ANOPHELES MICROBIOTA IN MALARIA VECTOR AND THE EFFECT ON PARASITE TRANSMISSION

Dita Paramytha Agustin[1)], Berlian Permata Dewi Erlambang[2)], Cizein Tri Cahyanti[3)], Syubbanul Wathon[4)], Rike Oktarianti[5)], Kartika Senjarini[6*)]
[1-6)] University of Jember
senjarini@unej.ac.id

Abstract

Malaria is a vector-transmitted disease with a high number of cases. Plasmodium parasites are transmitted from the body of the *Anopheles* mosquito to the host through several mosquito organs, including the salivary glands and the midgut. The salivary glands and the midgut, apart from being a breeding ground for parasites, are known from several studies that various types of microbiota inhabit these two organs. The group of bacteria is the most widely known to be associated with *Anopheles* mosquitoes. The bacteria found were dominated by Gram-negative bacteria, with Pseudomonas being the most common, followed by Aeromonas, Asaia, Comamonas, Elizabethkingia, Enterobacter, Klebsiella, Pantoea, and Serratia. The group of Gram-positive bacteria was represented by the genera Bacillus, Streptococcus, Lactobacillus, and Staphylococcus. Each species of *Anopheles* has a different composition of associated bacteria. *Anopheles*-associated bacteria currently receive much attention because of their role in fighting Plasmodium infection. The ability of malaria vector-associated bacteria to inhibit and fight Plasmodium infection is divided into three mechanisms. Bacteria can triger an immune response through the Immune-deficiency (IMD) pathway, which causes active anti-Plasmodium and *the production of antimicrobial peptides, increasing ROS synthesis and microbiota, which trigger hemocyte differentiation to protect against Plasmodium. This function related to inhibiting Plasmodium development needs further research so that later it can become an option for vector-based malaria control without damaging the sustainability of the environment.*

Keywords: *Microbiota, Anopheles, Plasmodium*

1. INTRODUCTION

Vector-transmitted diseases are still a health problem in several parts of the world. Malaria is an important vector-transmitted disease in tropical countries, with a death rate of 409,000 in 2019 (WHO, 2015). In Indonesia, based on the trend of positive malaria cases and the latest Annual Parasite Incidence (API) in 2019, 23% of the population still lives in endemic areas (Ministry of Health of the Republic of Indonesia, 2019). Malaria is a vector-borne disease in humans caused by the parasite Plasmodium sp. (Judge, 2011; Asmara, 2015).

Transmission of malaria begins when the blood-feeding process is carried out by female *Anopheles* mosquitoes (Pimenta *et al*., 2015). Plasmodium will be transmitted to the intestines (midgut) of mosquitoes through the sucked blood, spreads in the hemolymph and other tissues, and undergoes sexual development (Gendrin and Christophides., 2013). A week to 10 days later, the parasite will enter the mosquito's salivary gland to infect the next human host. The midgut and salivary glands are essential organs in the transmission of Plasmodium parasites.

The salivary glands and midgut, being a breeding ground for parasites, have been widely investigated in studies delving into various types of microbiota. The microbiota associated with mosquitoes consists of bacteria, fungi, protists, viruses, and nematodes (Minard *et al*., 2015; Thongsripong *et al*., 2017). Bacterial groups are the most widely known to be associated with mosquitoes, including the *Anopheles* mosquito as a malaria vector (Wilke *et al*., 2015). Bacteria can be found in several parts of the mosquito's body, including the head, salivary glands, reproductive organs (Sharma *et al*., 2014). Commensal bacteria in the midgut are known to suppress parasite

development and reduce the mosquito's ability to transmit parasites to new hosts, either by having an anti-plasmodial effect or by stimulating the mosquito's basal immune response to parasite development while in the mosquito's body (Osei *et al*., 2012; Sharma *et al*., 2014).

The presence of these bacteria is known to be one of the mosquito defense systems when the mosquitoes are infected with Plasmodium parasites and pathogens (Schneider *et al*., 2006; Kalappa *et al*., 2018). Immune activation of the immune system can also modulate mosquito defenses against malaria parasites (Meister *et al*., 2009; Bahia *et al*., 2013). Nevertheless, there is an overlap between the mosquito immune response in the form of antibacterial and antimalarial. As a result, some antibacterial immune genes have no impact on the development of Plasmodium.

