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16S Metagenomic Comparison of *Plasmodium falciparum*–Infected and Noninfected *Anopheles gambiae* and *Anopheles funestus* Microbiota from Senegal

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Abstract. In the context of the pre-elimination of malaria, biological control may provide an alternative or additional tool to current malaria control strategies. During their various stages of development, mosquitoes undergo subsequent changes in their associated microbiota, depending on their environment and nutritional status. Although *Anopheles gambiae* s.l. and *Anopheles funestus* are the two major malaria vectors in Senegal, the composition of their microbiota is not yet well known. In this study, we explored the microbiota of mosquitoes naturally infected or not by *Plasmodium falciparum* (*Pf*) using the 16S ribosomal RNA gene-based bacterial metagenomic approach. In both vector species, the microbiota was more diverse in *Pf*-infected samples than in the noninfected ones, although the total number of reads appeared to be higher in noninfected mosquitoes. Overall, the microbiota was different between the two vector species. Noteworthy, the bacterial microbiota was significantly different between *Pf*-positive and *Pf*-negative groups whatever the species, but was similar between individuals of the same infection status within a species. Overall, the phylum of *Proteobacteria* was the most predominant in both species, with bacteria of the genus *Burkholderia* outweighing the others in noninfected vectors. The presence of some specific bacterial species such as *Asaia bogorensis*, *Enterobacter cloacae*, *Burkholderia fungorum*, and *Burkholderia cepacia* was also observed in *Pf*-free samples only. These preliminary observations pave the way for further characterization of the mosquito microbiota to select promising bacterial candidates for potential use in an innovative approach to controlling malaria and overcoming the challenges to achieving a malaria-free world.

INTRODUCTION

Over the last 15 years, significant progress has been made against malaria around the world. Over this period, the annual number of malaria cases decreased by 18%, from 262 million in 2000 to 214 million in 2015. Similarly, annual malaria-related deaths decreased by 48%, from 839,000 deaths in 2000 to 438,000 in 2015. Over the same period, the prevalence of parasites among children aged between 2 and 10 years dropped from 33% to 16%, and malaria-related deaths among children younger than 5 years decreased from 723,000 to 306,000. Although most cases of malaria and associated deaths were reported in the WHO African region (88%), the region recorded the largest decrease in malarial burden.[1] These successes were the result of an integrated approach based on rapid diagnostic testing and treatment of clinical cases with artemisinin-based combination therapies, and preventive measures with core insecticide-based vector control interventions (long-lasting insecticide-treated nets and indoor residual spraying).[2] Although the gains have been massive in many parts of Africa, the increasing resistance of the parasite to the available drugs and vectors to insecticides may jeopardize the gains which have been made.

In the current situation, the development of innovative control tools and measures in addition to the current interventions is imperative to reduce malaria transmission and to reach the goal of malaria elimination. The use of the microbiota naturally inhabiting the mosquitoes' midgut to hamper the development of malarial parasites represents a potential strategy for malaria control. However, given the lack of comprehensive data on the mosquito's specific microbiota, with potential antagonistic effects on malarial parasites, primary studies to gather sufficient information are thus an important step in identifying bacteria that could negatively affect the development of parasites in the vectors. Exploration of the microbiota by the conventional method of bacteriology has many limitations, including non-cultured bacteria.[3] Therefore, few studies have attempted to inventory the mosquito microbiota using culture and isolation techniques.[4–6] The recent development of metagenomics approaches, targeting 16S ribosomal RNA (rRNA) has contributed toward overcoming these limitations.[7] This technique has enabled significant advances to be made in the understanding of the arthropod microbiota and its modifications related to insect feeding.[8]

Using the metagenomics approaches targeting 16S ribosomal RNA, this study aimed to characterize the microbiota naturally inhabiting wild populations of *Anopheles gambiae* s.l. and *Anopheles funestus* in Senegal. It then aimed to compare the microbiota of the naturally *Plasmodium falciparum* (*Pf*)–infected and noninfected mosquitoes to identify potential candidates for biological and/or paratransgenic control of malaria.

MATERIALS AND METHODS

Study sites. The study was conducted in the administrative regions of Fatick and Kedougou located in the center and southeast of Senegal, respectively. In Fatick, wild *Anopheles* populations were collected in the villages of Dielmo and Ndiop, whereas in Kedougou, the localities of Bandafassi and Tombonrokoto were chosen for sampling (Figure 1).

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Dielmo is located next to the marshy bank of a small permanent stream (the Nema), 280 km southeast of Dakar in the Sudan savannah region of central Senegal. The village, historically known as a malaria-endemic site, was selected in 1990 as part of a longitudinal study on natural malaria infection and the protective immunity mechanisms in a community living in a holoendemic area of Senegal.[9] The village of Ndiop, located 5 km from the Dielmo and with contrasting characteristics, was chosen 3 years later as a comparison site.[10] Belonging to the Sudan–Guinean savanna zone, Bandafassi and Tomboronkoto are located 700 km from Dakar in southeastern Senegal, in a region where the highest incidence of malaria has been recorded.[11]

Mosquito sampling and processing. Host-seeking females were caught monthly, indoors, and outdoors for three consecutive nights during the transmission season, using the human-landing collection method. The females captured were morphologically identified at the genera and species level as *A. gambiae*, *A. funestus*, other anopheline species, or other mosquito genera, using the morphological identification key described by Diagne et al.,[12] in 1994. Respectively, 21% and 90% of mosquitoes caught in Fatick and Kedougou were dissected on collection to estimate approximate age of each vector population.

