
AGAP011197	FBN9F	CCAAGATGTCGGGCAAGTAT	(Bahia *et al*., 2014)
	FBN9R	TTGTGGTACGTCAGCGAGTC	
AGAP010815	TEP1F	ATGCTCTGCTGTCGTTTGTG	(Bahia *et al*., 2014)
	TEP1R	TTCGTGTCCTCCGGTATTTC	
AGAP006348	LRIM1F	CATCCGCGATTGGGATATGT	(Bahia *et al*., 2014)
	LRIM1R	CTTCTTGAGCCGTGCATTTTC	
AGAP004581	HSP70F	ACGCCAACGGTATTCTGAAC	
	HSP70R	ACAGTACGCCTCGAGCTGAT	(Sim *et al*., 2007)

Accession no.	Gene acronym	Primer Sequences (5' → 3')	References
AGAP008212	CP6M2F	AGGTGAGGAGAGTCGACGAA	(Sim *et al*., 2007)
	CP6M2R	ATGACACAAACCGACAAGG	
AGAP011294	Defensin_1F	GCGGTTCCAAAGTTCCGACA	
	Defensin_1R	AGCGGGACACAAAATTGTTC	(Bahia *et al*., 2014)
AGAP006747	Rel2F	CGGAGAAGTCGAAGAAAACG	
	Rel2R	CACAGGCACACCTGATTGAG	(Bahia *et al*., 2014)

Appendix 2: Ethical clearance

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel: (254) (020) 2722541, 2713349, 0722-205901, 0733-400003, Fax: (254) (020) 2720030
E-mail: director@kemri.org, info@kemri.org, Website. www.kemri.org

KEMRI/RES/7/3/1 October 31, 2016

**TO: DR. JEREMY HERREN, (PRINCIPAL INVESTIGATOR),
INTERNATIONAL CENTRE OF INSECT PHYSIOLOGY AND ECOLOGY (ICIPE),
P.O BOX 30772-00100,
NAIROBI, KENYA**

Dear Sir,

RE: PROTOCOL NO. NON-KEMRI 545 (*RESUBMISSION OF INITIAL SUBMISSION*): SYMBIOTIC MICROBES AND VECTOR COMPETENCE: CHARACTERIZATION AND ISOLATION OF CANDIDATES FOR MALARIA TRANSMISSION BLOCKING

Reference is made to your letter dated 13th October 2016. The KEMRI/Scientific and Ethics Review Unit (SERU) acknowledges receipt of the revised study documents on the October 19, 2016.

This is to inform you that the Committee noted that the issues raised during the 255th Committee A meeting of the KEMRI Scientific and Ethics Review Unit (SERU) held on **13th September 2016** have been adequately addressed.

Consequently, the study is granted approval for implementation effective from **31st October 2016** for a period of one year. Please note that authorization to conduct this study will automatically expire on **October 30, 2017.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuation approval to the SERU by **September 18, 2017**.

You are required to submit any proposed changes to this study to SERU for review and the changes should not be initiated until written approval from SERU is received. Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of the SERU and you should advise the SERU when the study is completed or discontinued.

You may embark on the study.

Yours faithfully,

**DR. EVANS AMUKOYE,
ACTING HEAD,
KEMRI/SCIENTIFIC AND ETHICS REVIEW UNIT**

In Search of Better Health

Appendix 3: Consent Form

International Centre of Insect Physiology and Ecology (ICIPE)

PO Box 30, Mbita Point, Kenya

Mobile number: 0722 369 254

Email: psawa@icipe.org

Who should I contact if I have questions on my rights as a volunteer in this research study: If you have any question on your rights as a volunteer, you or your parent should contact.

The Secretary, Kenya Medical Research Institute, Scientific and Ethical Review Unit.