Therefore, research on the mosquito microbiota has received significant attention in recent years. A new concept of inhibiting the transmission of Plasmodium parasites mediated by microbiota is currently being investigated as an alternative to malaria control that brings about a decent impact on the environment. This study wanted to elaborate microbial symbiont in the body of the *Anopheles* mosquito vector and its potential role on the transmission of Plasmodium parasites.

2. RESEARCH METHOD

This study involved extensive literature reviews pertinent to various types of microbiota found in *Anopheles* mosquitoes and play a role in the transmission of pathogens. The data was obtained from several journals, articles, and reliable data sources such as the 2019 Ministry of Health Report, Google Scholar, the WHO (World Health Organization) website, and the CDC (Center for Disease Control and Prevention) through comprehensive and systematic library research.

3. RESULT AND DISCUSSION

Anopheles Mosquito and Malaria Transmission

The *Anopheles* mosquito is the primary vector of malaria. *Anopheles* mosquitoes are often found in tropical and subtropical climates. This mosquito species can also survive at moderate temperatures and even survive in areas with low temperatures, such as Antarctica. There are currently three known *Anopheles* genera and more than 500 species globally (Harbach, 2013), but only 70-80 species are known to transmit malaria to humans worldwide (Fitriany and Ahmad, 2018). *Anopheles* species are grouped into complex species, namely *An. Annularis* complex, *An. Barbirostris* complex, *An. culicifacies* complex, *An. dirus* complex, *An. fluviatilis* complex, *An. leucosphyrus* complex, *An. maculatus* complex, *An. minimal* complex, *An. Philippine-nivipes* complex, *An. punctulatus* complex, *An. sinensis* complex, *An. subpictus complex*, and *An. sundaicus complex* (WHO, 2007).

Naturally, malaria transmission occurs because of the interaction between the agent (Plasmodium parasite), the definitive host (*Anopheles* sp. mosquito), and the intermediate host (humans). In the human body, Plasmodium multiplies in the liver and then infects red blood cells. Plasmodium species in humans involve *Plasmodium falciparum*, *P. vivax, P. ovale*, and *P. malariae* (White, 2008; WHO, 2015). Malaria transmission is influenced by the presence and fluctuation of the vector population, namely the *Anopheles* spp. (Judge, 2011; Bates, 1990).

Plasmodium develops and grows by invading human blood in the human body so that humans infected with this parasite will experience anemia and other disorders. Plasmodium reproduces in two ways, namely sexual reproduction (in the mosquito body) and asexual reproduction (in the human body). The life cycle of the Plasmodium parasite (Figure 1) in the asexual reproduction stage begins with the bite of a female *Anopheles* mosquito carrying Plasmodium sporozoites. When a mosquito bites a human, in addition to the exchange of blood, the sporozoites in the mosquito's salivary glands will also enter the human body bitten by the mosquito. Sporozoites penetrating the body will invade red blood cells in liver tissue (Hakim, 2011).