Molecular analyses. *Anopheles* specimens caught in the field the day before were killed at −80°C, washed in a 70% ethanol bath to remove superficial environmental bacteria, and then dissected to separate the abdomen from the head–thorax, from which genomic DNA was immediately extracted using the cetyl-trimethyl-ammounium bromide 2% method.[13]

Quantitative polymerase chain reaction (qPCR) *design for* P. falciparum (Pf) *detection.* Specific qPCR primers (fwd: 5′- GGA CAT AAT AAA AGG TTT TTC TTC CA -3′, rev: 5′- CAA AAT ACA CAA AAT ACA GAA CCA AA -3′) and probes (6-FAM-CAT TAT GAT GTG ACG TGG TAG GAT GGG-TAMRA) targeting the 18S gene were designed for the detection of *Pf* infection.

A total reaction volume of 20 µL, containing 10 µL of master mix Takyon® (Eurogentec, Angers, France), 1 µL of each primer, 1 µL of probe, 2 µL of distilled water, and 5.0 µL of the DNA template, was used to amplify the *Pf* 18S gene using the CFX96 Touch detection system thermal cycler (Bio-Rad, Marnes-la-Coquette, France). The cycling conditions consisted of an initial denaturation at 95°C for 95 seconds, followed by 40 cycles of denaturation at 95°C for 10 seconds, and then annealing at 60°C for 45 seconds. A sample was considered positive when the cycle threshold value was less than 35 cycles.

Metagenomics. A total of 20 unfed parous females (10 *Pf*-infected and 10 noninfected) of each of the two vector species were randomly subsampled for metagenomic study based on the 16S rRNA gene. All the specimens of *A. funestus*, infected

FIGURE 1. Location of study sites. The study administrative regions and sampling villages are represented by red and blue spots, respectively. This figure appears in color at www.ajtmh.org.

or not, originated from the Fatick region, whereas *A. gambiae* *Pf*-infected females were from Kedougou and Fatick and the *Pf*-free from Fatick. The MiSeq sequencing technology (Illumina, Inc., San Diego, CA) with paired-end strategy construct according to the 16S Metagenomic Sequencing Library Preparation (Illumina, Inc.) was adopted. The sequencing libraries were to amplify the "V3–V4" region of the 16S rRNA gene, using the Kapa HiFi Hotstart ReadyMix 2× (Kapa Biosystems, Inc., Wilmington, MA), and the surrounding conserved region V3–V4 primers with overhang adapters (FwOvAd_341F TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG; ReOvAd_785R GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C).

After purification on AMPure beads (Beckman Coulter, Inc., Fullerton, CA), the concentration was measured using the High Sensitivity Qubit technology (Beckman Coulter, Inc.) and dilution at 0.2 ng/μL was performed. Using a subsequent limited cycle of polymerase chain reaction (PCR) on 1 ng of cleaned PCR product, Illumina sequencing adapters and dual-index barcodes were added to the amplicon. After purification on AMPure beads (Beckman Coulter, Inc.), the libraries generated were pooled for a total of 95 multiplexed samples. The global concentration was quantified by a Qubit High Sensitivity Kit (Life Technologies, Carlsbad, CA) to 31.1 ng/μL. Before loading for sequencing on MiSeq (Illumina, Inc.), the pool was diluted at 5 pM. Automated cluster generation and paired-end sequencing with dual-index reads was performed in a single 39-hour run in a 2 × 250 bp. A total of 7 Gb of information was obtained from a 754 K/mm^2 cluster density, with a cluster passing quality control filters of 84.8% (13,627,000 clusters). Within this run, the index representation was determined with an average of 0.47%. Paired reads with an average of 78,140 were filtered according to the read qualities. The raw data were saved into a FASTQ file format for R1 and R2 reads.

The paired-end sequences of the corresponding raw FASTQ files from Illumina Miseq were assembled into longer sequences by fast length adjustment of short reads (FLASH)[14] with a quality score cutoff value of 33. The low-quality reads were filtered out in quantitative insights into microbial ecology (QIIME).[15] Primers were removed and shorter sequences (< 200 nts) along with longer (> 1,000 nts) sequences were discarded. ChimeraSlayer was used to remove chimeric sequences. Operational Taxonomic Unit (OTU) clustering of all the sequences that passed all the quality controls were performed in UCLUST[16] using the de novo clustering method at 97% identity.

Taxonomic assignments. The OTUs were blasted[17] against the Silva reference database[18] (release 128) and the matches with ≥ 80% identity and 100% coverage were extracted from the reference database. The taxonomy was assigned by applying majority voting,[19,20] considering species level to be ≥ 98% identity.

