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The mosquito microbiota influences vector competence for human pathogens

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Abstract

The midgut of insect vectors of human disease contains not only pathogens harmful to human health, but also a diverse microbiota. This microbiota can influence insects' susceptibility to human pathogens, and the capacity to transmit them, through different mechanisms.

Understanding the interaction between the vector, its microbiota and transmitted pathogens will provide novel opportunities to limit disease transmission.

Keywords

Mosquito; Anopheles; Aedes; Plasmodium; dengue; innate immunity; microbiota

1. Introduction

Mosquito vector-borne diseases such as malaria, dengue and chikungunya represent a major global public health burden. In addition to disease-causing pathogens, mosquitoes are also continuously exposed to a variety of microorganisms from their ecosystem. Some microorganisms have co-evolved and developed symbiotic relationships with insects; other bacteria are commensal, likely acquired from the insect's breeding water or nectar sources, and they have adapted to persist within the insect. Early studies of insect symbionts suggest that they nutritionally benefit the insect, as in the case of cellulose digestion by the termite's gut microbiota [1,2]. Recent studies also suggest the importance of the insects' microbiota in maintaining its basal immune activity and immune priming [3–5]. The microbiota can modulate the mosquito immune response and influence vector competence to human pathogens. For example, the removal of the majority of midgut bacteria through antibiotic treatment results in a greater susceptibility of *Anopheles gambiae* and *Aedes aegypti* to *Plasmodium falciparum* and dengue virus (DENV) infection, respectively [6–8]. In addition to activating mosquito immune responses, bacteria can also influence insect mosquito competence by impairing pathogen infection through competition for resources or the secretion of anti-pathogen molecules [9–11].

Vector-borne disease transmission requires successful interactions between the human host, the pathogen, and the insect vector. Although the majority of research aimed at vector-borne disease control has thus far focused on the interactions between human and pathogen (i.e. vaccine and drug development), or between human and vector (i.e. insecticide development), the insect-pathogen interaction has evolved into a major area of study as we attempt to understand the importance of the tripartite interaction between vector, pathogen, and microbiota for disease transmission. This review will focus on recent advances in our understanding of the role of the mosquito microbiota in modulating infections with vector-borne pathogens, which could lead to alternative disease control strategies.

2. Mosquito immune responses to pathogens

In order to fight invading pathogens, insects mainly rely on their innate immune systems, which are to a significant degree controlled through different immune signaling cascades. Following recognition of pathogens through pattern recognition receptors (PRRs), immune pathway activation results in the systemic production of anti-microbial peptides (AMPs) and other anti-pathogen immune effectors, as well as other immune defense mechanisms such as melanization and phagocytosis [12–19]. Our current knowledge of insect immune signaling pathways is mainly based on research in *Drosophila* and mosquito models (reviewed in [20–22]). Classical immune pathways in insects, namely the Toll, immune deficiency (Imd), and Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathways, are activated in response to wide range of pathogens. The Toll pathway, an insect NF- κ B signaling pathway, is mainly activated by Gram-positive bacteria, fungi, and viruses [6,23–28]. Another NF- κ B signaling pathway, the Imd pathway, plays an important role in the insects' immune responses to bacteria, viruses, and the human *Plasmodium* parasite *P. falciparum* [29–32]. The insect JAK/STAT pathway is analogous to the vertebrate interferon (cytokine)-induced signaling pathway and has been shown to mediate insect anti-viral immunity as well as the mosquito defense against bacteria, fungi, and *Plasmodium* parasites [33–43]. Given the extensive overlap between the antibacterial, anti-parasitic, and antiviral immune responses, the microbiota can act through the immune system as a key determinant of vector susceptibility to human pathogen infection [6,7,44,45].