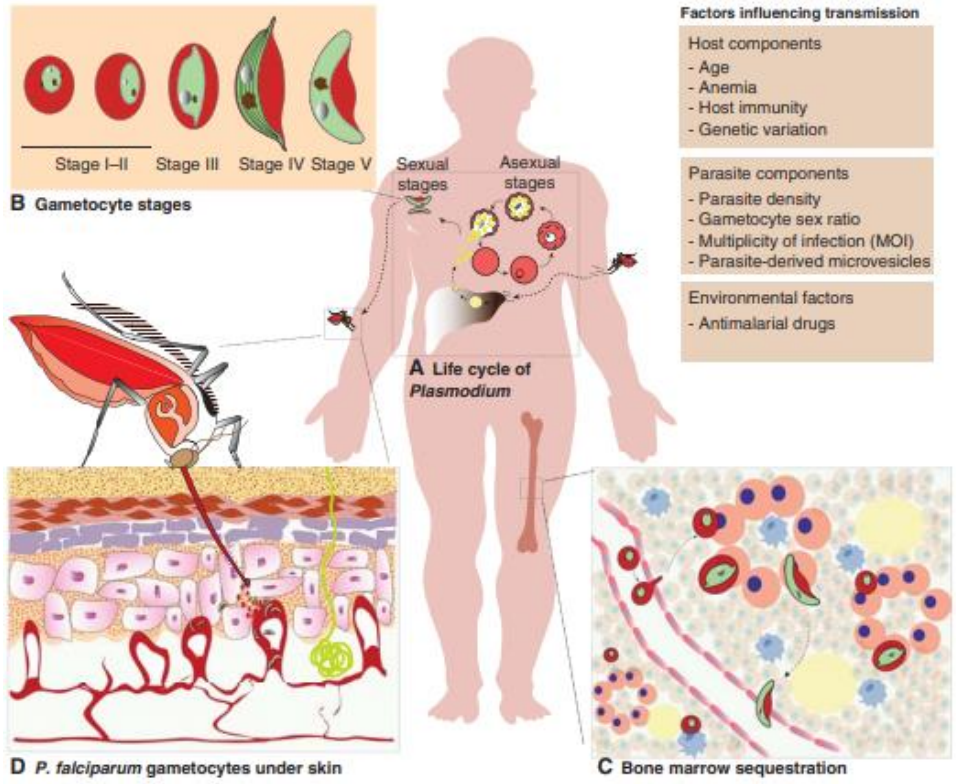

Figure 1. The Life Cycle of Plasmodium Parasites (Meibalan and Marti, 2017)

Sporozoites that successfully invade red blood cells will form gamete cells in the form of female gametocytes and male gametocytes. Gametocyte cells in the red blood cells of malaria sufferers will enter the female *Anopheles* mosquito when the mosquito sucks the blood of a malaria patient (Hakim, 2011).

The sexual reproduction stage is the stage after the mosquito sucks the blood of a malaria patient. The sucked blood also carries Plasmodium gamete cells. Plasmodium gamete cells that successfully enter the body of the female *Anopheles* mosquito will develop into sporozoites, after which these sporozoites will eventually reach the vector's salivary glands. The last stage is the transmission of Plasmodium by vectors. Mosquitoes that act as vectors will bite humans and transmit Plasmodium in their salivary glands. When mosquitoes suck blood, apart from spitting out saliva, they are also carried by Plasmodium, which enters the human body. Plasmodium will infect when the patient's immune system decreases (Judge, 2011).

Diversity of *Anopheles* Microbiota

Vital organs in the process of transmitting malaria parasites include the salivary glands and midgut of the *Anopheles* mosquito (Berhanu *et al*., 2019). Salivary and midgut glands play a role in mosquito defense through their diverse microbiota symbionts. The term microbiota denotes the microbial communities that live in contact with organisms' bodies, including bacteria, viruses, yeast, and protists (Gendrin and Christophides, 2013).

The microbiota composition has been studied in several *Anopheles* genera, especially by culture methods or molecular methods of sequencing the gene encoding 16S rRNA (Gendrin and Christophides, 2013). Forty-one genera are found in more than one species of *Anopheles*. *Pseudomonas* is the most prevalent genera, followed by *Aeromonas, Asaia, Comamonas, Elizabethkingia, Enterobacter*, *Klebsiella, Pantoea*, and *Serratia* (Gendrin and Christophides, 2013; Lindh *et al*., 2005). More details genera can be seen in Table 1.

Bacteria are microbiota commonly found in the body and are associated with the *Anopheles* mosquito (Gendrin and Christophides, 2013; Yilmaz *et al*., 2014). Generally, the bacteria found from Gram-negative bacteria are *Serratia marcescens, Klebsiella ozaenae, Pseudomonas aeruginosa, Escherichia coli*, and *Enterobacte*r spp. (Manguin *et al*., 2013). In addition, groups of gram-positive bacteria such as *Bacillus, Streptococcus* (Berhanu *et al*., 2019), *Lactobacillus* (Dong *et al*., 2009), and *Staphylococcus* (Ngo *et al*., 2016) were also identified. Each species of *Anopheles* has a different composition of associated bacteria. Currently, the bacteria associated with *Anopheles* as a malaria vector is getting a lot of attention because of their role in fighting Plasmodium infection.