C/o Kenya Medical Research Institute

P.O. Box 54840, Nairobi, Kenya

Tel. 254-20-2722541 or mobile 0717719477

Email: seri@kemri.org

IF THERE IS ANY PORTION OF THIS CONSENT AGREEMENT THAT YOU DO NOT UNDERSTAND, PLEASE TALK TO SOMEONE ON THE STUDY TEAM BEFORE SIGNING.

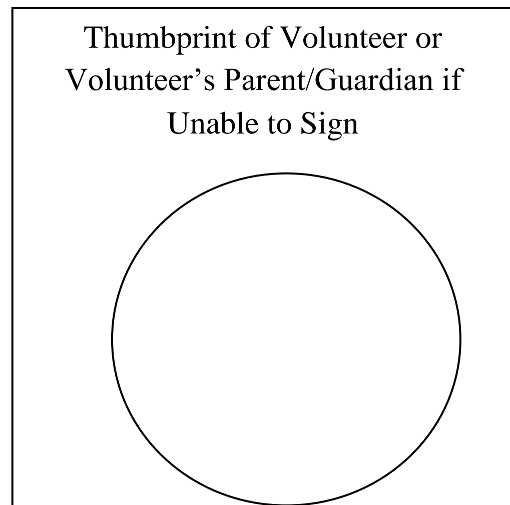
Subject's or Parent/Guardian's Signature: ____________________Date: ________

Permanent Address: ________________________________

Witness's Name: ___________________________________

Witness's Signature: ________________________________ Date: ________

Study Number: ______________


Thumbprint of Volunteer or Volunteer's Parent/Guardian if Unable to Sign

Person Administering Consent:

Name:____

Signature: ____

Informed consent agreement (blood collection)

What is the study called: **Symbiotic microbes and Vector Competence: Isolation and Characterization of Candidates for Malaria Transmission Blocking**

What is this study about: Malaria is transmitted by mosquitoes, however some mosquitoes are better at transmitting malaria than others. Scientist have found that mosquitoes can also be infected by other microbes and that these microbes may make mosquitoes unable to transmit malaria. We are trying to identify microbes that infect mosquitoes and make them unable to transmit malaria. We therefore need malaria infected blood to feed mosquitoes and determine if they can transmit malaria after being infected with these microbes.

Who is running the study: The study is being run by Dr Jeremy Herren, Dr. Daniel Masiga, JandouweVillinger and, Dr. Patrick Sawa (Head, St. Jude's Clinic, Thomas Odhiambo Campus, icipe). We will collaborate with other scientists at icipe, and MoPHS.

Do I have to participate: Participation in this study is voluntary. There is no penalty for refusing to participate. If you start the study you (your child) may discontinue your (your child's) participation at any time without any explanation. The principal investigators and co-investigators from ICIPE and MOPHS may decide to withdraw you (your child) from the study if we are unable to obtain a blood sample from you (your child).

What will happen to me if I participate in the study: You will be asked some questions, then tested for malaria. If you are found to have malaria in your blood, blood will be taken from a vein in your arm. We will then treat you for malaria at no cost to you. We will not test for HIV.

Are there any risks if I participate in the study: There is the possibility of mild discomfort, bruising and very rarely infection at the site where the blood is taken. But, should you (your child) be injured as a direct result of participating in this research project, you (your child) will be provided medical care, at no cost to you (your child), for that injury. You (your child) will not receive any injury compensation, only medical

care. You should also understand that this is not a waiver or release of your or your child's legal rights. You should discuss this issue thoroughly with the principal investigator before you or your child enroll in this study.

Are there any benefits from the study: The study can lead to a better understanding of the microbes that prevent malaria transmission. The information obtained will be used to develop new tools to control malaria transmission and hopefully contribute to a decline in malaria in the community and the continent as a whole. Volunteers will also find out if they are infected with malaria and if so receive treatment free of charge.

Will there be any compensation for being in the study: There is no compensation to volunteers for their participation.