Microbial components such as peptidoglycan (PGN) can establish basal immunity and induce genes with cross-reactivity against both bacteria and *Plasmodium* parasites [7,44]. Following ingestion of a blood meal, the bacterial population can expand 100-fold, and the transmembrane PGN recognition protein LC (PGRPLC) acts a sensor for recognition of both Gram-negative and Gram-positive bacteria and subsequent expression of AMPs [46–48]. Depletion of PGRPLC results in a bacteria-dependent increase in *Plasmodium* infection levels, suggesting that the presence of bacteria is essential for the PGRP-LC-mediated anti-*Plasmodium* immune response [48]. Other PRRs, the C-type lectins CTL4 and CTLMA2, have pleiotropic immune functions, acting as protective agonists preventing *P. berghei* melanization and regulating susceptibility to Gram-negative bacteria [49,50]. Shared immune signaling in response to different classes of microorganisms dictates that the presence, and likely the composition, of the microbiota influence susceptibility to infection.

3. Mosquito microbiota influences vector competence

The insect's microbial symbionts reside in various organs such as the gut, ovaries, Malpighian tubules, and hemocoel [51]. The gut microbiota is of particular interest because it represents the first site of most extensive exposure to pathogens. Insect gut microbiomes, especially those of mosquitoes, have been characterized by either classical cultivation methods or by metagenomic analyses based on 16S rRNA sequencing [52–57]. The bacterial composition of mosquitoes sampled from natural habitats is highly variable but often contain a core microbiome that is dominated by a small number of taxa that can, however, vary depending on the insect species, geographical origin, ecological niche, and source of food, as well as sex [52–54,56]. Members of the *Proteobacteria* class are often the most abundant and common in adult *A. gambiae* mosquitoes, with species of the *Enterobacter*, *Serratia*, and *Asaia* families commonly present [11,52,53]. Interestingly, the microbiota of immature stages is mainly comprised of *Cyanobacteria*, suggesting a shift in microbial communities occurs as the midgut ecosystem changes during development [58]. Despite their core microbiota, a high inter-individual variation in bacterial species composition is frequently found, even within the same mosquito colony and generation [7,52], suggesting that the midgut is predominantly composed of opportunistic bacteria that can tolerate the protease-rich restrictive environment.

An inverse correlation exists between the presence of certain Gram-negative bacteria in mosquitoes and *P. falciparum* infection rates, and several bacterial species have been shown to inhibit *Plasmodium* sporogonic development *in vivo* and *in vitro* [46,59–61]. Antibodies targeting Gram-negative bacteria within the mosquito midgut increase permissiveness to *Plasmodium* infection [62], and removal of *Ae. aegypti* endogenous bacteria results in an increased susceptibility to DENV infection [6]. Reintroduction of certain field-derived bacteria into the mosquito gut through a nectar meal increases the resistance to pathogen infection in both *A. gambiae* [46] and *Ae. aegypti* [45], although not all bacteria tested influence the susceptibility to infection. Our knowledge of the precise mechanisms by which certain bacteria can influence the mosquitoes' susceptibility to human pathogens is still limited. Below, we detail the multiple mechanisms through which the insect vector microbiota is likely to influence susceptibility to pathogens and summarize these effects in Table 1.

3.1. Resource Competition

Both bacterial symbionts and pathogens acquire certain essential nutrients from their insect hosts. *Plasmodium* oocysts internalize the mosquito lipoprotein lipophorin, suggesting a metabolic demand for vector-derived lipids [63]. Intracellular membrane remodeling is important for efficient virus replication and protein processing of DENV and WNV [64,65]. To modulate host membrane trafficking, DENV influences lipid composition, lipid homeostasis and genes involved in lipid trafficking in both vertebrate and invertebrate model [66–68]. Chemical inhibition of fatty acid synthase enzyme inhibited DENV, emphasizing the importance of lipids in virus replication [66,69]. Similarly to chemical treatment, the presence of symbionts can sequester such nutrients and thereby limit the development and infection of pathogens. For example, *Wolbachia*, the most prevalent

intracellular symbiont of insects, sequesters cholesterol and other lipids in insect cells, and these lipids, in turn, limit infections with DENV, Chikungunya virus (CHIKV), and the *Plasmodium* parasite [9,70]. Abundance of cholesterol mitigates the *Wolbachia* inhibition of *Drosophila* C Virus, further suggesting that resource competition can contribute to pathogen resistance [71].