Table 1. Bacterial Association of *Anopheles* Malaria Vector

Genus	***Class***	**Spesies *Anopheles***	
Microbacterium	*Actinobacteria*	*gambiae, stephensi*	Lindh *et al*., 2005
Rhodococcus	*Actinobacteria*	*arabiensis, stephensi*	Lindh *et al*., 2005
Elizabethkingia	*Flavobacteriia*	*gambiae, stephensi*	Kumar *et al*., 2010
Bacillus	*Bacilli*	*arabiensis, funestus, gambiae, stephensi*	Favia *et al*., 2007; d. Kajla *et al*.,2010; e. Chavshin *et al*., 2012
Clostridium	*Clostridia*	*gambiae*	Kambris *et al*., 2009
Enterococcus	*Bacilli*	*funestus, gambiae, stephensi*	Lindh *et al*., 2005
Lactobacillus	*Bacilli*	*gambiae, stephensi*	Kambris *et al*., 2009
Paenibacillus	*Bacilli*	*arabiensis, stephensi*	Kajla *et al*.,2010
Staphylococcus	*Bacilli*	*funestus, gambiae, maculipennis, quadrimaculatus, stephensi*	Kajla *et al*.,2010; Kumar *et al*., 2010
Streptococcus	*Bacilli*	*funestus, gambiae, stephensi*	Kumar *et al*., 2010
Acetobacter	*Alphaproteobacteria*	*stephensi*	Pumpuni *et al*., 1993
Asaia	*Alphaproteobacteria*	*coustani, funestus, gambiae, maculipennis, stephensi*	Kumar *et al*., 2010; Pumpuni *et al*., 1993; Kampfer *et al*., 2011
Citrobacter	*Gammaproteobacteria*	*darlingi, stephensi*	Kampfer *et al*., 2011
Comamonas	*Betaproteobacteria*	*dureni, funestus, gambiae, quadrimaculatus, stephensi*	Rani *et al*, 2009; k. Noden *et al*., 2011

Genus	***Class***	**Spesies *Anopheles***	
Enterobacter	*Gammaproteobacteria*	*albimanus, darlingi, funestus, gambiae, stephensi*	Lindh *et al*., 2005; Noden *et al*., 2011
Klebsiella	*Gammaproteobacteria*	*darlingi, funestus, gambiae, stephensi*	Noden *et al*., 2011; Osei *et al*., 2012
Methylobacterium	*Alphaproteobacteria*	*funestus, gambiae*	Kumar *et al*., 2010
Neisseria	*Betaproteobacteria*	*gambiae*	Kumar *et al*., 2010
Pantoea	*Gammaproteobacteria*	*albimanus, darlingi, funestus, gambiae, stephensi*	Kajla *et al*., 2010; Sharma *et al*., 2014
Pseudomonas	*Gammaproteobacteria*	*albimanus, darlingi, dureni, funestus, gambiae, maculipennis, quadrimaculatus stephensi*	Pumpuni *et al*., 1993; Kajla *et al*., 2010; Chavshin *et al*., 2012
Rahnella	*Gammaproteobacteria*	*stephensi*	Chavshin *et al*., 2012
Salmonella	*Gammaproteobacteria*	*funestus, gambiae*	Noden *et al*., 2011
Serratia	*Gammaproteobacteria*	*albimanus, dureni, gambiae, maculatus quadrimaculatus, stephensi, culicifacies*	Kajla *et al.*, 2010; Sharma *et al*., 2014
Yersinia	*Gammaproteobacteria*	*darlingi*	Kampfer *et al*., 2011

The Role of Microbiota in Mosquito Immunity

Anopheles sp. is similar to other mosquitoes with immune systems like other living things that function to kill, fight, or block the growth and development of pathogens in the body (Clayton *et al*., 2014). However, the pathogen that causes malaria, Plasmodium sp., must be able to survive in the mosquito body until the next blood-feeding process before being transmitted to humans. Based on this, pathogens that enter the mosquito's body through the blood-feeding process must go through a particular route, namely the midgut, hemocoel, and return to the salivary gland (Hillyer, 2010).

