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Interplay of *Asaia bogorensis* and fibrinogen related proteins (FREPs) in the management of *Plasmodium falciparum*

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Abstract
Over the years it has been known that Acetic Acid Bacteria (AAB) has a role in fermentation. These obligate aerobes oxidize variety of sugars and alcohols. But, despite this role, some acetic acid bacteria too have a role in controlling malaria by regulating the growth and development of *Plasmodium falciparum* in *Anopheles stephensi*. These AAB seem to play a role in the stimulation of the immune system and the protection of the host against pathogens. One among them is a α-Proteobacterium, *Asaia bogorensis* that belong to the Asaia family. Being present in the midgut of *Anopheles stephensi*, it lowers the load of *Plasmodium falciparum* pathogen by secreting anti-plasmodium factors like Drosomycin, Cecropin, Defensin and Gambicin. Consequently, it blooms and its growth is not hampered neither by the immune system of the host nor by the pathogen. It also maintains an interconnected relationship with FREP proteins that are present in the abdomen of the insect host to further lower the pathogen concentration by acting as two barriers. Genetically engineered *Asaia bogorensis* can be injected into male *Anopheles stephensi* mosquito. While mating, this genetically engineered bacterium gets transferred to the female *Anopheles stephensi* mosquito in the wild. Through trans-ovarian transmission, this genetically engineered bacterium also passes to the next generation male and female mosquitoes. Thus, this genetically engineered *Asaia bogorensis* can acts as a tool for the management of falciparum malaria by impeding the development of malaria parasite inside the vector. So, the vector might fail to attain the infective stage for transmission and thereby blocking its transmission to humans. Further studies in this regard are required to prove this hypothesis.

Keywords: AAB, Asaia bogorensis, malaria, FREP, mosquito, humans

1. Introduction
Malaria is one of the most common infectious diseases in the world and one of the greatest global public health problems. The *Plasmodium falciparum* causes approximately 500 million cases each year and over one million deaths in sub-Saharan Africa (Simon I Hay *et al* 2009) [2]. Falciparum malaria is also endemic in various parts of Asia including India, causing some colossal health problems (Bhattacharya Sajal 2010) [3]. More than 40% of the world's population is at risk of malaria. Strategic steps (like use of insecticide, biological control methods etc.) have been used to control the falciparum malaria vector. In the recent time, microorganisms are emerging and evolving fast with its increased usefulness. As previously defined by Anton de Bary, symbiosis is the living together of organisms belonging to different species. In the field of medical entomology, investigations of the interactions between arthropods and microorganisms, focused mainly on the negative interactions, i.e., on pathogenic microorganisms transmitted to vertebrate hosts or on microorganisms pathogenic to the arthropod itself. By analysing the symbionts of different insect hosts, *Wolbachia* has always been shown to encompass a wide molecular diversity. But recently it has been investigated that Acetic acid bacteria has gained importance. Acetic acid bacteria (AAB) are environmental bacteria that can be found in different niches, like the flower of the orchid tree *Bauhinia purpurea* or are involved in processes like wine fermentation or spoilage. Over the years, it has been known that several AAB were discovered in association with arthropods, like *Gluconobacter morbifer* associated with *Drosophila melanogaster* or *Acetobacter tropicalis* associated with the olive fruit fly *Bactrocera oleae* (Bessem Chouaia *et al* 2010) [8]. These Acetic acid bacteria (AAB) are obligate aerobes, and presently comprise 14 genera assigned to the family Acetobacteraceae, including Acetobacter (Ac.), Gluconobacter (Go.), Gluconacetobacter (Ga.), Granulibacter (Gr.), Komagataeibacter (Ko., former

Gluconacetobacter), Asaia (As.), Acidomonas (Am.), Kozakia, Swaminathania, Saccharibacter, Neoasaia, Tanticharoenia, Ameyamaea, and Neokomagataea, of which *Asaia* plays an important role. *Asaia* is a genus of Gram-negative, aerobic and rod-shaped bacteria from the family of Acetobacteraceae.