3.2 Immune priming by the microbiota

There is growing evidence for the importance of the insect microbiota for immune activation and maturation, ensuring that a robust anti-parasitic response occurs in adulthood. Gene expression analysis has revealed that aseptically reared *A. gambiae* and *Ae. aegypti* have lower levels of immune gene expression [6,7]. This finding, in conjunction with the greater susceptibility of aseptically reared mosquitoes to *Plasmodium* and DENV infection, has suggested a role for the mosquito midgut microbiota in controlling human pathogen infection by immune priming, especially through the Toll and Imd pathways [6–8].

The presence of mosquito midgut-dwelling bacteria can prime the immune response to pathogens in different ways. An *Acinetobacter* species that elicits strong induction of several Imd pathway-controlled anti-*Plasmodium* effectors inhibits *P. falciparum* infection in the mosquito gut to a much lesser degree when the Imd pathway is silenced, suggesting that its anti-parasitic activity is likely mediated through the activation of this anti-*Plasmodium* immune pathway [46]. *Enterobacter cloacae* induces the expression of the anti-*Plasmodium* serine protease inhibitor SRPN6, thereby priming the midgut epithelial cells for an elevated immune response [72]. In septic mosquitoes the heme peroxidase IMPer and the dual oxidase Duox form a dityrosine network between the peritrophic matrix and midgut epithelium, preventing immune elicitation by acting as a barrier to prevent the interaction of microbial elicitors with PRRs [73]. Formation of this barrier also prevents the interaction of *Plasmodium* elicitors with host immune receptors, leading to a decrease in immune effectors and increasing permissiveness to *Plasmodium* infection [73]. Silencing of IMPer and Duox results in an increased interaction of microbial elicitors and recognition receptors that culminates in increased expression of immune effectors and decreased infection levels [73]. The mosquito response, designed to prevent a bacteria-mediated overactivation of the immune system, influences the ability of *Plasmodium* to persist within the midgut. The field-derived bacterium *Proteus* sp. (Prsp_P) has been shown to inhibit DENV infection of the mosquito gut and also stimulate AMP gene expression, suggesting a causal link between these two events [45].

Genetic variation of mosquito vectors can shape the microbiota of individual mosquitoes, which can then influence the anti-pathogen response. *A. gambiae* genomic variability apparently determines the extent of the anti-bacterial response to the Enterobacterium *Serratia marcescens*, which has previously been shown to inhibit *Plasmodium* development [61,74,75]. Single nucleotide polymorphisms within a group of type III fibronectins (FN3Ds) have been identified, and silencing of these genes has been shown to increase the amount of *S. marcescens* and other *Enterobacteriaceae* within the microbiota [75]. It is intriguing to suggest that one consequence of genetic variation within immune genes could be an effect on the relative abundance of bacteria that negatively regulate *Plasmodium*.

infection. It is also important to consider bacterial genetic variation, since intra-specific variation within *S. marcescens* has been shown to determine the impact of the bacteria on susceptibility to *P. berghei* parasites [74]. These studies highlight the complex interaction that exists between the insect vector, its microbiota, and pathogens.