How long does the study last: This study requires completion of a short questionnaire and a rapid malaria test. If you are found to be infected with malaria you will be requested to go to the icipe TOC St. Jude's clinic to for a blood draw and to receive treatment. The entire process will take about 30 minutes.

Who can participate in this study: Children with the consent of a parent / legal guardian.

Who will be able to see my information or lab results: Any information about you (your child's) will remain confidential. Only the people involved in the study will be able to see your information. We will keep all files in locked cabinets when they are not in use, and all blood stored in locked freezers. Your (your child's) name will not be used in any report resulting from this study. Any report from this study will refer to you/your child only by a study identification number and not by a name. All blood samples collected will be labeled with a study identification number; no names will be used.

What will happen to my blood: Your (your child's) blood will be tested for malaria. If positive a small amount of this blood will be drawn for to study ways to block mosquitoes from transmitting malaria. A sample of your blood will be kept frozen in case we want to do more testing on it in the future. These samples will be labeled with only your study number. They will be secured in freezers at ICIPE facilities and only study investigators and their authorized staff will have access. All safeguards ensuring privacy and confidentiality that are in place during this study period will also continue to be in place for the long-term storage of samples and if samples are sent outside of

Kenya, no personal identifiers will be included. If you do not wish for your blood to be stored you can opt out of this.

If we do need to use the stored blood in the future we will first get permission from the Kenya National Ethical Review Committee.

Who can I contact about the study or my rights as a volunteer in this research study: If during the course of this study, you have questions concerning the nature of the research or you believe you have sustained a research-related injury, you should contact:

Who can I contact if I need information on the conduct of the study:

If you have any question you or your parent should contact:

Dr. Jeremy Herren

Molecular Biology and Bioinformatics Unit

International Centre of Insect Physiology and Ecology (ICIPE)

Duduville, Kasarani

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International Centre of Insect Physiology and Ecology (ICIPE)

PO Box 30, Mbita Point, Kenya

Mobile number: 0722 369 254

Email: psawa@icipe.org

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C/o Kenya Medical Research Institute

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Email: seri@kemri.org

IF THERE IS ANY PORTION OF THIS CONSENT AGREEMENT THAT YOU DO NOT UNDERSTAND, PLEASE TALK TO SOMEONE ON THE STUDY TEAM BEFORE SIGNING.

Subject's or Parent/Guardian's Signature: ___________Date: ________

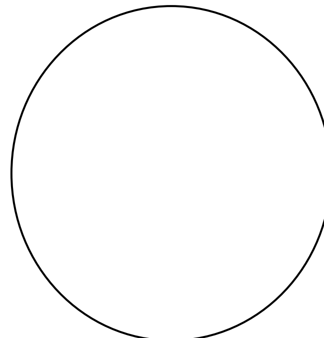
Permanent Address: ______________________________

Witness's Name: _________________________________

Witness's Signature: _________________________Date: ________

Study Number: _____________

Thumbprint of Volunteer or Volunteer's Parent/Guardian if Unable to Sign



Person Administering Consent:

Name:____

Signature: ____

Appendix 4: Individual informed consent agreement

Individual informed consent agreement

I, _______________________(name of parent/legal guardian) being the legal representative of (name of the participant)_______________________, certify giving hereby my consent for the child to participate in the research project titled: Symbiotic microbes and Vector Competence: Isolation and Characterization of Candidates for Malaria Transmission Blocking. I understand that I may feel some discomfort during the procedure and I understand that although the risks are minimal. I am aware that there will be no benefit apart from those described in the first part of this form to either my child or myself personally.

I have read the foregoing information, or it has been read to me. I have been given the opportunity to ask questions concerning this project. Any such questions have been answered to my full satisfaction. I have been provided with the name of the Project Coordinator who I may contact if any further questions arise concerning the rights of the child.

I consent voluntarily to participate in this study and I understand that I have the right to withdraw from the study at any time without penalty or loss of benefits.