Statistical analysis. Both the *Pf*-infected and *Pf*-free samples used in this study were randomly selected using a random stratified sampling method function in the Excel program. The Excel software was also used for the correlation analysis between bacterial genera and the presence or absence of *Pf* in anopheline vectors. Potential variations within and inter-species/sample were assessed to ascertain by comparing individuals of the two vector species taking into account their infectious status. The similarity and difference in microbiomes of *Pf*-infected and uninfected of both vectors were evaluated at the genera level using a Venn diagram. The principal component analysis (PCA) was performed with the Spad 5.5 software (version 5.5, Coheris) to determine the variation of the microbiota according to the species, the infectious status, and the collection site of mosquito. The bacterial genera were used as input variables. The alpha diversity for each group was estimated using the Shannon–Weaver index based on the OTU matrix. The beta diversity was estimated using the eigenvalue histogram to select the two first axes explaining the most of the observed variance. The variables PCA graph was drawn to identify correlated bacterial community characterizing each vectors group according to their *Pf* infection status and their origin. Finally, the individuals PCA plot was used to determine which samples aggregate because of the similarities in their infection status, bacterial community, and/or site of collection. All the analyses were performed by considering only variables with an OUT greater or equal to cutoff equal to 10% of the total of OTUs.

RESULTS

Mosquito collection. A total of 1,214 host-seeking female anophelines were sampled, including 305 *A. gambiae* s.l. and 432 *A. funestus* from the Fatick, and 477 *A. gambiae* s.l. from Kedougou. In Fatick, the parity rates were 97% and 94.4% for *A. funestus* and *A. gambiae*, respectively. In Kedougou, the parity rate for *A. gambiae* was about 51%. In Fatick, respectively, 5.1% (22/432) and 2.6% (8/305) females of *A. funestus* and *A. gambiae* were found infected by *Pf*. In Kedougou, where only *A. gambiae* specimens were tested, 4.6% (22/477) females were infected.

Composition of microbial communities in anopheline mosquitoes. All the 40 samples were processed using the 16S RNA–based metagenomic approach to generate a total number of 1,930,396 sequences reads. After quality filtering and data cleaning, a total number of 782,072 (40.51%) sequences were generated at the phylum level. Among these reads, more than 99% (780,571/782,072) were successfully assigned: 229,798 reads for *Pf*-positive *A. funestus*, 129,524 reads for *Pf*-free *A. funestus*, 33,019 reads for *Pf*-positive *A. gambiae*, and 388,230 reads for *Pf*-free *A. gambiae* (Table 1).

The identified reads belong to 25 phyla, dominated by *Proteobacteria* (53%) followed by *Firmicutes* (31.34%) when comparing the relative proportions of all phyla (Figure 2). Further analysis showed that the microbial fauna of both vectors varied according to species and infectious status. Indeed, some bacterial genera were specific to one group or shared between the four groups of vector infection status (Figure 3). In particular, five phyla were shared by the four groups regardless of their different infectious status, whereas only the phylum of *Proteobacteria* was absent from *A. gambiae* s.l. carrying *Pf*.

TABLE 1
Number of mapped and unmapped by species and infection status

Species	*Pf*-infection	Reads		
		Mapped	Unmapped	Total
Anopheles funestus	*Pf-positive*	229,798	866	230,664
	Pf-negative	129,524	61	129,585
Anopheles gambiae s.l.	*Pf-positive*	33,019	163	33,182
	Pf-negative	388,230	411	388,641
Total		780,571	1,501	782,072

Pf = Plasmodium falciparum.

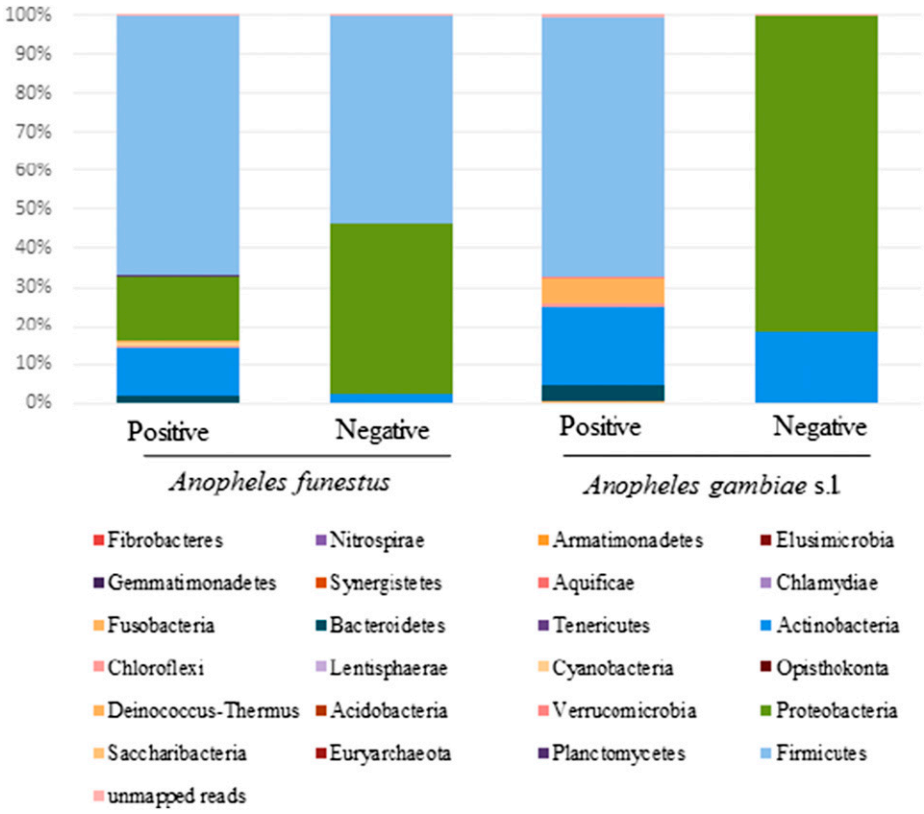

FIGURE 2. Composition (relative %) of the microbiota of *Anopheles gambiae* s.l. and *Anopheles funestus* positive and negative for *Plasmodium falciparum* at the bacterial phylum level (total: 25). This graph is a representation of microbiota at the phylum level by vector species and infection status. Mosquito species are presented according to their infection status. Each phylum is represented by a single color with height corresponding to the proportion of a phylum over the total number of the phyla found in each group. The proportion of unmapped reads is also represented. This figure appears in color at www.ajtmh.org.