These AAB incompletely oxidize a variety of sugars and alcohols (Mikihiko Kawai *et al*, 2015) [5]. Surprisingly, these AAB seem to play a role in the stimulation of the immune system and the protection of the host against pathogens (Bessem Chouaia *et al.* 2010) [8]. AAB of the genus *Asaia* (*Asia bogoriensis*), a α-proteobacterium, have recently been found associated with different insect species, like the malaria mosquito vectors *Anopheles stephensi* (*An. stephensi*) and *Anopheles gambiae* (*An. gambiae*) or the virus vector *Aedes aegypti* (*Ae. aegypti*) (Bessem Chouaia *et al* 2010) [8]. Much of the work hasn't been performed on *Asaia* sp. As an efficient inducible colonizer of mosquitoes, that transmit *Plasmodium* sp., *Asaia* sp. may be a potential candidate for malaria control (Guido Favia *et al*, 2007) [8].

2. Location and diversity

Asaia bogorensis occur in tropical plants. Flower and plant derived materials are the natural habitats of *Asaia* species. They have also been isolated from bottle fruit flavoured drinks (Guido Favia *et al*, 2007) [8]. As a micro symbiont, it is also found in the body of certain hosts. In anopheline mosquito species, it is found as an extracellular bacterium in the gut (of female *Anopheles stephensi*), the salivary glands, and reproductive organs (of both male and female *Anopheles stephensi*). Thus, *Anopheles stephensi* mosquitoes can take up *Asaia* bacteria from the environment, either from the natural habitats or through mating. 16S rRNA gene copies constitutes a mean of 41%, 25%, and 20% of the total 16S rRNA gene copies of the bacterial population in the gut, salivary glands, and female reproductive system, respectively, showing that this Acetic acid bacterium represents the dominant bacterium in the gut and this has been found more profoundly in *Anopheles stephensi*. It is transmitted vertically to the progeny by egg smearing and venereally from males to females, and then to the offspring. *Asaia bogorensis* is also transmitted horizontally, through the co-feeding of mosquitoes on the same food source (Bessem Chouaia *et al*, 2010) [8]. Because of diverse mode of transmission of *Asaia* spp. among mosquitoes, it indicates that these insects are potentially exposed to diverse strains, suggesting that differently symbiont form transmitted only (or predominantly) vertically. Several strains could coexist in different population of host species despite of the complexity of the harbouring host and even within single insect individuals. This is particularly due to difference in the expression of genes present on the surface of Asaia bogorensis. (Bessem Chouaia *et al.*, 2010) [8].

3. Barriers to *Plasmodium falciparum*

After the pathogen, *Plasmodium falciparum* enters into the body of *Anopheles stephensi* vector through proboscis, its movement is impeded by two barriers. First, by the naturally occurring *Asaia bogorensis* population that thrives in the midgut and second, by the Fibrinogen related proteins (FREPs) that mostly works in the abdomen, where naturally occurring Asaia population is absent.

