Chapter 17

# The *Anopheles* Mosquito Microbiota and Their Impact on Pathogen Transmission

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## 1. Introduction

An ecosystem is composed of a biological community and its physical environment. A unique ecosystem is the metazoan digestive tract, which contains and interacts with many microorganisms, e.g. a single human gut contains $10^{13}$-$10^{14}$ bacteria belonging to hundreds of species [4, 5]. These microorganisms are important for the host physiology, particularly in shaping the mucosal immune system [6] and protecting the host against infections by colonization resistance [7].

The term microbiota defines the microbial communities that live in contact with the body epithelia. They are composed of bacteria, viruses, yeasts and protists. To date, the bacterial component of the microbiota is the most studied and best characterized. Studies from *Drosophila* to mice have revealed that the microbial flora is tightly regulated by the immune system and that failures in this can have detrimental effects on the host [8, 9]. The microbiota composition and numbers undergo significant changes during a host's lifetime, in particular upon changes of the environment and feeding habits.

*Anopheles* mosquitoes are of great importance to human health. They transmit pathogens including malaria parasites, filarial worms and arboviruses (arthropod-borne viruses). These pathogens infect the mosquito gut when ingested with a bloodmeal, disseminate through the hemolymph (insect blood) to other tissues and are transmitted to a new human host upon another mosquito bite some days later. The time pathogens spend in mosquitoes is known as extrinsic incubation period. The malaria parasite, *Plasmodium*, undergoes sexual reproduction in the midgut lumen and develops into a motile form that, approximately 24h after infection, traverses the gut epithelium establishing an infection on the basal side that is bathed in the hemolymph [10]. A week to 10 days later, parasites travel to the salivary glands where they become infectious to man. Similarly, after shedding their protective sheath in the mosquito midgut lumen, the elephantiasis nematodes *Wuchereria* and *Brugia* microfilariae migrate

through the midgut epithelium to the thoracic muscles where they embark on larval development [11]. Some 10-14 days later, infectious larvae emerge from the mosquito cuticle or the proboscis and infect the human host via a skin wound, such as that caused by the mosquito bite. The O'Nyong Nyong virus (ONNV), the only arbovirus known to be transmitted exclusively by *Anopheles*, mosquitoes infects the muscle bands of the midgut and other visceral tissues after dissemination from infected gut cells [12, 13]. The next steps of the virus migration through the mosquito are not well characterized but it is thought that, as shown for its cousin Chikungunya virus, it infects the salivary glands from where it can be transmitted to the human host. Thus, for all three types of pathogens, the *Anopheles* mosquito midgut is an obligatory gateway to infection and transmission.

The mosquito gut microbiota has recently emerged as an important factor of resistance against pathogens. In particular, midgut bacteria have been shown to have a substantial negative impact on malaria parasite burden through colonization mechanisms involving either direct *Plasmodium*-microbiota interactions or bacteria-mediated induction of the mosquito immune response [1, 2, 14]. Equivalent effects of the microbiota on infection with the Dengue virus and *Brugia* microfilariae are shown in the mosquito *Aedes aegypti* [15-17]. Therefore, the research field of mosquito microbiota has received great attention in the last years and new concepts of microbiota-mediated transmission blocking are currently investigated. These studies face an important challenge: the microbiota of a female mosquito changes considerably as the mosquito shift environments during metamorphosis, from the aqueous developing larva to an air-living adult, and yet during adulthood as its feeding behaviour alternates between flower-nectar feeding and blood feeding [18, 19]. The diversity of the bacterial community is shown to decrease during mosquito development and after the first bloodmeal, whereas bacteria massively proliferate, with a 10 to 900-fold increase registered 24h to 30h after a bloodmeal [18, 20, 21].

In this chapter, we provide an overview on the current knowledge of the composition of the *Anopheles* mosquito microbiota, including important findings from recent high-throughput sequencing studies. We then review studies about the impact of the microbiota on mosquito physiology and infection, focusing in particular on resistance to infection by human pathogens. Finally, we discuss the potential use of this knowledge toward reducing the mosquito vectorial capacity and transmission blocking.

## 2. The diversity of the *Anopheles* microbiota

The microbiota composition has been studied in several anophelines mainly by culturing or sequencing of the 16S rRNA [14, 18, 20, 22-41]. Together, studies on field-collected or laboratory-reared mosquitoes identified as many as 98 bacterial genera excluding genera of low abundance identified by high-throughput sequencing analyses (Table 1). Of these, 41 genera were found in more than one *Anopheles* species while 9 were reported in at least 7 of these 23 studies and thus appear to be frequently associated with *Anopheles*. *Pseudomonas* was the most frequent of those genera, detected in 16 studies, followed by *Aeromonas*, *Asaia*, *Comamonas*, *Elizabethkingia*, *Enterobacter*, *Klebsiella*, *Pantoea* and *Serratia*, detected in 7-10 studies. No single bacterial genus was found in all the studies, even if culture-dependent studies are not consid-

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ered – as culturing techniques might be an issue. Thus, there is presumably no obligate symbiont in the *Anopheles* genus, as is the case of some other blood-sucking insects such as the Tsetse fly that hosts *Wigglesworthia spp.*, an obligatory bacterial symbiont important for fly fecundity [42] or the head louse that hosts *Riesia pediculicola* [43]. As the most frequent genera are present in both laboratory and field-collected mosquitoes, it is suggestive that laboratory colonies retain bacterial communities established prior to laboratory colonisation (Table 1 and [18]). There are, however, substantial differences between field-collected and laboratory-reared mosquitoes, as reflected by the loss of microbiota species richness in laboratory-reared mosquitoes [18, 22].