At the genus level, after data cleaning, a total of 1,722,267 generated reads were assigned to 373 genera. Overall, the genus *Burkholderia* (49.96%) was the most common genus, followed by *Sphingomonas* (14.5%), *Bacillus* (11.20%), *Pseudomonas* (4.24%), *Microbacterium* (3.88%), and *Cupriavidus* (3.45%). The remaining genera accounted for less than 2%. The microbial composition was different between *Anopheles* species and the infectious status of *Pf*. Indeed, comparison of the bacterial microbiota between the two vector species revealed that the most frequent five bacteria genera among the *Pf*-free samples were *Burkholderia*, *Sphingomonas*, *Microbacterium*, *Bosea*, and *Bradyrhizobium* with significantly different frequencies ($P < 0.001$) except for *Bradyrhizobium* ($P = 0.9275$). Among the *Pf*-infected group, the top five were represented by the genera of *Geobacillus*, *Bacillus*, *Methylobacterium*, *Cupriavidus*, and *Pantoea*, present with significant difference in both vector species ($P < 0.001$) (Figure 4). In *A. funestus*, the microbiota was more diverse among the *Pf*-infected samples (352,452 OTU and 541 species) than in the noninfected samples (407,598 OTU and 240 species). Similarly, the microbiota of *A. gambiae* carrying *Pf* parasites was more complex (67,320 OTU and 264 species) than in the *Pf*-free group (898,220 OTU and 154 species). At least 342 bacterial species were common to *Pf*-infected specimens for both vector species; 147 OTU were specific to *A. funestus*, whereas only 29 OTU were specific to *A. gambiae*. A similar trend was also noticed for the *Pf*-free samples, with 202 bacteria common to both vector species, 54 specific to *A. funestus*, and 38 to *A. gambiae*.

Bacterial flora and mosquito susceptibility to *Pf*. It is noteworthy that carriage of some specific bacterial species was negatively correlated with *Pf* infection, and some bacteria were mostly associated with *Pf*-free samples, although they were also found in specimens carrying *Pf* in both vector species.

In *A. funestus*, the presence of six bacterial genera was negatively correlated with the carriage of *Pf*. With the respective correlation coefficients of –0.50 and –0.44, *Bartonella* and *Burkholderia* were significantly less associated with *Pf* infections. However, the presence of some genera such as *Microvirga* (+0.82), *Micrococcus* (+0.80), *Corynebacterium* (+0.73), and *Cupriavidus* (+0.73) is likely to be positively associated with *Pf* infection. In *A. gambiae*, only four bacterial genera (*Bosea* [–0.90], *Bradyrhizobium* [–0.90], *Sphingomonas* [–0.73], and *Burkholderia* [–0.65]) were negatively correlated with the presence of *Pf*, whereas three, namely, *Pantoea* (+0.70), *Aeribacillus* (+0.58), and *Ensifer* (+0.50), were positively correlated with the carriage of *Pf*.

Microbial diversity in *Pf*-infected and noninfected mosquitoes. Table 2 presents the Shannon alpha diversity index for all taxa represented in each of the *Pf*-infected/free vector species combinations. Although the number of OTUs was higher in noninfected samples, species diversity was higher in *Pf*-infected vectors ($df = 9$, $P < 0.05$). At the phylum level, the diversity of the microbiota was the lowest for *Pf*-negative samples. In *A. gambiae* s.l., the phylum of *Actinobacteria* was well represented, whereas the phylum of *Proteobacteria* was the sole not