Print Name and Signature ____________________________

Date___________________

Day/month/year

--

To opt out of blood storage for future use:

I consent voluntarily to participate in this study and I understand that I have the right to withdraw from the study at any time without penalty or loss of benefits. I do not wish to have my blood stored for future use

Print Name and Signature ____________________________

Date___________________

Day/month/year

--

If illiterate

I have witnessed the accurate reading of the informed consent form to the parent/legal guardian of the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Print name and Signature of Witness______________________ Thumb print of parent

Date______________________

Day/month/year

--

I have accurately read or witnessed the accurate reading of the informed consent form to the parent/legal guardian of the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Print Name of Researcher____________________ Signature ____________________

Date_______________________

Day/month/year

A copy of this Informed Consent Form has been provided to the parent or legal guardian of the participant ____________________ (initialed by researcher/assistant)

Appendix 5: Individual Recruitment information

Site/Location ----------------- Date of screening: ------------------- Serial Number -----------
-

Identification code: --------------------------

IDENTIFICATION

First Name: --------------------------------- Surname: ---

Sex: --------- Age: ----------------- or Date of Birth ---

District: ----------------------------- Locality: --

Parent/Guardian: --

CLINICAL PRESENTATION

ID number: ------------------- Weight: ------------------ Body temperature ---------------------

I-Major complains and symptoms: (yes = 1, No = 0)

Shiver ------- Abdominal pain -------- Vomiting -------- Dhiarrea -------- Headache --------

Constipation ------- Convulsion ------- Aching pains -------- Cough ------- Asthnia --------
-

Fever --------- Initial date of feverache ---------------------------- Anemia ---------------------
-

Previous drug history: date ------------------ type of drugs ------------------- dosage ---------
--

(within the last two weeks)

Other complains and signs ---
-

-

II- Parasitological presentation

Thick blood film (Positive = +, Negative = 0) -------------- Species --------------------------
-

Asexual parasitemia (relative) --------------------- (absolute) --------------------
parasite/mm3

Gametocytemia (relative) -------------------------- (absolute) ------------------------gcts / mm3

TREATMENT

Drug: 1) -- dosage --

2) -- dosage --

Rendez-vous: --

LABORATORY EXPERIMENTS

Blood collection (yes = ok, No) ----------------- Volume collected: ------------------------ml

Experimental infections ---

Observations --

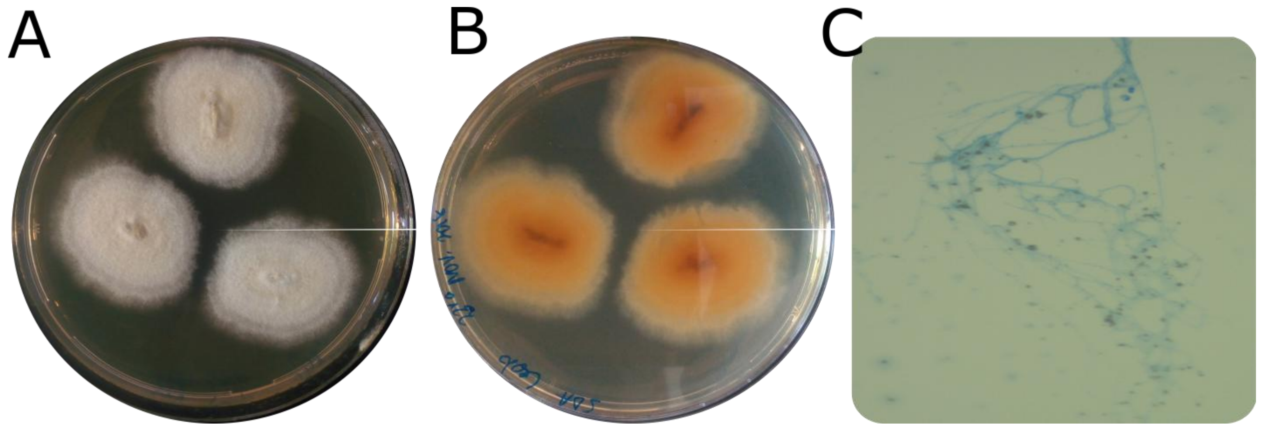
Appendix 6: Macroscopic description of isolated fungi colonies