The endosymbiont *Wolbachia* influences pathogen susceptibility in multiple insects, predominantly through activation of the host immune response. For example, *Drosophila* and *Ae. aegypti* harboring wMel *Wolbachia* are more resistant to DENV infection [76]. The *Wolbachia* wAlbB strain induces production of reactive oxygen species (ROS) in *Ae. aegypti*, which, in turn, activate the Toll pathway and limit DENV infection [77]. This wAlbB *Wolbachia* also stimulates ROS production in *A. stephensi* and decreases susceptibility to *P. falciparum* infection [77,78]. However, *Wolbachia*-infected *D. melanogaster* did not show significant changes in immune activation, suggesting that the anti-DENV mechanism in the fruit fly is independent of the immune system [76]. In addition, various *Wolbachia* strains either increase (wAlbB) or decrease (wMelPop) the susceptibility of *A. gambiae* to *P. berghei* infection [79]. These results suggest that *Wolbachia* interacts with vectors and influences pathogen susceptibility through multiple mechanisms, including priming the insect immune response. As *Wolbachia* continues to be evaluated in field trials [80,81], elucidating the exact mechanisms undergirding its interaction with mosquito pathogens becomes increasingly important.

In addition to the studies of mosquito vectors, data from the tsetse fly (*Glossina morsitans*) symbiont *Wigglesworthia glossinidia* suggest that this symbiont plays an important role in regulating PGRP-LB expression and controlling trypanosome infection through the Imd pathway [82]. The presence of symbionts during developmental (larval) stages influences the basal level of immune activity in the adult tsetse fly. The presence of *W. glossinidia* during larval development appears to be required for healthy immune responses to microbial challenge, including the expression of AMPs, prophenol-oxidase activity, melanization and increase in the number of hemocytes [83,84]. These data suggest that certain symbionts are required in particular developmental stages for insect immune system maturation.

3.3. Production of secondary metabolites

The impact of the microbiota is not only mediated through the mosquito immune system; the microbiota can also have a direct effect, independent of the mosquito vector. Three bacteria isolated from *Ae. albopictus*, *En. ludwigii*, *Pseudomonas rhodesiae*, and *Vagococcus salmoninarum* have been shown to inhibit La Crosse virus *in vitro*, suggesting that these bacteria may secrete anti-viral metabolites [85]. A recently identified *Enterobacter* strain (*Esp_Z*) from wild *Anopheles* mosquitoes is able to directly influence *Plasmodium* infection, independent of the mosquito, through a mechanism that involves reactive oxygen species (ROS). *In vitro Plasmodium* inhibition assays with bacteria and bacterial culture supernatants have demonstrated a mosquito-independent inhibition of *Plasmodium* within the mosquito midgut [11]. A study of a panel of naturally occurring bacteria has revealed that some species inhibit the malaria parasite through the immune system, while others directly inhibit the parasite, but not through the same ROS-generation mechanism used by *Esp_Z* [46]. This finding raises the intriguing possibility that multiple, independent and

direct anti-*Plasmodium* functions are present in naturally occurring commensal bacteria. Harnessing these direct effects through bioprospecting and isolation of bacteria-produced anti-pathogenic natural products represent a promising line of investigation in the development of novel therapeutic and transmission-blocking agents.

4. Current status of the insect microbiota studies and their application

The extensive impact of the mosquitoes' gut microbiota on the human pathogens they transmit has only recently come to light. The role of the microbiota in immunity has been extensively studied in the *Drosophila* model; for example, the gut microbiota triggers the activation of the Imd and dual oxidase (DUOX) pathways to maintain a healthy gut environment (reviewed in [86,87]). Further study of these systems in a vector-pathogen interaction context could enable the development of strategies to manipulate vector microbiota in order to limit disease transmission.

Past vector microbiota studies have rarely considered the pathogen infection status of the insects. However, a positive correlation has been observed between *Enterobacteriaceae* and *Plasmodium* infection in Cameroon [52]. Interestingly, in a contrasting separate study, bacteria isolated from mosquitoes in Cameroon reduced the mosquitoes' susceptibility to *P. falciparum* infection [88]. These studies highlight the importance of determining infection status when performing metagenomic studies.