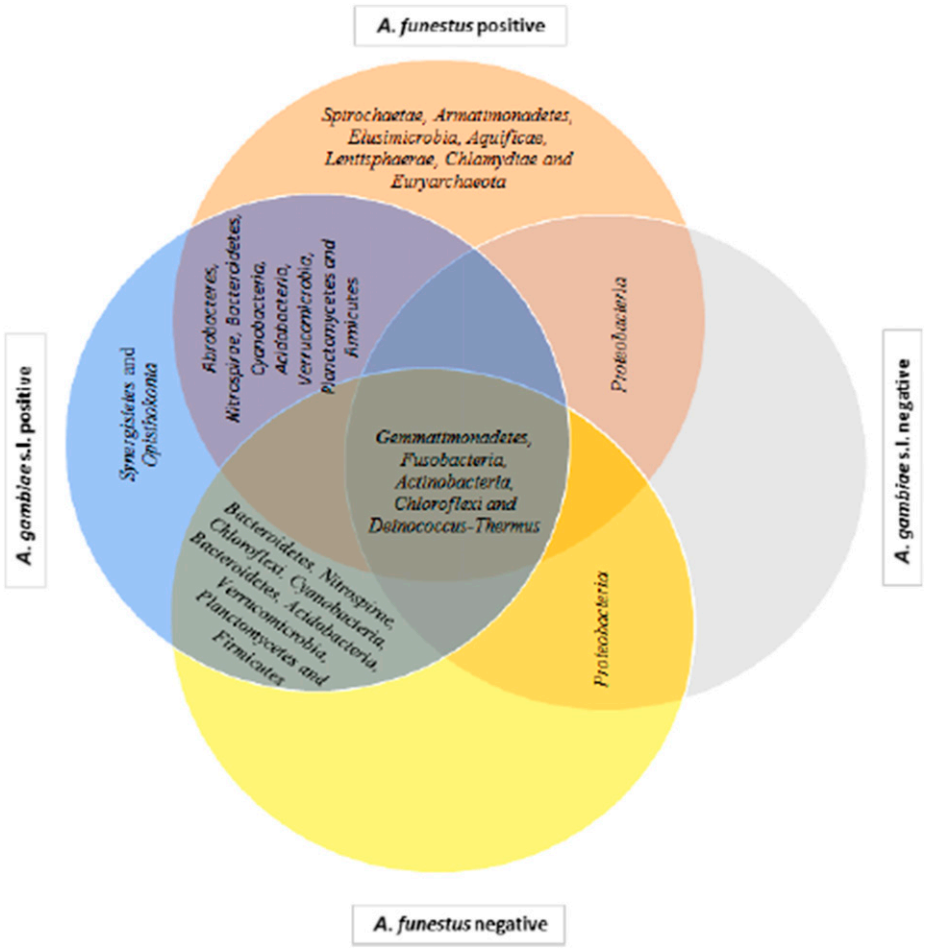

FIGURE 3. Microbial similarities at the phylum level between *Plasmodium falciparum* (*Pf*)-infected and uninfected groups. The Venn diagram circles with different colors represent the different vector/infection status groups. The overlapping areas show shared phyla, whereas the non-overlapping one report phyla that are specific to each group. Only five phyla are shared by all the four groups, and the phylum of *Proteobacteria* is absent from the microbiota of the *Pf*-infected group of *Anopheles gambiae* s.l. This figure appears in color at www.ajtmh.org.

found in the *A. gambiae* s.l. *Pf*-positive group. Conversely, for *A. funestus*, the microbiota was essentially composed (97.62%) of *Firmicutes* and *Proteobacteria*.

The graph 5 shows that several bacterial combinations contributed to individuals grouping across the factorial plan. Notably, the genera of *Asaia*, *Bosea*, *Bradyrhizobium*, *Burkholderia*, *Microbacterium*, *Sphingomonas*, and *Williamsia* are positively correlated and associated to *Pf*-free specimens of both vector species (Figures 1 and 2), whereas the genera of *Paracoccus*, *Methylobacterium*, *Corynebacterium*, *Microvirga*, and *Nocardioides* among others were associated to the *A. funestus Pf*-positive group. The lower right quadrant, where one of the *Pf*-infected *A. gambiae* specimens is located, is strongly influenced by the presence of bacterial genera, including mainly *Streptococcus*, *Halomonas*, *Gemella*, and *Delftia*. Most of the remaining *Pf*-positive *A. gambiae* cluster in the upper left quadrant. All the *Pf*-free specimen of both species cluster near the center of inertia in the lower part of the individual PCA graph. Geographic location probably contributed less to individual clustering. At the species level, the *Pf*-positive specimens of both vector species harbor different bacterial communities compared with the *Pf*-negative individuals (Figures 5 and 6). Furthermore, the bacterial composition was less diverse within the *Plasmodium*-free group of both vector species compared with the infected samples (Table 2).

Identification of bacterial species. A 402-bp-long sequence of a portion of *Bartonella bovis* was identified from three individuals of *A. funestus* with a range of 13–23 reads per sample. A basic local alignment search tool (BLAST) search showed that the sequence is identical to several *B. bovis* strains recently isolated from cattle in Dielmo[21] (Genbank accession numbers KU859919–KU859922).

Bartonella mastomydis is a potential new species within the *Bartonella* genus associated with *Mastomydis erythroleucus* from Senegal.[22] Approximately 16 and 28 reads belonging to this bacterium were identified from one *A. gambiae* and one *A. funestus*.

Rickettsia sp. was identified in two *A. funestus* individuals. Unfortunately, the 16S rRNA gene is too conservative among *Rikettsiae* to allow species identification. The amplified sequence has 99% identity with several pathogenic (*Rickettsia australis* and *Rickettsia felis*) and nonpathogenic (*Rickettsia bellii* and insect-associated endosymbiotic rickettsiae) species.

Asaia aff. bogorensis, identical to the strain recently isolated from *A. gambiae* collected in Dielmo (*Asaia* sp. GD01), was identified in three *A. funestus* and two *A. gambiae*.