Isolate Code (GenBank Accession no.)	Related Taxa (GenBank Accession no.)	Colony description Compartment isolated
Isolate 2	*Hyphopichiaburtonii*(KY103604)	White mycelium
Isolate 4	*Hyphopichia sp.* (KY103604)	White mycelium
Isolate 5	*Penicillium georgiense*(NR_121325)	Spherical blue colonies in clusters
Isolate 6	*Marasphaeriumgattermannii*(HQ322127)	White mycelium
Isolate 8	*Lichtheimiahyalospora*(NR_111440)	Black/dark mycelium
Isolate 11	*Periconia sp.* (LN813031)	White hyphae
Isolate 13B	*Leptosphaerulinachartarum*(KJ863505)	Septate pseudo hyphae
Isolate 16	*Cladosporium cladosporioides*(KP689176)	Black/dark mycelium
Isolate 19	*Marasphaeriumgattermannii*(HQ322127*)*	Black/dark mycelium
Isolate 20	*Marasphaeriumgattermannii*(HQ322127)	Black/dark mycelium
Isolate 22	*Marasphaeriumgattermannii*(HQ322127)	Black/dark mycelium
Isolate 23	*Cladosporium cladosporioide*(KF159973)	Black/dark mycelium
Isolate 24	*Aspergillus niger*(KX426976*)*	Black/dark conidia, filamentous
Isolate 25	*Cladosporium sp. Strain LCZ2* (KY643766)	Black/dark spores

Isolate 29	*Marasphaeriumgattermannii*(HQ322127)	Black/dark mycelium
Isolate 30	*Hasegawazymalactosa*(FJ515208)	Maroon colonies
Isolate 34	*Cladosporium tenuissimum*(MF319902)	Black/dark mycelium
Isolate 43	*Aspergillus tamari* (NR_135325)	Brown/yellowish colonies, spore forming
Isolate 44	*Fungal sp.* (KY404943)	White spores, filamentous
Isolate 48	*Dothideomycetessp*(KX909022)	Black/dark mycelium
Isolate 55	*Epicoccumsp*.(KX965725)	Yellow/orange colonies darkens with age
Isolate 50	*Alternaria alternata*(MF099865)	Hyphae, darkened spores, septate
Isolate 57	*Alternaria brassicae*(KU844330)	Hyphae, darkened spores, septate
Isolate 58	*Lichtheimiahyalospora*(NR_111440)	Darkened spores
Isolate 61	*Marasphaeriumgattermannii*(HQ322127)	Black/dark mycelium

Suppl Fig 1: colony appearance of isolated fungi

Appendix 7: Morphology of *Leptosphaerulina*sp fungus isolated from semi-field mosquitoes.



The isolate is characterized by white colonies (viewed from culture surface) that form septate-hyphae and spore when stained**.** Pure isolates obtained from sub-cultured isolates of the midgut dissection were maintained on Saboroud Dextrose Agar media (**A** is top view of culture on a plate, **B** is the bottom view). Cultures were stained in Lactophenol blue stain for morphological visualization under microscope (**C**).