The immune system in the *Anopheles* midgut consists of physical and physiological defenses. Physical defense is a defense system that first plays a role in protecting the mosquito body when exposed to pathogens (Kumar *et al*., 2018). This defense system includes the cuticle on the exoskeleton, the epithelial tissue that makes up the epidermis, trachea, and midgut (Belachew, 2018; Kumar *et al*., 2018). Epithelial tissue in the midgut will secrete the peritrophic matrix, which functions in digesting blood-feeding in the posterior part (Kumar *et al*., 2018).

Pathogens that escape physical defense mechanisms in the midgut will face physiological defense mechanisms. This defense system is a process in the mosquito body that occurs due to pathogen infection as an immune response (Kumar *et al*., 2018). Immune response in *Anopheles* sp. can be divided into humoral and cellular immune responses. Humoral immune response in the form of synthesis of LRIM1 (leucine-rich repeat protein 1), APL1 (*Anopheles* Plasmodium-responsive leucine-rich repeat protein 1), or TEP1 (thioester-containing protein 1) cut complex which functions in

killing Plasmodium in the ookinete phase in the midgut. (Hillyer, 2010; Belachew, 2018), while the cellular response includes phagocytosis, melanization, and lysis by immune cells (Kumar *et al*., 2018; Belachew, 2018).

Immune cells in mosquitoes are contained in hemolymph and are called hemocytes (Belachew, 2018). These cells are transported throughout the body through the hemocoel. Hemocytes in *Anopheles* mosquitoes can be divided into three types based on their morphology: granulocytes, oenocytes, and prohemocytes (Dedkhad *et al*., 2019). Belachew (2018) stated that hemocytes also play a role in producing reactive oxygen species (ROS) and antimicrobial peptides (AMPs) when a pathogenic infection occurs. ROS in the mosquito body plays a role in killing Plasmodium in the ookinete phase through melanization and lysis mechanisms (Kumar *et al*., 2018), AMPs found in *Anopheles* mosquitoes include Defensins (Def1-5), Cecropins (Cec1-4), Gambicin (Gamb), and Attacin, also play a role in killing pathogens, but the mechanism is carried out by disrupting the integrity of the cell membrane [44]. AMPs in the mosquito body are synthesized through transcription and translation steps. AMPs transcription can be induced through three stages, namely the Toll pathway, Imd (Immune deficiency) or REL2 pathway, and JAK-STAT (Janus Kinase and Signal Transducers and Activators of Transcription) pathway (Clayton *et al*., 2014) (Figure 2).