***Actinobacteria***

| Genus | Family | Class | Example | Conditions | stage | *Anopheles* species | Deep seq | Culture | Non-culture |
|---|---|---|---|---|---|---|---|---|---|
| *Agromyces* | *Microbacteriaceae* | *Actinobacteria* | JX186590 | F* | L | *gambiae* | [17] | | |
| *Brevibacterium* | *Brevibacteriaceae* | *Actinobacteria* | FJ608062 | F | L | *stephensi* | | | [38] |
| *Corynebacterium* | *Corynebacteria-ceae* | *Actinobacteria* | GQ109703 | F, F* | A | *funestus, gambiae* | [17, 36] | | |
| *Janibacter* | *Intrasporangiaceae* | *Actinobacteria* | NR_043218 | F | A | *arabiensis* | | [22] | |
| *Kocuria* | *Micrococcaceae* | *Actinobacteria* | HQ591424 | F | L | *stephensi* | | [23] | |
| *Microbacterium* | *Microbacteriaceae* | *Actinobacteria* | HQ591431 | F, L | L | *gambiae, stephensi* | | [11, 23] | |
| *Micrococcus* | *Micrococcaceae* | *Actinobacteria* | FJ608230 | F, L | A | *gambiae, stephensi* | | [38, 37] | |
| *Propionibacterium* | *Propionibacteria-ceae* | *Actinobacteria* | GQ003306 | F, F* | A | *funestus, gambiae* | [17, 36] | | |
| *Rhodococcus* | *Nocardiaceae* | *Actinobacteria* | AY837749 | F | L, A | *arabiensis, stephensi* | | [22, 23] | |
| ***Bacteroidetes*** | | | | | | | | | |
| *Chryseobacterium* | *Flavobacteriaceae* | *Flavobacteriia* | HQ591432 | F, F*, L | L, P, A | *coustani, funestus, gambiae, stephensi* | [17, 36] | [11, 38, 23] | [38] |
| *Dysgonomonas* | *Porphyromonada-ceae* | *Bacteroidia* | FJ608061 | F | L | *stephensi* | | | [38] |
| *Elizabethkingia* | *Flavobacteriaceae* | *Flavobacteriia* | EF426434 | F*, L | A | *gambiae, stephensi* | [17, 21] | [22, 37] | [38, 27, 32] |
| *Flavobacterium* | *Flavobacteriaceae* | *Flavobacteriia* | | F, L | A | *albimanus, funestus, gambiae, stephensi* | | [19] | [30] |
| *Flexibacteraceae* | | *Cytophagia* | FJ608195 | F | A | *stephensi* | | | [38] |
| *Myroides* | *Flavobacteriaceae* | *Flavobacteriia* | HQ832872 | F | L, A | *stephensi* | | [23] | |
| *Prevotella* | *Prevotellaceae* | *Bacteroidia* | JN867317 | F* | A | *gambiae* | [21] | | |
| *Sediminibacterium* | *Chitinophagaceae* | *Sphingo-bacteriia* | FJ915158 | F* | A | *gambiae* | [21] | | |
| *Sphingobacterium* | *Sphingobacteria-ceae* | *Sphingo-bacteriia* | EF426436 | L | P, A | *gambiae* | | [35] | |
| ***Firmicutes*** | | | | | | | | | |
| *Bacillus* | *Bacillaceae* | *Bacilli* | AY837746 | F, L | L, A | *arabiensis, funestus, gambiae (ss, sl), stephensi* | | [11, 38, 22, 24] | [38, 27, 30] |
| *Clostridium* | *Clostridiaceae* | *Clostridia* | JN391577 | F* | L | *gambiae* | [17] | | |
| *Enterococcus* | *Enterococcaceae* | *Bacilli* | HQ591441 | F | L, A | *funestus, gambiae, stephensi* | [36] | [23] | |
| *Exiguobacterium* | *Bacillales Family XII. Incertae Sedis* | *Bacilli* | HQ591439 | F | L | *stephensi* | | [38, 23] | |

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|---|---|---|---|---|---|---|---|---|---|
| *Lactobacillus* | *Lactobacillaceae* | *Bacilli* | FJ608053 | F, F* | L, A | *gambiae, stephensi* | [17] | | [38] |
| *Lysinibacillus* | *Bacillaceae* | *Bacilli* | GU204964 | F | L | *maculipennis, stephensi* | | [24] | |
| *Paenibacillus* | *Paenibacillaceae* | *Bacilli* | EF426449 | F | A | *arabiensis, stephensi* | | | [38, 22] |
| *Staphylococcus* | *Staphylococcaceae* | *Bacilli* | FJ608067 | F, F*, L | L, A | *funestus, gambiae, maculipennis, quadrimaculatus, stephensi* | [21, 36] | [25, 38, 40] | [38, 26] |
| *Streptococcus* | *Streptococcaceae* | *Bacilli* | FJ608047 | F, F* | L, A | *funestus, gambiae, stephensi* | [21, 36] | | [38] |

***Proteobacteria***

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *Acetobacter* | *Acetobacteraceae* | *Alpha-proteobacteria* | | L | A | *stephensi* | | | [26] |
| *Achromobacter* | *Alcaligenaceae* | *Beta-proteobacteria* | FJ608301 | F | A | *stephensi* | | | [38] |
| *Acidovorax* | *Comamonadaceae* | *Beta-proteobacteria* | AY837725 | F | A | *arabiensis* | | | [22] |
| *Acinetobacter* | *Moraxellaceae* | *Gamma-proteobacteria* | FJ608267 | F, F*, L | L, A | *albimanus, funestus, gambiae, stephensi* | [17, 21, 36] | [19, 38] | [38, 26] |
| *Aeromonas* | *Aeromonadaceae* | *Gamma-proteobacteria* | FJ608130 | F, F*, L | L, A | *coustani, darlingi, funestus, gambiae, maculipennis, stephensi* | [17, 36] | [19, 38, 23, 24] | [22, 33] |
| *Agrobacterium* | *Comamonadaceae* | *Beta-proteobacteria* | FJ607997 | L | A | *stephensi* | | [38] | [38] |
| *Alcaligenes* | *Alcaligenaceae* | *Beta-proteobacteria* | HQ832875 | F | A | *funestus, stephensi* | | [23] | [30] |
| *Anaplasma* | *Anaplasmataceae* | *Alpha-proteobacteria* | AY837739 | F | A | *arabiensis* | | | [22] |
| *Aquabacterium* | *Burkholderiales Genera incertae sedis* | *Beta-proteobacteria* | | F | A | *gambiae* | | | [26] |
| *Asaia* | *Acetobacteraceae* | *Alpha-proteobacteria* | FN821398 | F, F*, L | L, A | *coustani, funestus, gambiae, maculipennis, stephensi* | [21, 36] | [11, 26-28, 37] | [26, 28] |
| *Azoarcus* | *Rhodocyclaceae* | *Beta-proteobacteria* | FJ608071 | F | L | *stephensi* | | | [38] |
| *Bordetella* | *Alcaligenaceae* | *Beta-proteobacteria* | HQ832874 | F | A | *stephensi* | | [23] | |
| *Bradyrhizobium* | *Bradyrhizobiaceae* | *Alpha-proteobacteria* | AB740924 | F* | A | *gambiae* | [21] | | |
| *Brevundimonas* | *Caulobacteraceae* | *Alpha-proteobacteria* | GU204962 | F | L, A | *funestus, stephensi* | | [24] | [30] |
| *Burkholderia* | *Burkholderiaceae* | *Beta-proteobacteria* | AY391283 | F, F*, L | A | *gambiae, stephensi* | [21] | | [26, 27] |
| *Buttiauxella* | *Enterobacteriaceae* | *Gamma-proteobacteria* | | F | A | *darlingi* | | | [33] |
| *Cedecea* | *Enterobacteriaceae* | *Gamma-proteobacteria* | DQ068869 | F, F*, L | A | *funestus, gambiae (ss, sl), stephensi* | [21] | [19, 29] | [30] |
| *Citrobacter* | *Enterobacteriaceae* | *Gamma-proteobacteria* | FJ608234 | F | A | *darlingi, stephensi* | | [38] | [33] |
| *Comamonas* | *Comamonadaceae* | *Beta-proteobacteria* | EF426440 | F, F* | P, A | *dureni, funestus, gambiae, quadrimaculatus, stephensi* | [17, 21] | [38, 35, 39, 40] | [30] |