a. Midgut: First Barrier to *Plasmodium falciparum*

Parasite development in the vector starts when a mosquito ingests an infected blood meal containing *Plasmodium* sexual forms, known as gametocytes. Gametocytes round-up (in case of *Plasmodium falciparum*), egress from the red blood cell (RBC) and differentiate into gametes. This takes place within 15 minutes. Male gametocytes undergo a drastic transformation known as exflagellation. This is the process by which the DNA replicates to 8N followed by the formation of eight haploid microgametes. Microgametes then detach from the exflagellation centre and actively search for female gametes to fertilise. Fertilisation gives rise to a diploid zygote that undergoes one round of DNA replication to become tetraploid. The resulting zygotes differentiate into motile ookinetes that migrate in the blood bolus to invade and traverse the mosquito midgut epithelium. The ookinete may traverse multiple epithelial cells before emerging from the basal side facing the haemocoel, where it lodges beneath the basal lamina (BL) and differentiates into a round oocyst. Within the next 10-14 days, each oocyst grows in size and undergoes sporogony to produce thousands of sporozoites. Upon oocyst maturation, these sporozoites are released into the haemolymph where they circulate with the haemolymph and subsequently invade the salivary glands. Following invasion, sporozoites lodge in the lumen of the salivary gland. When an infected mosquito feeds on a human host, sporozoites are released with the saliva and deposited in the skin. This results in the closing of transmission cycle. During this process, malaria parasites undergo dramatic losses during their development in the mosquito vector. Reduction in the population of malarial parasites occurs at each developmental step, from the formation of gametocytes in the human host to oocyst formation, resulting in very low parasite numbers. This reduction in number in the midgut of mosquito is mediated in part by the transition of the parasite from an intracellular (RBC) to extracellular forms, thus exposing the parasites to both human and mosquito components that are deleterious to the parasite. Out of the thousands of gametocytes that a female Anopheles mosquito typically ingests in a blood meal, only 50-100 develop into ookinetes and only around five survive to form oocysts. In the entire *Plasmodium* life cycle (in both human and mosquito hosts), parasite numbers are lowest during the ookinete stage and then quickly expand when each ookinete releases thousands of sporozoites via oocyst stage. For this reason, the midgut stages of parasite development constitute prime targets for strategies aiming to block malaria transmission (After Ryan C Smith *et al.*, 2014) [6].

In midgut, these pathogens are regulated by the residing micro symbionts i.e. *Asaia bogorensis.* In *Anopheles stephensi*, the ingested blood meal carries higher loads of Plasmodium. This is known to elicit a strong immune response, that acts both in the lumen (e.g. through the action of AMPs, nitric oxide and other effector molecules) and at the gut wall. The first barrier is presented by the micro symbiont present at the midgut by the production of these Anti-Malarial Proteins (AMPs) like Drosomycin, Cecropin, Defensin and Gambicin. Interestingly, the Anopheles midgut micro biota is negatively affected by this immune response. The bacterial load shows a growth after the blood meal, detectable at 24 h and exponentially increasing during the next two days, reaching about a tenfold quantity in both Plasmodium-infected and-uninfected mosquitoes. Therefore, there is evidence that: i) *Asaia*

population blooms in the mosquito midgut after blood ingestion and ii) the bacterial blooming is not affected negatively by the presence of *Plasmodium falciparum* in the blood (Aida Capone *et al.*, 2013) [7]. The evidence that Plasmodium presence at a high load does not interfere with the blooming of bacterial symbiont (i.e. Asaia) is a novel result. Thus, the immune reaction triggered by *Plasmodium falciparum* does not interfere with the presence of *Asaia bogorensis* in the insect. Alternatively, parasite infection in the mosquito does not seem to modify the kinetics of Asaia populations after the blood meal. The amount of bacteria in the midgut of infected mosquitoes can be considered at least comparable to that of uninfected specimens, and is higher than in the control group. There is a drop in *Plasmodium falciparum* intensity during Asaia replication in the time period analysed: while the average parasite numbers dropped from 270 (24 h), to 134 (48 h) and eventually to 94 (72 h), there was an approximately eight-fold increase of Asaia population at 72 h compared to 48 h (Aida Capone *et al*, 2013) [7]. Thus, from this evidence it can be stated that there is a strong relation between Asaia population and *Plasmodium falciparum*.