Studies of the mosquito gut microbiota have been biased toward prokaryotic symbionts. Although eukaryotic microbiota members such as *Pichia* yeast and *Candida* fungus have been isolated from mosquitoes, the impact of these eukaryotic microbes on insect vector competence has not been addressed [89–91].

The use of insect microbiota for the development of disease control strategies is a relatively new concept that, like other novel approaches, carries some ecological and safety concerns that need to be addressed. The use of microbes as biopesticides does, however, have a precedent for vector control. The entomopathogenic bacteria *Bacillus thuringiensis* and the fungus *Beauveria bassiana* have been evaluated in field trials for the control of mosquito larvae [92–94]. *B. bassiana* not only kills mosquitoes but also reduces susceptibility to DENV infection through immune pathway activation [43]. The use of *B. bassiana* for control of human disease can however raise ecological and safety concerns because the fungus will be applied in close contact to humans compared to the previous use in agricultural application, especially for the anthropophilic mosquito *Ae. aegypti* [95–97]. The most advanced application of insect symbionts in controlling the transmission of human disease is represented by the “Eliminate Dengue” research program (<http://www.eliminatedengue.com>), which aims at using *Wolbachia*-infected *Ae. aegypti* to control DENV transmission [80,81,98]. In addition to the ecological safety advantage of *Wolbachia* (being a species-specific endosymbiont), this organism can suppress a broad spectrum of pathogens, including DENV, CHIKV, yellow fever virus (YFV), West Nile virus (WNV), filarial nematodes, and *Plasmodium* parasites [9,10,80,81,98–101]. Some *Wolbachia* strains also shorten the lifespan of the insects, reducing the chance that the pathogens will complete the transmission cycle [102]. The most recent follow-up from the Eliminate Dengue

program found that field-caught *Wolbachia*-infected *Ae. aegypti* still maintain their refractoriness to DENV [103]. More recently, a *Wolbachia* strain related to, but distinct from other arthropod-associated strains has been identified in Burkina Faso *A. gambiae* populations [104]. This novel strain, that is maternally transmitted, warrants future investigation to determine its prevalence and impact upon *P. falciparum* development.

5. Concluding remarks

Alternative approaches for insect and arbo-pathogen control are needed to reduce the global public health burden from arthropod-borne diseases. The insect's immune system largely contributes to the insect's vector competence, and recent findings have pointed to the importance of the mosquito microbiota for disease transmission. The dissection of the tripartite relationships between mosquito, its microbiota, and the transmitted human pathogens may lead to novel powerful tools that can complement current strategies for disease control.

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Highlights

- The midgut of mosquito disease vectors contains a diverse microbial flora
- The microbiota can modulate the mosquito immune response and influence vector competence to human pathogens.
- The microbiota can alter susceptibility to infection through either immune priming or direct inhibition.
- The tripartite relationship between mosquito, its microbiota, and human pathogens is critical to disease transmission.
- Dissection of this tripartite interaction may lead to novel powerful tools that can complement current strategies for disease control.

Table 1

Impact of selected components of the insect microbiota that are detailed within the text on vector competence