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|---|---|---|---|---|---|---|---|---|---|
| *Delftia* | *Comamonadaceae* | *Beta-proteobacteria* | EF426438 | L | P | *gambiae* | | [35] | |
| *Ehrlichia* | *Anaplasmataceae* | *Alpha-proteobacteria* | | F | A | *arabiensis* | | | [22] |
| *Enterobacter* | *Enterobacteriaceae* | *Gamma-proteobacteria* | HQ832863 | F, F*, L | L, A | *albimanus, darlingi, funestus, gambiae (ss, sl), stephensi* | [17] | [11, 38, 23, 30, 31] | [38, 23, 26] |
| *Erwinia* | *Enterobacteriaceae* | *Gamma-proteobacteria* | FJ816023 | F, L | A | *darlingi, funestus, gambiae* | | [37] | [30, 33] |
| *Escherichia-Shigella* | *Enterobacteriaceae* | *Gamma-proteobacteria* | FJ608223 | F, F*, L | A | *arabiensis, darlingi, funestus, gambiae (ss, sl), stephensi* | [21, 36] | [11, 38, 30] | [30, 33] |
| *Ewingella* | *Enterobacteriaceae* | *Gamma-proteobacteria* | | L | A | *stephensi* | | [25] | |
| *Gluconacetobacter* | *Acetobacteraceae* | *Alpha-proteobacteria* | FN814298 | F*, L | A | *gambiae* | [21] | [27] | |
| *Gluconobacter* | *Acetobacteraceae* | *Alpha-proteobacteria* | | F, L | A | *funestus, stephensi* | | | [26, 30] |
| *Herbaspirillum* | *Oxalobacteraceae* | *Beta-proteobacteria* | FJ608162 | F, L | A | *gambiae, stephensi* | | [11] | [38] |
| *Hydrogenophaga* | *Comamonadaceae* | *Beta-proteobacteria* | FJ608063 | F, F* | L | *gambiae (ss, sl), stephensi* | [17] | | [38, 30] |
| *Ignatzschineria* | *Xanthomonada-ceae* | *Gamma-proteobacteria* | FJ608103 | F | L | *stephensi* | | | [38] |
| *Klebsiella* | *Enterobacteriaceae* | *Gamma-proteobacteria* | HQ591433 | F, F*, L | L, A | *darlingi, funestus, gambiae (ss, sl), stephensi* | [17] | [23, 30, 37, 39] | [38, 30, 33] |
| *Kluyvera* | *Enterobacteriaceae* | *Gamma-proteobacteria* | | F | | *funestus, gambiae* | | [19] | [30] |
| *Leminorella* | *Enterobacteriaceae* | *Gamma-proteobacteria* | FJ608283 | F | A | *stephensi* | | | [38] |
| *Leptothrix* | *Burkholderiales Genera incertae sedis* | *Beta-proteobacteria* | FJ608083 | F | L | *stephensi* | | | [38] |
| *Morganella* | *Enterobacteriaceae* | *Gamma-proteobacteria* | | F | A | *gambiae sl* | | | [30] |
| *Methylobacterium* | *Methylobacteria-ceae* | *Alpha-proteobacteria* | AB673246 | F, F* | A | *funestus, gambiae* | [21, 36] | | |
| *Methylophilus* | *Methylophilaceae* | *Beta-proteobacteria* | FJ517736 | F* | P | *gambiae* | [17] | | |
| *Neisseria* | *Neisseriaceae* | *Beta-proteobacteria* | JX010905 | F* | A | *gambiae* | [21] | | |
| *Novosphingobium* | *Sphingomonada-ceae* | *Alpha-proteobacteria* | JX222980 | F* | A | *gambiae* | [17] | | |
| *Pantoea* | *Enterobacteriaceae* | *Gamma-proteobacteria* | JF690934 | F, L | L, A | *albimanus*, darlingi, funestus, gambiae (*) (ss, sl), stephensi (*)* | | [11, 19, 24, 35] | [38, 30, 33] |
| *Pelagibacter* | *SAR11 cluster* (no family) | *Alpha-proteobacteria* | GQ340243 | F* | A | *gambiae* | [17] | | |
| *Phenylobacterium* | *Caulobacteraceae* | *Alpha-proteobacteria* | | F | A | *gambiae* | | | [26] |
| *Phytobacter* | *Enterobacteriaceae* | *Gamma-proteobacteria* | | L | A | *gambiae* | | [11] | |
| *Porphyrobacter* | *Erythrobacteraceae* | *Alpha-proteobacteria* | JQ923889 | F* | L | *gambiae* | [17] | | |