Appendix 8: Manuscripts published or accepted

PLOS ONE

RESEARCH ARTICLE

The fungus *Leptosphaerulina* persists in *Anopheles gambiae* and induces melanization

Godfrey Nattoh[1,2]*, **Joel L. Bargul**[1,3], **Gabriel Magoma**[2,3], **Lilian Mbaisi**[1], **Hellen Butungi**[1,4], **Enock Mararo**[1], **Evan Teal**[1], **Jeremy Keith Herren**[1]

1 International Centre of Insect Physiology and Ecology, Nairobi, Kenya, **2** Pan African University Institute for Basic Sciences Technology and Innovation, Nairobi, Kenya, **3** Department of Biochemistry, Jomo Kenyatta University of Science and Technology, Nairobi, Kenya, **4** Wits Research Institute for Malaria, University of the Witwatersrand, Johannesburg, South Africa

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OPEN ACCESS

Citation: Nattoh G, Bargul JL, Magoma G, Mbaisi L, Butungi H, Mararo E, et al. (2021) The fungus *Leptosphaerulina* persists in *Anopheles gambiae* and induces melanization. PLoS ONE 16(2): e0246452. https://doi.org/10.1371/journal.pone.0246452

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Abstract

Anopheles mosquitoes are colonized by diverse microorganisms that may impact on host biology and vectorial capacity. Eukaryotic symbionts such as fungi have been isolated from *Anopheles*, but whether they are stably associated with mosquitoes and transmitted trans-stadially across mosquito life stages or to subsequent generations remains largely unexplored. Here, we show that a *Leptosphaerulina* sp. fungus isolated from the midgut of *An. gambiae* can be stably associated with *An. gambiae* host and that it imposes low fitness cost when re-introduced through co-feeding. This fungus is transstadially transmitted across *An. gambiae* developmental stages and to their progeny. It is present in field-caught larvae and adult mosquitoes at moderate levels across geographical regions. We observed that *Leptosphaerulina* sp. induces a distinctive melanotic phenotype across the developmental stages of mosquito. As a eukaryotic symbiont that is stably associated with *An. gambiae* *Leptosphaerulina* sp. can be explored for paratransgenesis.

Front. Microbiol., 28 July 2021 | https://doi.org/10.3389/fmicb.2021.647183

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Horizontal Transmission of the Symbiont *Microsporidia MB* in *Anopheles arabiensis*

Godfrey Nattoh[1,2†], Tracy Maina[1†], Edward E. Makhulu[1], Lilian Mbaisi[3], Enock Mararo[4], Fidel G. Otieno[1], Tullu Bukhari[1], Thomas O. Onchuru[1,5], Evan Teal[1], Juan Paredes[1], Joel L. Bargul[1,2], David M. Mburu[6], Everline A. Onyango[7], Gabriel Magoma[2,8], Steven P. Sinkins[9] and Jeremy K. Herren[1*]

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Abstract
Importance Statement

The recently discovered *Anopheles* symbiont, *Microsporidia MB*, has a strong malaria transmission-blocking phenotype in *Anopheles arabiensis*, the predominant *Anopheles gambiae* species complex member in many active transmission areas in eastern Africa. The ability of *Microsporidia MB* to block *Plasmodium* transmission together with vertical transmission and avirulence makes it a candidate for the development of a symbiont-based malaria transmission blocking strategy. We investigate the characteristics and efficiencies of *Microsporidia MB* transmission between *An. arabiensis* mosquitoes. We show that *Microsporidia MB* is not transmitted between larvae but is effectively transmitted horizontally between adult mosquitoes. Notably, *Microsporidia MB* was only found to be transmitted between male and female *An. arabiensis*, suggesting sexual horizontal transmission. In addition, *Microsporidia MB* cells were observed infecting the *An. arabiensis* ejaculatory duct. Female *An. arabiensis* that acquire *Microsporidia MB* horizontally are able to transmit the symbiont vertically to their offspring. We also investigate the possibility that *Microsporidia MB* can infect alternate hosts that live in the same habitats as their *An. arabiensis* hosts, but find no other non-anopheline hosts. Notably, *Microsporidia MB* infections were found in another primary malaria African vector, *Anopheles funestus s.s.* The finding that *Microsporidia MB* can be transmitted horizontally is relevant for the development of dissemination strategies to control malaria that are based on the targeted release of *Microsporidia MB* infected *Anopheles* mosquitoes.

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