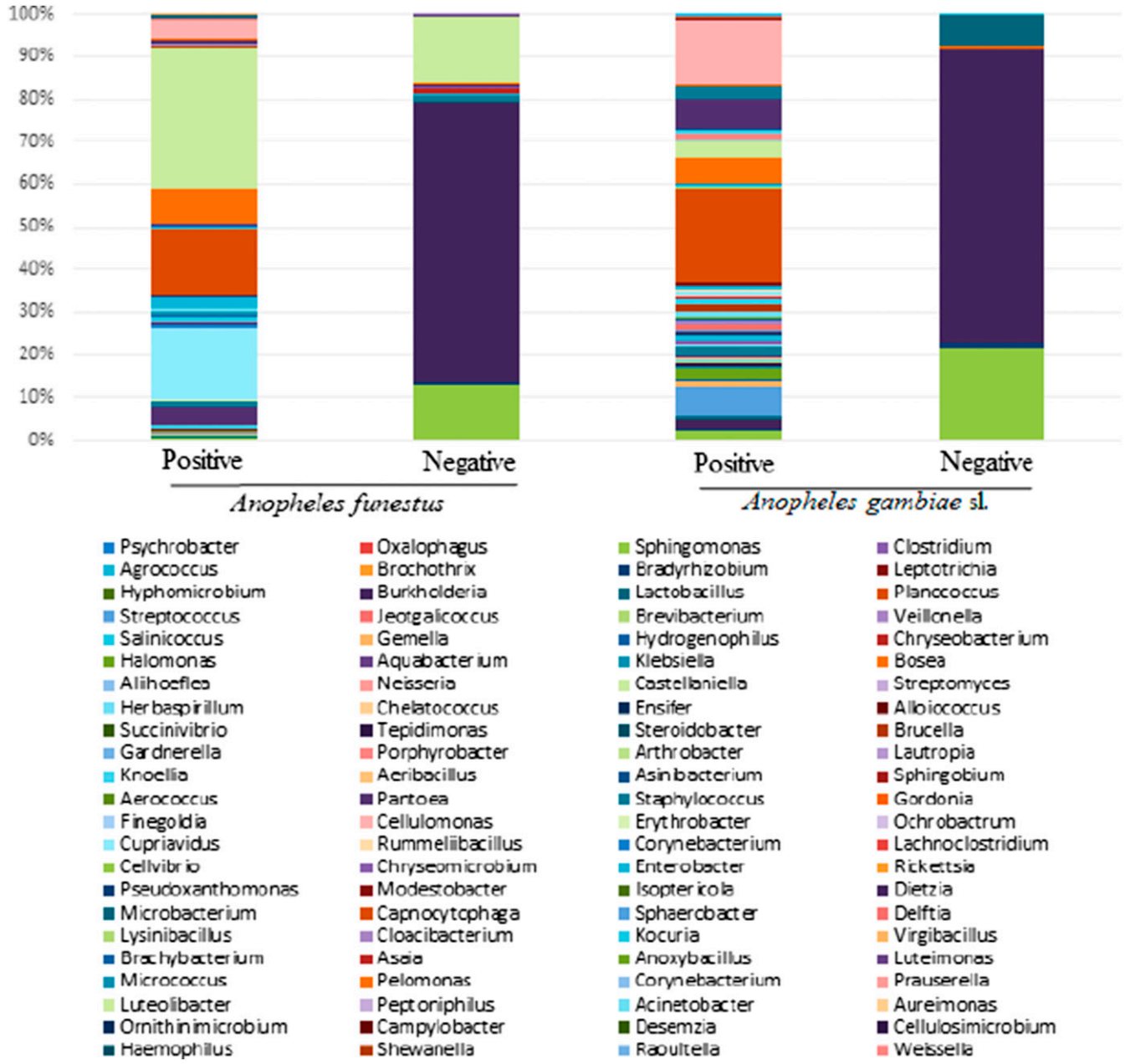

FIGURE 4. Composition (relative %) of the microbiota of *Anopheles gambiae* s.l. and *Anopheles funestus* positive and negative for *Plasmodium falciparum* at the bacterial genus level (total: 373). This graph shows a representation of microbiota at the genus level. Mosquito species are presented according to their infection status. Each genus is represented by a single color with height corresponding to the proportion of a genus over the total number of genus found in each group. This figure appears in color at www.ajtmh.org.

Sodalis sp., closely related to the secondary endosymbionts of many insects including *Glossina* spp. tsetse flies, was found in one *A. funestus*.

TABLE 2

Total OTUs and Shannon index by anopheline species and infection status

Species	*Plasmodium falciparum*	Total OTUs	Shannon index
Anopheles funestus	Positive	352,452	3.56
A. funestus	Negative	407,598	1.66
Anopheles gambiae s.l.	Positive	67,320	4.50
A. gambiae s.l.	Negative	898,220	1.31

OTUs = operational taxonomic units.

Brucella canis was identified in two *A. gambiae* mosquitoes. Several bacterial species which are pathogenic for humans, including *Burkholderia cepacia*, *Elizabethkingia meningoseptica*, *Haemophilus influenzae*, *Moraxella catarrhalis*, *Stenotrophomonas maltophilia*, *Propionibacterium acnes*, and *Klebsiella pneumoniae* were regularly identified in both mosquito species.