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|---|---|---|---|---|---|---|---|---|---|
| *Pseudomonas* | *Pseudomonadaceae* | *Gammaproteobacteria* | EF426444 | F, F*, L | L, P, A | *albimanus, darlingi, dureni, funestus, gambiae (ss, sl), maculipennis, quadrimaculatus stephensi* | [17, 21, 36] | [11, 19, 22-24, 29, 35, 38, 39, 40] | [38, 26, 30, 33] |
| *Rahnella* | *Enterobacteriaceae* | *Gammaproteobacteria* | GU204974 | F | L | *stephensi* | | [24] | |
| *Ralstonia* | *Burkholderiaceae* | *Betaproteobacteria* | AY191852 | F* | A | *gambiae* | [21] | | |
| *Raoultella* | *Enterobacteriaceae* | *Gammaproteobacteria* | HQ811336 | F* | A | *gambiae* | [17] | | |
| *Rhizobium* | *Rhizobiaceae* | *Alphaproteobacteria* | DQ814410 | F* | L | *gambiae* | [17] | | |
| *Salmonella* | *Enterobacteriaceae* | *Gammaproteobacteria* | | F | | *funestus, gambiae sl* | | | [30] |
| *Schlegelella* | *Comamonadaceae* | *Betaproteobacteria* | FR774570 | F* | A | *gambiae* | [21] | | |
| *Serratia* | *Enterobacteriaceae* | *Gammaproteobacteria* | FJ608101 | F, F*, L | L, A | *albimanus, dureni, gambiae, maculipennis,quadrimaculatus, stephensi* | [17, 21] | [11, 19, 25, 31, 37-40] | [38] |
| *Shewanella* | *Shewanellaceae* | *Gammaproteobacteria* | HQ591421 | F | L | *stephensi* | | [23] | |
| *Sphingobium* | *Sphingomonadaceae* | *Alphaproteobacteria* | GU940735 | F* | A | *gambiae* | [17] | | |
| *Sphingomonas* | *Sphingomonadaceae* | *Alphaproteobacteria* | GU204960 | F, F*, L | L, A | *funestus, gambiae, stephensi* | [21, 36] | [11, 24] | [26] |
| *Stenotrophomonas* | *Xanthomonadaceae* | *Gammaproteobacteria* | EF426435 | F, F* | A | *arabiensis, funestus, gambiae* | [17, 21] | [35] | [22, 30] |
| *Thorsellia* | *Enterobacteriaceae* | *Gammaproteobacteria* | NR_043217 | F, F* | L, A | *gambiae, stephensi* | [17] | [38, 22] | [38, 34] |
| *Vibrio* | *Vibrio* | *Gammaproteobacteria* | FJ608116 | F | L, A | *arabiensis* | | [38, 22] | |
| *Xenorhabdus* | *Enterobacteriaceae* | *Gammaproteobacteria* | FJ608329 | F | A | *stephensi* | | | [38] |
| *Yersinia* | *Enterobacteriaceae* | *Gammaproteobacteria* | | F | A | *darlingi* | | | [33] |
| *Zymobacter* | *Halomonadaceae* | *Gammaproteobacteria* | FR851711 | F | A | *funestus, gambiae* | [36] | | |
| ***Others*** | | | | | | | | | |
| *Bacillariophyta (Eukaryota: Diatom)* | | | JQ727029 | F* | L | *gambiae* | [17] | | |
| *Chlorophyta (green algae)* | | | EF114678 | F* | L | *gambiae* | [17] | | |
| *Calothrix* | *Rivulariaceae* | (no data) | FJ608095 | F | L | *stephensi* | | | [38] |
| *Deinococcus* | *Deinococcaceae* | *Deinococci* | FJ608089 | F | L | *stephensi* | | [38] | [38] |
| *Mycoplasma* | *Mycoplasmataceae* | *Mollicutes* | AY837724 | F | A | *arabiensis* | | | [22] |
| *Spiroplasma* | *Spiroplasmataceae* | *Mollicutes* | AY837733 | F | A | *funestus* | | | [22] |
| *Cyanobacteria-GpI* | | | HM573452 | F* | P | *gambiae* | [17] | | |
| *Cyanobacteria-GpIIa* | | | JQ305084 | F* | L | *gambiae* | [17] | | |
| *Cyanobacteria-GpV* | | | AB245143 | F* | L | *gambiae* | [17] | | |
| *Fusobacterium* | *Fusobacteriaceae* | *Fusobacteriia* | JX548360 | F* | A | *gambiae* | [17, 21] | | |

**Table 1.** List of bacterial genera associated with *Anopheles* mosquitoes reported in the following studies: [11, 17, 19, and 21-40]. For high-throughput sequencing studies; only genera found to represent at least 1% of the total population in at least one study/condition are listed. Genera are classified by phyla, which are indicated in bold. In column "Conditions", F, F* and L indicate field, semi-natural and laboratory conditions, respectively. In column "Stage", L, P and A indicate larvae, pupae and adults, respectively. Column "Example" shows NCBI accession number of a sequence example for each genus (first hit after BLAST). Columns "Deep seq", "Culture", "Non culture" list studies based on 16S rRNA gene deep sequencing, culture-dependent methods, conventional sequencing (including 16S rRNA gene libraries and DGGE) and gas chromatography, respectively. In the line "*Pantoea*", * refers to what was identified in [19] as *Enterobacter agglomerans*, since then renamed *Pantoea agglomerans*.

Three metagenomics studies were recently carried out using 16S RNA from bacteria found in the *Anopheles* gut [18, 22, 37]. Wang and co-workers examined the microbiota composition throughout the mosquito life cycle, using a laboratory colony of *A. gambiae* mosquitoes (the main vector of malaria in sub-Saharan Africa) reared in semi-natural microcosms in Kenya [18]. The microcosms contained local rainwater and topsoil and were kept outside to allow microbial colonization. Boissière and co-workers investigated the microbiota of adult *A. gambiae* mosquitoes in Cameroon and how these microbiota may be related to *Plasmodium* infection [22]. They collected larvae from the field, reared them to adulthood in the laboratory and monitored the microbiota composition of individual mosquitoes 8 days after infection with *Plasmodium falciparum* sampled directly from gametocytemic patients. Finally, Osei-Poku and co-workers collected adult mosquitoes in Kenya and analysed the microbiota of individual mosquitoes of 8 different species, including 3 species of *Anopheles* (*A. coustani*, *A. funestus* and *A. gambiae*) [37].

These studies led to 5 main observations. First, the microbiota diversity is high: when defining species as $OTU_{97\%, V1\text{-}V3}$[1], Wang et al. detected more than 2,000 species in a pool of 30 adult *A. gambiae* [18]. The highest diversity was registered in larvae and pupae, with an estimate of 4,000-8,000 species in a pool of 30 individuals of each stage. Diversity decreased during adulthood to 2,000-4,000 species upon emergence and dropped further to 600-900 species after a bloodmeal. As all of these high-throughput sequencing studies used bacterial DNA, which is a very stable molecule, an important question is whether these results genuinely reflect the *Anopheles* gut communities or include environmental contaminants. By direct sampling of the larval aquatic environment, Wang et al. indeed showed that the microbial communities differed from those in the larvae, suggesting that – at least in this study – bacteria were able to persist in, if not colonise, the mosquito host (Figure 1A).

Second, this diversity is partially explained by significant diversity within a single mosquito [22, 37], varying from 5 to 71 $OTUs_{97\%, V3}$ per individual (median: 42 $OTUs_{97\%, V3}$) [37]. Diversity is higher than what observed by metagenomics studies in other insects such as the honeybee which hosts 8 dominant species ($OTU_{97\%, V6\text{-}V8}$), the estimated species richness within a colony being 9-10 [44], and *Drosophila* where 31 $OTUs_{97\%,V2}$ were observed in a pool of 50 females [45]. Nevertheless, a single $OTU_{97\%, V3}$ represents on average 67% of a mosquito bacterial community and the median mosquito gut species richness is only 17% to that of humans, where an individual hosts 150-300 $OTUs_{99\%, whole\ 16S}$ [4, 37].