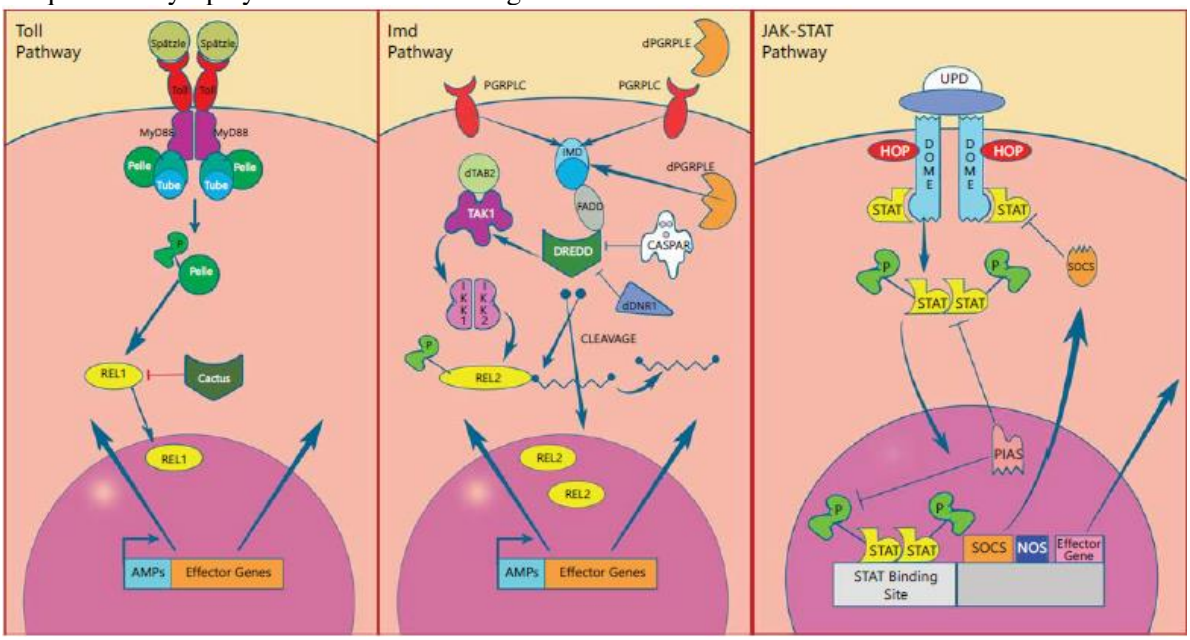

Figure 2. Mechanism of Toll pathway, Imd (Immune deficiency) or REL2 pathway, and JAK-STAT (Janus Kinase and Signal Transducers and Activators of Transcription) pathway (Source: Clayton *et al*., 2014)

These three stages are classified as molecular-based mosquito immunological defense mechanisms (Kumar *et al*., 2018). Based on Figure 2, the Toll pathway will take place if the pathogen, Plasmodium spp. In the ookinete phase, it binds to molecules in the mosquito's body, forming a ligand which then binds to the Toll receptor on the cell membrane. The binding between the ligand and the receptor causes a molecular reaction in the cell that activates REL1, then REL1 will translocate to the cell nucleus and cause the cell to undergo transcriptional regulation in the form of an increase in the transcription rate of genes encoding AMPs to kill pathogens (Clayton *et al*., 2014).

Another transcriptional step of AMPs is the Imd (Immune deficiency) or REL2 pathway. This stage has the same target killing as the Toll pathway, namely the

Plasmodium spp. ookinete, but the Imd pathway is considered more efficient in fighting Plasmodium falciparum infection in mosquitoes. The Imd pathway occurs when Plasmodium or bacteria binds to the PGRP-LC receptor on the cell membrane (Clayton *et al*., 2014). This mechanism causes a reshuffle of molecules in the cell until REL2 is activated (Clayton *et al*., 2014), but the process of REL2 activation is hardly known (Zakovic *et al*., 2017).

The final step of AMPs transcription, the JAK-STAT pathway, occurs when an unpaired (UPD) cytokine ligand binds to a domeless receptor (DOME) on the cell membrane. This binding causes STAT to translocate to the nucleus. STAT in the nucleus will attach to the STAT binding set region so that gene transcription can take place. This transcription process will produce AMPs and reactive nitrogen species (RNS) (Clayton *et al*., 2014). Based on the results, the genes transcribed in the JAK-STAT pathway are genes encoding AMPs and nitric oxide synthase enzymes. This process happened because the RNS in the mosquito's body will be formed when the nitric oxide synthase enzyme oxidizes L-arginine compounds. The enzyme nitric oxide synthase also plays a role in lysing *Plasmodium* spp. ookinetes in the mosquito midgut (Kumar *et al*., 2018). Several defense mechanisms in mosquitoes can be activated when initiated by bacterial symbionts. The symbiotic bacteria will activate several defense mechanisms in the midgut if there is an infection with a pathogen (Scolari *et al*., 2019).

The role of symbiotic bacteria as part of the mosquito immune system, especially in the midgut, is to inhibit infection and the incubation phase of pathogens through direct or indirect mechanisms (Guegan *et al*., 2018; Gao *et al*., 2020). In general, the ability of malaria vector-associated bacteria to inhibit and fight Plasmodium infection takes place through three mechanisms (Figure 3). First, bacterial growth triggers an immune response through the Immune-deficiency (IMD) pathway, which causes active anti-Plasmodium and also the production of antimicrobial peptides (Meister *et al*., 2009). Second, the Enterobacter (EspZ) strain isolated from An. arabiensis has been shown to directly affect the development of Plasmodium in the mosquito gut through increased ROS synthesis (Cirrimotich *et al*., 2011). Third, the microbiota will trigger hemocyte differentiation to protect against Plasmodium (Ngwa *et al*., 2013).