b. Abdomen: **Second barrier for *Plasmodium falciparum***

In spite of the general inter relationship between *Asaia bogorensis* and the pathogen *Plasmodium falciparum*, *Asaia bogorensis* also shows a missing link with mosquito immune system in controlling *Plasmodium falciparum* pathogen. It has been stated above that Asaia population is found specifically in the midgut of female mosquito that harbours *Plasmodium falciparum* pathogen. Presence of these bacteria might control the pathogen. Moreover, the innate immune system of mosquito might also control the growth of *Plasmodium falciparum* via inter connecting with micro symbiont, *Asaia bogorensis*. The mosquito innate immunity plays an important role in the interaction between the pathogen and the insect vector. This interaction determines the vectorial capacity of mosquito. This immune defense system is comprised of cellular and humoral mechanisms that are activated upon recognition of invading pathogens by the mosquito sensing receptors called Pattern Recognition Receptor (PRR) molecules. Recognition of pathogen-associated molecular patterns (PAMPs) can indirectly trigger a variety of defense mechanisms through the activation of serine protease cascades and intracellular immune signalling pathways that control the transcription of effectors. It can directly invoke killing mechanisms such as encapsulation and phagocytosis. They do this through the production of certain FREP (fibrinogen related) proteins. A common pattern recognition receptor gene family in invertebrates is the Fibrinogen-related proteins (FREPs) gene family. This is also known as Fibrinogen Domain Immunolectin (FBN). There are ~200 amino acid residues in a FBN. These FREPs contain a pathogen-binding FBG domain at their C-terminus, and an N-terminal sequence that is particularly involved in interactions with the N termini of other FREPs, thereby resulting in the formation of multimeric protein bundles. These protein bundles are loaded with potentially increased affinity and specificity for the pathogens (Yuemei Dong *et al.*, 2008) [9]. Among all the FREP protein genes, eight *FBN* genes (4, 5, 8, 24, 25, 31, 32, and 36) are specifically expressed in male Anopheles, and the expression of *FBN36* is the most abundant in male mosquitoes. These members are less likely to play major roles in the defence against *Plasmodium*, since only female mosquitoes transmit malaria parasites. Ten genes are specifically expressed in the females, of which *FBN2* is the most abundant followed by *FBNs* 37,29, 19, 39, 20, 7, 12, 33, and 11. These FREP proteins are highly abundant in the thoracic part, cell line, fat body and abdomen where the Asaia population does not accumulate. Moreover, the expression of *FBN39*, a previously identified anti-Plasmodium molecule, is only induced by *P. falciparum* challenge and not by other pathogens. This gene is female-specific and is located on the X chromosome (Yuemei Dong *et al*, 2008) [9]. Although the midgut is the primary site of the response to invading ookinetes and parasitic infections, most of the FREPs are not found in the midgut tissue but rather in the abdominal parts, which contain hemolymph and hemocytes. Hence, the observed anti-*Plasmodium* activities originate from the fat body, hemocytes, and other tissues (Yuemei Dong *et al*, 2008) [9]. One midgut enriched FREP i.e. FBN9 forms a dimer with the midgut micro symbionts to act synergistically to inactivate the *Plasmodium falciparum* pathogen. Gene silencing of individual *FREPs* (*FBN8*, *FBN9*, and *FBN39*) resulted in an increased permissiveness to *P. falciparum* infection, as indicated by a significant 58–81% increase in oocyst number. Whereas, targeting these genes together, including *FBN6*, resulted in a 95% increase in oocyst number. Thus, the effect of individually silencing genes was not comparable to that obtained when simultaneously silenced multiple genes was, suggesting that FREP proteins are functioning synergistically in the defence against *Plasmodium* (Yuemei Dong *et al*, 2008) [9].

4. The possible Inter-relationship between *Asaia bogorensis* and FREP proteins in controlling *Plasmodium falciparum*

With infected blood, the pathogen, *Plasmodium falciparum* enters into the body of *Anopheles stephensi*. After it crosses the peritrophic matrix, it enters the mid gut and then traversed its way to the blood thereby entering fully into circulatory system. Its journey is impeded by two agents: one, by the *Asaia bogorensis* population in the mid gut and second, by the FREP proteins in the abdomen. When the pathogen first enters the gut, its growth gets prevented by the Asaia population (interacting with FBN9) and when it enters the abdomen, it is prevented by the synergistic action of FREP proteins due to the interplay of host innate immunity. As the respective locational role of two agents have been seen, it might be possible to develop an idea that the two agents i.e. *Asaia bogorensis* and FREP proteins, interact with the pathogen in a network thereby regulating the concentration of the pathogen, *Plasmodium falciparum* in the vector. This network shows that the growth of *Plasmodium falciparum* is prevented in two different regions (Midgut and Abdomen) of the body of the vector by two different agents (Micro symbiont and innate immunity) in a complementary way.