Third, another component of the observed biodiversity lies within the high variability in microbial communities between individuals. This is quantified by calculating the UniFrac distance between mosquitoes. UniFrac varies from 0 when two mosquitoes have exactly the same microbiota to 1 when there is no phylogenetic overlap between the microbiota of two mosquitoes. The mean UniFrac distance between individuals is high, 0.72 and 0.74 in *A. funestus* and *A. gambiae*, respectively [37]. This variability is almost as high between *Anopheles* individuals of the same species as between mosquitoes of different species and/or genera [37].

1 As not all the studies were based on the same region of 16S or the same threshold of differences, we refer here to OTU97%, V1-V3 as the operational taxonomic unit with more than 97% identity in the V1-V3 regions of 16S rRNA gene sequences.

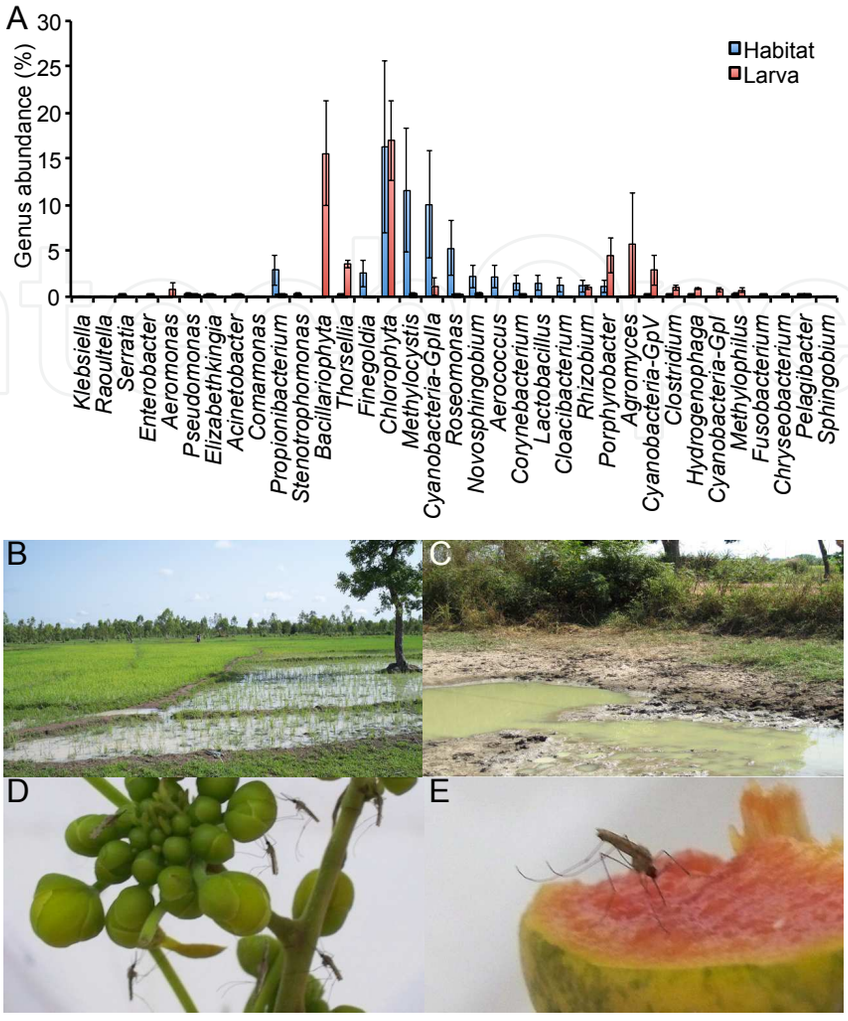


**Figure 1.** Anopheles microbiota and environment. A: Abundance of bacterial genera in larval habitat and in larvae found in [17]. B, C: Natural habitat of *A. gambiae*. Permanent habitats such as rice fields (B) are colonized with M molecular form of *A. gambiae* and temporary water ponds (C) with S plus M forms (mostly S). D, E: Mosquitoes feeding on *Senna siamea* flowers (D) and papaya fruit-*Carica papaya* (E).

Fourth, the microbiota composition partly reflects the larval origin but bacteria acquired during adulthood may affect the microbiota composition to the extent that the geographic origin cannot be traced. Osei-Poku and co-workers did not observe any correlation between geographic location and microbiota composition in their Kenyan adult collections [37]. This is in sharp contrast to the Boissière et al. observations that microbiota were more similar between adults derived from larvae breading in the same pond than between adults derived from larvae of different geographic origins [22]. These results are, however, not contradictory if we consider differences in experimental designs of these studies. The latter study focused almost exclusively on bacteria transmitted from larvae to adults since larvae from the field were sampled and adults where fed with sterile sugar upon emergence, while the former study additionally sampled bacteria acquired during adulthood, and related to presumably diverse adult life histories. Together, these studies suggest that the acquisition of new strains of bacteria

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during adulthood can potentially increase the inter-individual diversity and mask similarities linked to the larval origin. However, this hypothesis requires further investigation, as mosquitoes from the two geographical origins reported in the Boissière et al. study belonged to the M and S molecular forms of *A. gambiae*, respectively, which are thought to be emerging species breading in different types of aquatic environments, i.e. permanent and temporary (rain-dependent) water pools, respectively (see Figures 1B, C) [22]. These environments are likely to contain different microbiota that largely determine the mosquito enterotype. Additionally, genetic differences between the two molecular forms may also partly account for the observed differences in microbiota composition.

Fifth, when considering the *Plasmodium* infection status, Boissière and co-workers found that the abundance of bacteria of the *Enterobacteriaceae* family was higher in *P. falciparum*-infected mosquitoes than in non-infected mosquitoes fed with the same infectious bloodmeal. This observation may indicate that *Enterobacteriaceae* favour *P. falciparum* infection or, conversely, that *P. falciparum* infection influences the composition of microbiota to the benefit of *Enterobacteriaceae* [22].

## 3. Bacterial colonization of mosquitoes

In addition to metagenomics studies, factors determining the composition of the adult mosquito microbiota were also investigated by conventional methods. Evidence that mosquitoes are colonized by bacteria both found in the environment and transmitted between individuals or developmental stages was revealed, but the relative contribution of these transmission routes to the microbiota diversity remains largely unknown. Laboratory studies investigated the vertical (from parent to progeny), transstadial (between developmental stages) and horizontal (between individuals of the same stage) transmission of specific bacterial strains. In particular, horizontal transfer of *Asaia sp*. is found to occur both by feeding and by mating (from male to female), but it is yet unclear whether vertical transmission occurs via egg spreading or by contamination of the environment during egg-laying [27]. Transstadial transmission of *Pantoea stewartii* is shown to occur from larvae to pupae but not from pupae to adults [36]. This is likely due to gut sterilization during metamorphosis; bacterial counts are high in the gut of fourth instar larvae, decrease after final larval defecation, increase again during pupal development and are very low or null in newly emerged adults [46].