The *Anopheles* mosquito symbiont bacteria still have some known mechanisms to fight or inhibit Plasmodium that causes malaria. It is acknowledged that the genus Enterobacter (Esp_Z) has been shown to inhibit the formation of öokinet, oocyst, and sporozoites of *P. falciparum* in *Anopheles gambiae* up to 99% (Cirrimotich *et al*., 2011). In Ngwa's study (2013), another isolate, *Elizabethkingia meningoseptica*, contained ethyl acetate with antiplasmodial activity with an inhibitory concentration of 50% [IC50] and 0.25 mg/ml against *P. falciparum*. It exhibited antigametocidal activity and reduced the density of P. falciparum at a dose of IC50. Other bacteria, such as *Pantoea agglomerans* and *Asaia* spp., are known to have been successfully converted into antimalarial drugs (anti-plasmodial effector protein) (Wang *et al*., 2012). This effect can be a paratransgenic means to prevent malaria transmission, namely a strategy of blocking parasite transmission.

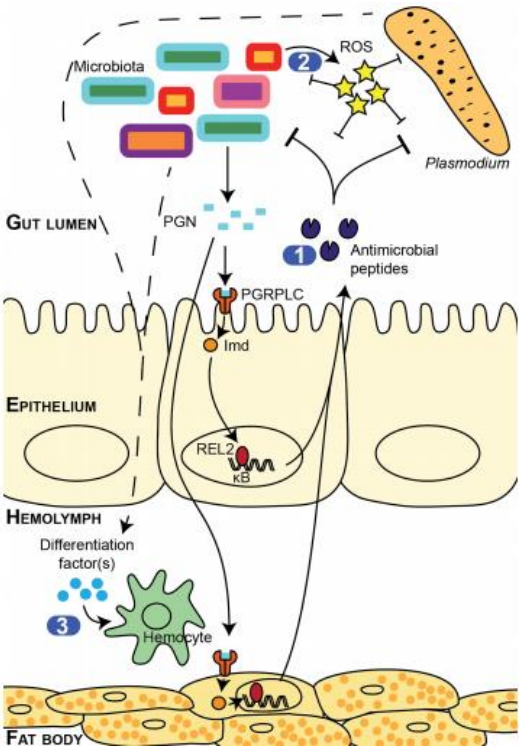

Figure 3. Mechanism of colonization resistance of *Anopheles* microbiota against *Plasmodium* infection: (1). The direct effect through ROS synthesis by strain; (2) Indirect effect through induction of NFkB antibacterial response; and (3) hemocyte differentiation by hemolymph (Gendrin and Christophides, 2013).

Other bacterial genera such as Serratia are also known to initiate active anti-Plasmodium. The Serratia Y1 strain studied by Bai *et al*. (2019) caused several genes (TEP1) regulated by the Toll and IMD pathways to become active and produce anti-Plasmodium substances. In addition, Serratia Y1 isolate is also known to produce C-types Lectin protein (CTLs) and serine protease CLIP which can interfere with Plasmodium development by limiting the number of ookinetes. Briefly, the mechanism of the microbiota in the mosquito midgut against Plasmodium infection (Figure 3). *Serratia marcescens* inhibits the development of *P. vivax* oocysts in *Anopheles albimanus* (Gonzales *et al*., 2003). The bacterial microbiota in *Anopheles* and its influence on the development of Plasmodium parasites as a potential means of controlling malaria transmission have been reported in various *Anopheles* species. However, the exact mechanism of their inhibitory activity is still poorly understood.

4. CONCLUSION

Based on literature research in the present study, some of the microbiota found in the *Anopheles* mosquito is known to be interrelated. This microbiota has the advantage of conducting more in-depth testing related to its ability to fight or inhibit the Plasmodium parasite that causes malaria. Not all of the bacterial species found have known resistance mechanisms against Plasmodium. With all the advances in science and technology, this study predicts that the microbiota found can be used as an option for effective and environmentally friendly malaria control in the future.

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