DISCUSSION

Over the last 15 years, considerable efforts have been made to control malaria around the world, particularly across sub-Saharan Africa. The decline in the malaria burden in many

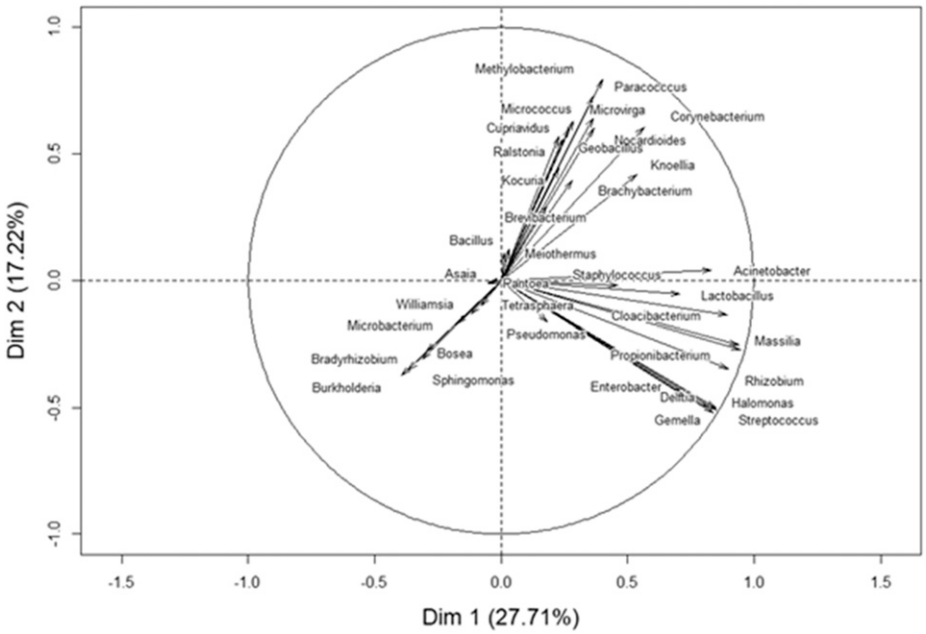

FIGURE 5. Principal component analysis (PCA) plot of the variables contributing to the two first dimensions. This is the variable PCA graph showing the correlation between variables and their contribution in shaping the individuals clustering into a two-dimensional plan drawn with the two first dimensions contributing mainly to the observed variance between individuals. Variables represented are the bacterial genus and their correlation compared in the different axes.

parts of Africa was made possible through the integration of all existing effective measures and tools.[1] The overall decline in the global malaria burden thus offers an unprecedent opportunity for local pre-elimination and elimination in eligible areas, where local transmission has been reduced to its very low level.[23] However, some of the challenges impeding progress toward elimination include the emergence of parasite resistance to antimalarial drugs and mosquito resistance to insecticides, as well as increasing behavioral changes in vector populations, avoiding the contact with treated surfaces.[2] To tackle these challenges, in May 2012, the WHO released the Global Plan for Insecticide Resistance Management in malaria vectors and strongly recommended the use of "*innovative approaches for sustainable vector control at global scale*."[24,25] One of the potential promising approaches would be the use of symbiotic microorganisms naturally associated with mosquitoes, to impede the development of *Plasmodium* during the sporogonic cycle inside the mosquito.[26] However, preliminary

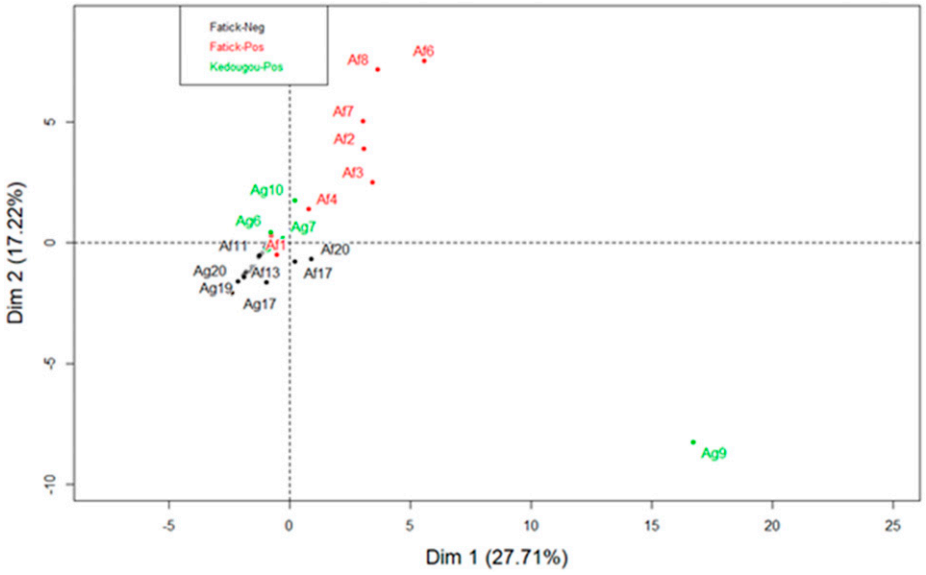

FIGURE 6. Individual principal component analysis plot considering the vector species, their infection status, and collection sites. Each individual in the graph is represented by a single dot in the space drawn using the factors which mainly contribute to explain the observed trend. The figure shows how individuals are clustered considering the similarity of bacterial microbiota between individuals. The quadrants delimit the spread of individuals across the first factorial plane. The quadrant sizes are drawn by default by the program which automatically assigns the adequate size to each of the quadrants based on the inertia and the barycenter of the projected area weighted by the contributions of each individual used to draw each of the axes. This figure appears in color at www.ajtmh.org.

studies are needed to better characterize the mosquito's microbiota and to identify potential bacterial candidates to control malaria.