5. Possible Application

The evidence that the ingestion of a blood meal carrying a high load of Plasmodium does not interfere with *Asaia bogorensis* growth suggests that this bacterium is not affected by the immune defence system of the mosquito triggered by a malaria parasite challenge. Thus, *Asaia bogorensis*is is resistant to AMPs (Anti-Malarial Proteins) and phagocytosis. *Asaia bogorensis* induces expression of certain Anti-Malarial

Proteins like Defensin, cecropin, gambicin and drosomycin in *Anopheles stephensi*. So, Asaia population is resistant to the mosquito immune effector molecules. Therefore, adaptation and survival of Asaia population is not dependent upon the mosquito immune system. Alternatively, it thrives in the host in achieving resistance from the immune system. In addition, the *Asaia bogorensis* is less phagocytised than E. coli by *Anopheles stephensi* haemocytes. This shows *Asaia bogorensis* is a symbiont of anopheline mosquitoes. Thus, genetic engineering of this *Asaia bogorensis* could be promising against malaria (Chris M.cirimotich *et al.*, 2011) [1]. Genetically engineered *Asaia bogorensis* may be injected into male *Anopheles stephensi* in the laboratory. This Anopheline mosquito when mate with female *Anopheles stephensi* mosquitoes in the wild, it venerally transfer the genetically engineered bacteria along with the naturally harbouring *Asaia bogorensis* into the female. This is possible as the bacterium is also naturally found in the reproductive organs of both male and female *Anopheles stephensi* mosquitoes. Now, both the natural and genetically engineered bacteria are also transferred to the progeny through transovarian transmission. Thus, the natural and genetically engineered bacterium passes to the next generation male and female mosquitoes. So, the mosquitoes now harbour both natural and genetically engineered *Asaia bogorensis* population. While sucking blood from the infected human hosts, the *Plasmodium falciparum* pathogen in its gametocyte stage enters into the proboscis of female mosquito vector thereby entering into the midgut. As it has been stated already that the mid gut is the repository of natural micro symbiont (*Asaia bogorensis*), they will act on the pathogen during its ookinete stage, preventing it from traversing the midgut and becoming oocyst by producing or inducing anti-malarial peptides (AMPs). Also, the genetically engineered *Asaia bogorensis* will act on the pathogen producing same or more AMPs depending on the level of chromatin modification done through engineering. As a result, the pathogen will experience a heightened dual dose of AMPs from the natural and genetically engineered Asaia population. Thus, the extent to which *Plasmodium falciparum* would have been prevented from growing might now increase thereby reducing the load of ookinete to traverse the mid gut as oocysts. If crossed, it will again experience the action of FREP proteins in the abdomen as a result of innate immunity (Based on Chris M.cirimotich *et al*, 2011) [1].

Currently, no such laboratory interventions based on native micro symbiont i.e. *Asaia bogorensis* in controlling falciparum malaria by the regulation of growth and development of *Plasmodium falciparum* have been carried out. As new strategies are developing in medical entomology, it might be a way to examine this native microbiota and their impact on disease transmission as part of an integrated strategy for the management of falciparum malaria.

6. Conclusion

Despite of being an Acetic Acid Bacteria (AAB), *Asaia bogorensis* is involved in the management of Malaria by the regulation of growth and development of *Plasmodium falciparum* in the vector, *Anopheles stephensi*. It acts as a micro symbiont in the vector, accumulating abundantly in the midgut of female *Anopheles stephensi* along with its traces in the salivary gland and reproductive organs of both male and female. Also, it is resistant to the innate immunity of the vector. Together, with the FREP proteins of mosquito innate immune system, it further reduces the pathogen load while acting in a circuit. Genetically engineered *Asaia bogorensis* can be used to infect female *Anopheles stephensi* thereby using as a tool for the management of falciparum malaria by impeding the development of malaria parasite inside the vector. So, the vector might fail to attain the infective stage for transmission of falciparum malaria and thereby blocking its transmission to humans. Further studies in this regard are required to prove this hypothesis.

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