Two mechanisms are thought to be involved in gut sterilization during adult emergence [46]. Firstly, bacteria are enclosed in the degenerated larval midgut, the meconium, enveloped by 2 meconial peritrophic matrixes and egested during molting. Secondly, during emergence, adults ingest exuvial liquid that has bactericidal properties. Nevertheless, sterilisation is thought to be incomplete, thus allowing some direct transmission from pupae to adults [46] and being responsible for the contribution of the larval/pupal breading sites to the adult microbiota, as mentioned earlier [22]. Moreover, emerging adults have been reported to ingest water and uptake bacteria during or shortly after emergence, with colonization efficiencies depending on the bacterial strains, e.g. *Elizabethkingia anophelis* (previously thought to be *E.*

*meningoseptica)* is more successful than *Pantoea stewartii* [33, 36]. During adulthood, mosquitoes take sugar-meals of floral and extra-floral nectar, sap, ripe fruit and honeydew (Figure 1D, E) [47-49]. These meals potentially provide new bacterial species and are likely to affect the relative growth of existing species or strains depending on their properties, such as the concentration of each sugar type, typically glucose, fructose or gulose [50]. This might well be the case for *Asaia* and *Gluconacetobacter*, two genera usually found in flowers, and which have been identified as part of the adult *Anopheles* microbiota [22, 27].

The *Anopheles* tissue specificity of *Asaia sp.* was studied using a bacterial strain expressing GFP (green fluorescent protein) [27]. *Asaia* was found in the female gut and salivary glands, two tissues of particular interest to vector biology, but also in the male reproductive tract and the larval gut, which are potentially important tissues for the bacterial spread [27]. The microbiome of *Anopheles* other tissues than the gut has not yet been characterized. Interestingly, *Wolbachia sp.*, a maternally transmitted intracellular bacterium able to colonize multiple tissues in other insects, has not yet been found in any *Anopheles* species. This is of particular interest, as this endosymbiont colonizes around half of the insect species including several *Culex* and *Aedes* mosquito species [51]. Reasons for the apparent incompatibility between *Anopheles* and *Wolbachia* are unknown, but the generation of *Wolbachia*-infected *Anopheles* colonies is currently being pursued. Laboratory infection has been achieved for *Ae. aegypti* [52, 53], where *Wolbachia* is a promising candidate for reducing the vector competence (see below). To our knowledge, no endosymbiont has been described in *Anopheles* to date.

Non-bacterial members of the *Anopheles* microbiota are poorly understood. Such studies are of special interest, as these microorganisms can potentially interact directly with the bacterial microbiota as well as the human pathogens and are likely to affect the mosquito physiology. An initial study, based on sequencing a 18S-library, identified 6 fungal clones related to *Candida sp.*, *Hanseniaspora uvarum*, *Pichia sp.*, *Wallemia sebi*, *Wickerhamomyces anomalus* and uncultured fungi in laboratory-reared *A. stephensi* [54]. *W. anomalus* is also found in wild and laboratory-reared *A. gambiae* [55]. TEM observation of mosquito tissues revealed the presence of yeasts in the female midgut and of actively dividing yeasts in the male gonoduct of *A. stephensi* [54, 55].

## 4. Impact of microbiota on *Anopheles* physiology and pathogen transmission

The studies reviewed above suggest that *Anopheles* mosquitoes do not host any particular obligate symbiont. However, bacteria as a whole appear to be essential for mosquito physiology. In particular, it has not been possible to date to maintain *Anopheles* colonies on conventional laboratory diet in axenic conditions. In addition, *A. stephensi* larval development is slowed down in the presence of antibiotics and putatively blocked at the 3$^{rd}$ or 4$^{th}$ instar, but an antibiotic-resistant strain of *Asaia* is sufficient to revert this effect [56]. Although the mechanism involved in this dependence is unknown, several lines of experimental evidence point to the important nutritional role of gut commensals. First, the development of aseptic

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*A. stephensi* mosquitoes was achieved from sterilized eggs to adults in a custom aseptic medium [57], although no mention is made about adult fertility under these conditions. Second, a delay in the development was also observed in *Drosophila melanogaster* raised in axenic conditions under protein deprivation, which was rescued by the addition of live *Lactobacillus plantarum* in the fly medium [58]. *L. plantarum* was shown to promote larval growth under poor dietary conditions by enhancing nutrient sensing in a TOR-dependent manner, thus acting on ecdysone and insulin-like-peptide pathways [58]. Third, larval mortality was reported in the clothing louse deprived of its bacterial symbionts and can be avoided by supplementing the blood with B-vitamins (ß-biotin, pantothenate and nicotinic acid) [59]. The *Anopheles* microbiota may also participate in metabolism, as adult mosquitoes fed with radiolabelled-Glycine *Pseudomonas* displayed radioactive signal throughout their body [40]. Interestingly, *Plasmodium* oocysts and sporozoites developing in these mosquitoes also contained radioactive compounds, suggesting that bacteria also participate in parasite nutrition [40].

*Anopheles* females appear to also sense bacterial presence in the water, which influences oviposition in a bacterial strain dependent manner [60]. The underlying stimuli are not known but they are likely semiochemicals, i.e. messenger molecules produced by bacteria [60]. A principal component analysis of volatiles emitted by 17 bacterial strains, including 6 oviposition-inducing strains, failed to identify compounds shared between all oviposition-inducing bacterial strains, suggesting that such semiochemicals are acting as cocktails [60].

An aspect of the *Anopheles* microbiota that received great interest recently is the colonisation resistance effect towards *Plasmodium* infection, as depicted in Figure 2. First, bacterial growth after a bloodmeal is reported to trigger an immune response via the Immune-deficiency (Imd) pathway, which causes synthesis of antimicrobial peptides and other immune effectors [2]. These effectors target bacterial populations in the mosquito midgut and exert antiparasitic effects. Second, an *Enterobacter* strain (*EspZ*) isolated from wild *A. arabiensis* mosquitoes is shown to directly affect *Plasmodium* development in the mosquito gut via elevated synthesis of ROS (reactive oxygen species) [1]. Third, microbiota-dependent immune priming is reported upon *Plasmodium* infection. This effect protects mosquitoes from subsequent *Plasmodium* infections and is likely to be mediated by hemocyte differentiation [3].

As mentioned above, *Anopheles* mosquitoes are also vectors of filarial worms and ONNV (anophelines are also secondary vectors of West Nile virus). The effect of gut microbiota on infection with these pathogens has not been thoroughly investigated to date, but feeding *A. quadriannulatus* with an antibiotic/antimycotic mixture is shown to increase *Brugia malayi* infection [61]. In *Ae. aegypti*, antibiotic treatment increases the susceptibility of mosquitoes to Dengue virus via a decrease in antimicrobial gene transcription [53]. This can be reverted by addition of bacterial strains such as *Proteus sp.* and *Paenibacillus sp.* [62]. The role of *Anopheles* microbiota upon viral infections is still unclear, but our unpublished observations suggest that antibiotic treatment of *A. gambiae* increases significantly the prevalence of infection with ONNV.