Despite extensive work on local malaria vector populations in Senegal, the composition of their microbiota is not yet well known. This is the first attempt to characterize the bacterial flora of two main malaria vectors in Senegal. In sum, the results showed the predominance of bacteria of the *Proteobacteria* phylum among the bacterial community of both *A. gambiae* s.l. and *A. funestus*, as previously reported in several other contexts.[27–29] Our results suggest that the *Pf* infection status of the studied vector population of both species is likely more influenced by the microbiota they harbor than the collection site. Notably, there were inter- and intraspecies differences between the microbial communities harbored by the different infected/uninfected groups, even in the same location. These observations corroborate those previously reported on the bacterial flora of larval and the adults of malaria vectors. Noteworthy, only one specimen of infected *A. gambiae* is segregated from the clusters of *Pf*-infected individuals grouping both species, whatever their origin. This may be an erroneous characterization due to possible contamination of this sole individual. Data reported here, although preliminary, revealed differential microbial composition for the two major malaria vectors and their *Pf* infection status. This difference may be explained by the presence of the species-specific microbial communities associated with each of the anopheline species and the different characteristics of larval breeding sites. Akorli et al.[28] found that the composition of malaria vectors' microbiota was strongly influenced by the microbial flora in larval breeding sites and/or their diet. More recently, Dieme et al.[30] discussed the limitless microbial repertoires to which wild anopheline larval populations are exposed during their aquatic life in their breeding sites. The authors hypothesized that larval microbial exposure and, particularly, the carriage of certain bacterial communities might impact their vectorial competence for *Plasmodium*. Our results are consistent with the above observations with several bacterial species negatively being correlated with *Pf* infection. Indeed, several classes of α, β, γ, and δ *Proteobacteria* phylum identified here may potentially impede the development of *Plasmodium* in *Anopheles*.[31–33] Among the identified *Proteobacteria* group, bacteria belonging to the *Burkholderia* genus were the most abundant in both anopheline species. The genus consists of more than 30 described species, of which the members of the *B. cepacia* complex have been the most studied because of their pathogenicity to both human and plant pathogens, as well as environmentally significant bacteria. For instance, some *Burkholderia fungorum* strains, such as the LB400 and DBT1, are good biodegraders and are considered to be promising candidates for the bioremediation of polycyclic aromatic hydrocarbons.[34] Moreover, some *Burkholderia* spp. are known for their antifungal actions.[35] To the best of our knowledge, this is the first time the genus *has been reported with such a high proportion within* two malaria vector microbiota and with a potential negative impact on *Pf* parasites. The exclusive presence of *Burkholderia* spp. within the *Plasmodium*-free specimens of both vectors, with low bacterial diversity, suggests broader antiparasitic/antibacterial activity as well as known antifungal activity.[35] The parasite spectrum of this potential antiparasitic effect may include the malarial agent, *Pf*, which was noticeably absent in all individuals carrying *Burkholderia* spp. In addition to *Burkholderia* spp., several other bacteria with a potential impact on the sporogonic cycle of *Pf* in vectors such as *Asaia* spp.,[31,36] *Wolbachia* sp.[37–39] and *Enterobacter* spp.,[33] were associated with *Pf*-free specimens of the two study species.

Interestingly, bacterial flora was more diverse in mosquitoes infected by *Pf*. This observation contrasts with previous metagenomic-based studies of vertebrates (mostly humans) showing a lower microbial diversity and/or richness among the patient group versus the control group for certain respiratory pathologies for instance.[40,41] This could be related to the carriage of some bacterial genera such as *Burkholderia*, which was well represented in *Pf*-free specimens of both species. Moreover, patients often present an increasing proportion of pathogenic bacteria.[42] From the current data alone, it is not clear whether the differences in the bacterial community are secondary to *Plasmodium* infection or factors facilitating the infection in mosquitos.

The identification of several nonhuman vertebrate bacteria in mosquitoes suggests a fairly wide range of host spectra for blood feeding. Rodents and bovine-associated bartonellae (*B. mastomydis* and *B. bovis*), as well as the dog pathogen, *B. canis*, may have been acquired by *Anopheles* mosquitoes during blood feeding on these animals. Although the role of mosquitoes is not yet known in the transmission cycle of such bacteria, the preliminary data presented here raise an interesting question about their probable involvement.

Until recently,[39,43] *Anopheles* mosquitoes were considered not to harbor primary or secondary bacterial endosymbionts. Here, we identified the bacterial genera of *Sodalis* and *Wolbachia*. This confirms the presence of *Wolbachia* sp. in wild *A. gambiae* populations and describes, for the first time, its presence in *A. funestus*, one of the major malaria vectors in several sub-Saharan African countries.[44–46] Additional studies are ongoing to characterize and estimate the frequencies of the *Wolbachia* genus among the natural Senegalese population of *A. gambiae* as well as *A. funestus* and to evaluate their direct impact on the sporogonic development of *Pf*.

This first study of the microbiota of the two main vectors of malaria in Senegal highlighted a strong association between *B. fungorum* with *Pf*-free samples. Although more studies are urgently needed to further assess its impact against human malaria parasites, *B. fungorum*, because of its antifungal and potential antiparasitic properties, seems to be a good biological antimalarial candidate. Notably, the detection of stable *Wolbachia* infection in wild *A. gambiae* and *A. funestus* populations strengthens its attractiveness as a potential novel tool to control both endo- and exophagic populations of malaria-transmitting anophelines. However, additional studies are urgently needed to screen the presence of *Wolbachia* sp. and *Burkholderia* sp. in a wide range of anopheline mosquitoes, particularly those involved in malaria transmission, and to better understand the mechanisms involved in combating *Pf*.

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