Bacteria	Impact upon vector competence	References
<i>EspZ</i>	Generation of ROS results in an increased resistance to <i>Plasmodium</i> infection in <i>A. gambiae</i>	11 (Cirimotich et al. 2011)
<i>Serratia marcescens</i>	Blocks sporogonic development of <i>P. vivax</i> parasites in <i>A. albimanus</i> Intra-specific diversity determines the level of <i>P. berghei</i> inhibition in <i>A. stephensi</i> Reduces susceptibility of <i>A. stephensi</i> to <i>P. falciparum</i> infection	61 (Gonzalez-Ceron et al. 2003) 74 (Bando et al. 2013) 59 (Pumpuni et al. 1996)
<i>Enterobacteriaceae</i> sp	<i>P. falciparum</i> infected <i>A. gambiae</i> harbor a greater abundance of <i>Enterobacteriaceae</i>	52 (Boissière et al. 2012)
<i>Enterobacter cloacae</i>	Elevates the expression of <i>A. stephensi</i> SRPN6 leading to a heightened immune response to <i>P. falciparum</i> infection	72 (Eappen et al. 2013)
<i>Proteus</i> sp	Introduction of a <i>Proteus</i> species into <i>Ae. aegypti</i> results in an increased resistance to DENV, potentially as a result of increased AMP expression.	45 (Ramirez et al. 2012)
<i>Acinetobacter</i> sp	Increases resistance of <i>A. gambiae</i> to <i>Plasmodium</i> development, in part through induction of Imd pathway anti- <i>Plasmodium</i> factors	46 (Bahia et al. 2014)
<i>Wigglesworthia glossinidia</i>	PGRP-LB mediated tolerance of <i>W. glossinidia</i> impacts the tsetse fly anti-trypanosome immune response Presence of the tsetse fly symbiont <i>W. glossinidia</i> is required for proper immune function in adulthood	82 (Wang et al. 2009) 83,84 (Pais et al. 2008, Weiss et al. 2011)
<i>Xanthomonas maltophilia</i> <i>Pseudomonas aeruginosa</i> <i>Escherichia coli</i> <i>Cedecea lapagei</i> <i>Ewingella americana</i>	Gram negative bacteria reduce <i>P. falciparum</i> infection of <i>A. stephensi</i>	59, 60 (Pumpuni et al. 1993, Pumpuni et al. 1996)
<i>Enterobacter cloacae</i> <i>Enterobacter amnigenus</i>	Inhibits <i>P. vivax</i> infection in <i>A. albimanus</i>	61 (Gonzalez-Ceron et al. 2003)
<i>Bacillus pumilus</i> <i>Comamonas</i> sp <i>Escherichia coli</i> <i>Enterobacter</i> sp <i>Pseudomonas stutzeri</i> <i>Serratia marcescens</i>	Exposure to bacteria isolated from <i>A. gambiae</i> in Cameroon results in a decreased susceptibility to <i>P. falciparum</i> infection	88 (Tchioffo et al. 2013)
<i>Enterobacter ludwigii</i> <i>Pseudomonas rhodesiae</i> <i>Vagococcus salmoninarum</i>	Exposure to the bacteria results in inhibition of La Cross virus <i>in vitro</i>	85 (Joyce et al. 2011)

Bacteria	Impact upon vector competence	References
<i>Wolbachia</i> - <i>wMel</i>	Increases <i>Ae. aegypti</i> resistance to DENV infection Increases <i>Ae. aegypti</i> resistance to CHIKV	76, 81 (Rancès et al. 2012, Walker et al. 2011) 99 (Van den Hurk et al. 2012)
<i>Wolbachia</i> - <i>wAlbB</i>	Induces ROS mediated activation of the TOLL pathway leading to reduced DENV titers in <i>A. aegypti</i> Decreases <i>A. stephensi</i> susceptibility to <i>P. falciparum</i> , potentially through ROS generation Increase susceptibility to <i>P. berghei</i> infection in <i>A. gambiae</i>	77 (Pan et al. 2012) 78 (Bian et al. 2013) 79 (Hughes et al. 2012)
<i>Wolbachia</i> - <i>wMelPop</i>	Decreases <i>P. berghei</i> infection level in <i>A. gambiae</i>	79, 100 (Hughes et al. 2012, Kambris et al. 2010)
<i>Wolbachia</i> - <i>wMelPop-CLA</i>	Reduces susceptibility of <i>Ae. aegypti</i> to DENV, CHICKV and <i>P. gallinaceum</i> Reduces <i>Ae. aegypti</i> susceptibility to DENV Reduces <i>Ae. aegypti</i> susceptibility to YFV	9 (Moreira et al. 2009) 81 (Walker et al. 2011) 99 (Van den Hurk et al. 2012)