Vertically-transmitted *Wolbachia* endosymbionts are under special focus as promising candidates to stop pathogen transmission. Research in this field has advanced in *Ae. aegypti*, where stable infections of *Wolbachia* strains have been established in laboratory colonies [52, 53]. The

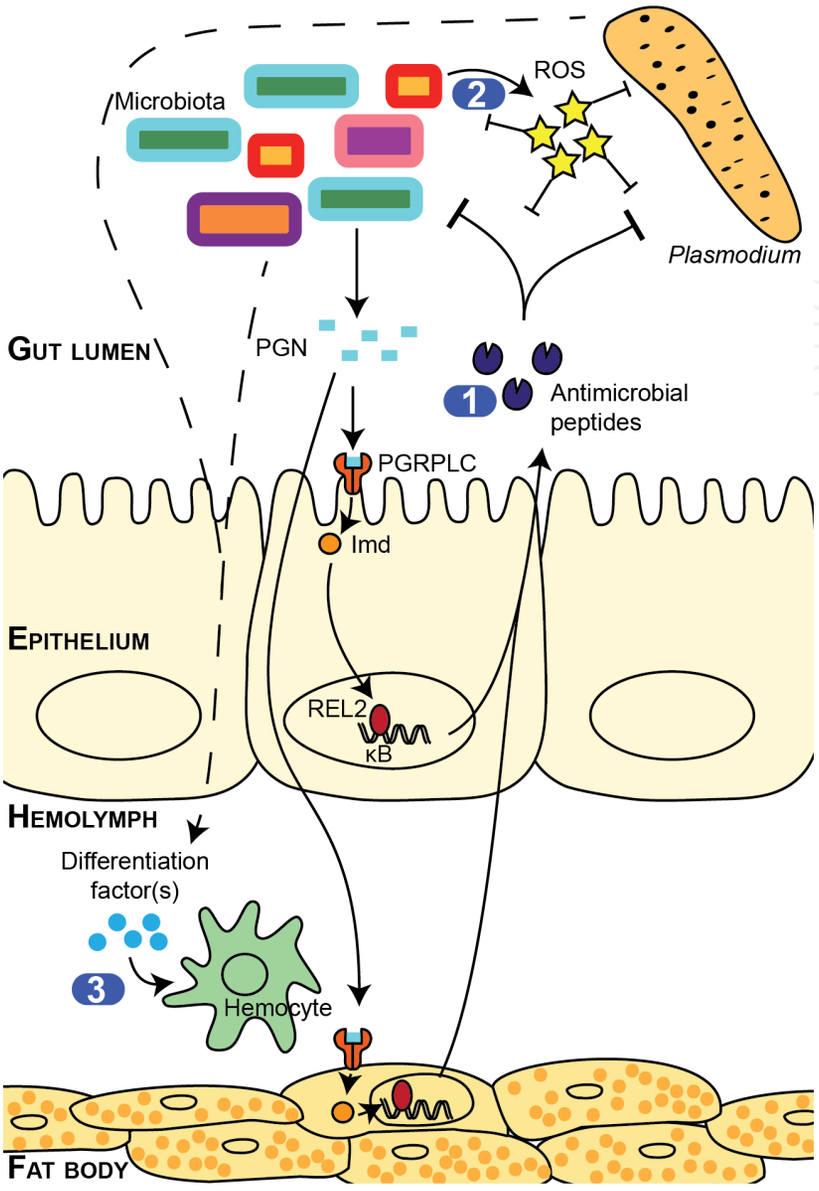


**Figure 2. Mechanisms of colonization resistance conferred by *Anopheles* microbiota against *Plasmodium* infection.** 1 — Direct effect via synthesis of ROS by the *Enterobacter EspZ* strain [1]. 2 — Indirect effect via induction of NF-κB antibacterial responses that have antiparasitic effects [2]. This is likely to be the most general mechanism. 3 — Induction of hemocyte differentiation by unknown soluble hemolymph factors during *Plasmodium* infection, which has a priming effect against asubsequent *Plasmodium* infection [3].

fast growing wMelPop strain of *Wolbachia* halves the mosquito lifespan, thus potentially affecting the capacity of mosquitoes to transmit pathogens with long extrinsic incubation periods [52]. It also induces a constitutively elevated immune response that negatively impacts on the infection prevalence and intensity of *Brugia pahangi* microfilariae, Chikungunya and Dengue viruses and the avian parasite *Plasmodium gallinaceum* [15, 17]. wAlbB and wMel, which naturally infect the Asian tiger mosquito *Aedes albopictus* and *D. melanogaster*, respectively, also render *Aedes* mosquitoes resistant to Dengue virus when introduced into laboratory

populations [16, 63, 64]. Moreover, wMel is shown to successfully spread into wild *Ae. aegypti* populations in North-Eastern Australia [65] and is a strong candidate for Dengue biocontrol. When injected into *Anopheles* mosquitoes, *Wolbachia* seems to positively or negatively impact on *Plasmodium* infection depending on the *Wolbachia*/*Plasmodium* strain/species combination [66-68].

The immune system of *Anopheles* is known to control the microbiota population, by both resistance and tolerance mechanisms. On the one hand, the Imd pathway is shown to control the midgut bacterial numbers, especially after a bloodmeal [2], together with the production of ROS [21]. The melanization reaction might also contribute to limiting the bacterial numbers, as shown in the hindgut of the silkworm *Bombyx mori* [69]. On the other hand, induction of the Duox-IMPer (Dual oxidase - Immunomodulatory peroxidase) pathway after a bloodmeal leads to the formation of a dityrosine-linked mucus layer in the space between the peritrophic membrane and the midgut epithelium that reduces the permeability to immune elicitors. This tolerance mechanism leads to increased bacterial and *Plasmodium* loads [21]. Interestingly, such protection from oxidative stress is also identified in *Ae. aegypti*, where blood heme induces a protein kinase C-dependent mechanism leading to decreased ROS production and bacterial proliferation [70]. In *Drosophila*, several negative regulators of the Imd pathway are involved in tolerance to gut bacteria, but equivalent tolerance mechanisms have not yet been described in *Anopheles*. In particular, PGRP-LB and PGRP-SC1A/B degrade peptidoglycan into non-immunogenic fragments and Pirk downregulates the activity of the PGRP-LC and PGRP-LE receptors [71-76]. Orthologs of these regulators PGRPs, but not of Pirk, are present in *Anopheles* [77, 78].

In several insect species, microbiota are shown to also impact on host behavior. Notably, *Drosophila* mating preference is influenced by the microbiota composition [79]. *Klebsiella oxytoca* is proposed as a probiotic able to rescue the loss of copulatory performance that follows male sterilization by irradiation in medfly (*Ceratitis capitata*), by restoring the *Klebsiella*/*Pseudomonas* ratio to its normal levels [80]. In termites, a Rifampicin treatment is shown to reduce the queen oviposition rate and to decrease longevity and fecundity of termite reproductives [81]. As *Anopheles* mosquitoes are able to sense the presence of bacteria in water as well as on human skin and modulate their oviposition rate and feeding behavior accordingly [60, 82], the microbiota composition could also influence the mosquito social and/or reproductive behavior and feeding preference. This may prove to be of particular importance to vector control.

## 5. Potential exploitations to reduce *Anopheles* vector competence

Reduction of the *Anopheles* competence to transmit human pathogens, especially malaria, will have great implications on public health. Any perspective of reducing vector competence should affect at least one of the parameters of the Ross-McDonald model of disease transmission [83]. These parameters include the mosquito-to-man ratio, the mosquito biting rate, the probability of successful man-to-mosquito and mosquito-to-man transmission, the mosquito

daily survival probability, the days needed for the parasite in the mosquito to become infective and the daily rate at which humans become non-infectious to mosquitoes. From studies carried out to date and reviewed in preceding sections, it is evident that the mosquito microbiota can potentially affect most of these parameters except those referring only to disease progression in the vertebrate host. The most important of these parameters are mosquito longevity, feeding behavior and capacity to support pathogen development and/or replication.

A direct way to reduce vector competence using our current knowledge of the *Anopheles* microbiota would be to use bacterial strains that are naturally incompatible with pathogen development and/or replication. Potential candidates are either natural microbiota such as the EspZ strain of *Enterobacter* that causes resistance to *Plasmodium* [1] or artificially introduced bacteria such as *Wolbachia*, which apparently induce a wide spectrum of resistance to human pathogens [15]. The great advantage of the latter is its ability to spread into populations by manipulating insect reproduction in several ways. In particular, *Wolbachia* induces death of young embryos laid by *Wolbachia*-free females mated with infected males; *Wolbachia*-infected females are always fertile independently of the male infection status [84]. This so-called cytoplasmic incompatibility confers a reproductive benefit to *Wolbachia*-infected females and leads to propagation of *Wolbachia* even if it bears small fitness cost to the host, including reduced fecundity (discussed in [85, 86]). The challenge of this approach is the fact that *Wolbachia* and *Anopheles* seem to be incompatible in nature and introduction of the endosymbiont in laboratory colonies of *Anopheles* has not yet been achieved. Screening of *Wolbachia* strains able to infect the *Anopheles* reproductive tissues, when cultured *ex vivo*, has been reported [87]. Alternatively, preadaptation of *Wolbachia* strains by long-term culturing in mosquito cell lines has been suggested as a strategy to infect new hosts, as shown successfully for *Aedes* [52, 88]. As previously reported in *Aedes* [15-17], *Wolbachia* might impact both on mosquito longevity and successful development and/or replication of all three taxa of *Anopheles*-borne pathogens, i.e. *Plasmodium*, viruses and nematodes.

An alternative approach is paratransgenesis, the introduction of genetically modified bacteria into the vector, which would confer resistance to pathogens. *Pantoea agglomerans*, a natural *Anopheles* symbiont, is a candidate for this approach and has been successfully engineered to express and secrete proteins that either inhibit midgut invasion by *Plasmodium*, such as $[EPIP]_4$ (*Plasmodium* enolase-plasminogen interaction peptide) that competes with *Plasmodium* EPIP for plasminogen binding, or by directly targeting the parasite, such as the scorpion-derived antiplasmodial scorpine [89, 90]. Green fluorescent protein (GFP)-tagged *P. agglomerans* persists and grows in the *Anopheles* gut, while transgenic *P. agglomerans* confers resistance against *P. falciparum* infection in both *A. stephensi* and *A. gambiae* without affecting the mosquito lifespan [90]. Applicability to more than one mosquito species is particularly advantageous for a transmission blocking approach. *Asaia* has also been proposed as a candidate for paratransgenesis, as it is quite frequent in *Anopheles* microbiota and can be successfully transformed [27]. Interestingly, this genus has been found in all of the 30 individuals assessed in the metagenomics study of Boissière et al. suggesting that it can easily spread into field populations [22]. *Asaia* can be transmitted both horizontally and vertically presenting an additional advantage for the spread of a

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transgenic strain into mosquito populations [27]. The introduction of such microbiota into mosquito populations could be achieved by using baiting stations, i.e. clay jars containing cotton balls soaked with sugar and bacteria, around malaria endemic villages, but this approach requires further investigation [90].

Finally, transmission-blocking interventions could involve drugs or other interventions that would impact on the microbiota, thus affecting mosquito homeostasis and efficiency of pathogen development. For example, the effects of antibiotics in the human blood could significantly impact the mosquito microbiota upon blood feeding, indirectly influencing mosquito physiology and infection with pathogens. Depending on its spectrum, an antibiotic could influence the microbiota composition and thus have a positive or negative impact on pathogen development and/or replication.

## 6. Conclusion

Recent high-throughput sequencing studies of the *Anopheles* microbiota have revealed the extent of the microbiota diversity, mostly in field or semi-natural conditions. A diverse range of bacteria is able to colonize the *Anopheles* gut, and there is a vast diversity of microbiota between mosquitoes. To some extent, this diversity needs to be considered at the bacterial strain level, as different strains of one species may have diverse effects on the mosquito physiology and other microbes of the gut ecosystem. Although bacteria may be the most abundant and important members of the gut microbiota, characterization of the viral, fungal and protist communities could prove insightful into the understanding of the homeostasis of this complex biological system (e.g. phage predation is thought to regulate bacterial populations [91]) and its effects on pathogen transmission. An important question that may arise from further studies is whether variability and/or discrepancies in experimental findings about the interactions between mosquitoes and pathogens could be attributed to differences in the microbiota between laboratories. Toward exploiting the knowledge on *Anopheles* microbiota to reduce vector competence, research is currently at its infancy, but some bacteria such as *Pantoea* and *Asaia* already emerge as promising candidates of paratransgenesis. The use of *Wolbachia* to reduce *Aedes* vectorial capacity and fitness may be of particular importance, if this technology can be effectively transferred to *Anopheles*. Finally, the possibility to use drugs such as antibiotics to target specific mosquito microbiota and affect vector competence or fitness is a new concept that merits further investigation.

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