Diversity of the bacterial microbiota of Anopheles Mosquitoes from Binh Phuoc province, Vietnam
Chung Ngo, Sara Romano-Bertrand, Sylvie Manguin, Estelle Jumas-Bilak

To cite this version:
Chung Ngo, Sara Romano-Bertrand, Sylvie Manguin, Estelle Jumas-Bilak. Diversity of the bacterial microbiota of Anopheles Mosquitoes from Binh Phuoc province, Vietnam. Frontiers in Microbiology, Frontiers Media, 2016, 7, pp.2095. 10.3389/fmicb.2016.02095. hal-01814232

HAL Id: hal-01814232
https://hal.archives-ouvertes.fr/hal-01814232
Submitted on 12 Oct 2018

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Distributed under a Creative Commons Attribution 4.0 International License
Diversity of the Bacterial Microbiota of Anopheles Mosquitoes from Binh Phuoc Province, Vietnam

Chung T. Ngo1,2, Sara Romano-Bertrand3,4*, Sylvie Manguin1 and Estelle Jumas-Bilak3,4

1 Institut de Recherche pour le Développement France, UMR-MD3, Faculté de Pharmacie, Montpellier, France, 2 National Institute of Veterinary Research, Hanoi, Vietnam, 3 UMR 5569 Hydrosciences, Équipe Pathogènes Hydriques, Santé et Environnements, Faculté de Pharmacie, Université de Montpellier, Montpellier, France, 4 Département d’Hygiène Hospitalière, Centre Hospitalier Universitaire de Montpellier, Montpellier, France

The naturally acquired microbiota of Anopheles can influence vector’s susceptibility to Plasmodium and its capacity to transmit them. Microbiota modification is a new challenge to limit disease transmission but it still needs advanced knowledges on bacterial community in Anopheles, especially in wild and infected specimens from diverse origin and species. Bacterial culture and 16S rRNA gene-PCR associated to Temporal Temperature Gradient Electrophoresis (TTGE) were applied to explore the bacterial diversity in the abdomen of 100 wild specimens (eight Anopheles species) collected in the Binh Phuoc Province, Vietnam. Culture and PCR-TTGE were complementary. The bacterial richness of the mosquito collection encompassed 105 genera belonging to seven phyla, mostly Proteobacteria, Firmicutes, and Actinobacteria. Staphylococcus, Clostridium, and Bacillus in Firmicutes were the most prevalent genera. However, Proteobacteria represented by 57 genera was the most diversified phylum in Anopheles microbiota. The high overall of Anopheles-associated bacteria is confirmed with, to our knowledge, 51 genera described for the first time in Anopheles microbiota. However, the diversity per specimen was low with average diversity index and the average Shannon–Wiener score (H) of 4.843 and 5.569, respectively. The most represented bacterial genera were present in <30% of the specimens. Consequently, the core microbiota share by Anopheles from Binh Phuoc was very narrow, suggesting that Anopheles microbiota was greatly influenced by local environments. The repertory of bacterial genera in two specimens of An. dirus and An. pampanai naturally infected by Plasmodium vivax was also described as preliminary results. Finally, this study completed the repertory of bacteria associated to wild Anopheles. Anopheles associated-bacteria appeared specimen-dependent rather than mosquitoes species- or group-dependent. Their origin and the existence of Anopheles-specific bacterial taxa are discussed.

Keywords: Anopheles mosquitoes, abdomen bacterial microbiota, 16S rRNA PCR – TTGE, malaria, Binh Phuoc, Vietnam
INTRODUCTION

Despite some success in controlling malaria, this disease continues to be a major health burden in many countries around the world with 438,000 deaths reported in 2015, particularly in sub-Saharan Africa recording 395,000 deaths (90%), but also in forested regions of Southeast Asia with 32,000 deaths (7%; WHO, 2015). Until now, vector control has been one of the key elements for controlling this disease based on the use of insecticides, such as DDT, pyrethroids, organophosphates, carbamates (Verhaeghen et al., 2009; Kabula et al., 2013). However, this strategy of insecticide use has many serious side-effects, in particular on human health and the environment (Dabrowski and Balderacchi, 2013; Singleton et al., 2013; Dabrowski et al., 2014), having also a direct impact on the increase of Anopheles resistance to insecticides which has now been reported in 64 countries (WHO, 2012; Chang et al., 2013; Kabula et al., 2013; Tangena et al., 2013). Therefore, new and innovative approaches to control Anopheles vectors for reducing malaria transmission on a more durable and safer manner for human health are, hence, needed.

In Vietnam, malaria remains the most important vector-borne parasitic disease with a strong prevalence in forested regions, in particular along the international borders with Cambodia, where Binh Phuoc Province is located. This province, situated in central-south Vietnam, is considered as having the highest malarial transmission of all (Abe et al., 2009; Tran et al., 2012). The local malaria situation reported both resistance of Plasmodium to anti-plasmodial drugs and Anopheles mosquitoes to the insecticide used in this province (Huong et al., 2001; Nguyen et al., 2003; Thanh et al., 2010; Verhaeghen et al., 2010; Tran et al., 2012). However, there is a lack of information on the distribution and vector competence of the local Anopheles vectors.

The impact of the microbiota on mosquito infection and more specifically on Anopheles resistance to malaria pathogens is showing great potential toward reducing the mosquito vector competence and blocking transmission of several infectious diseases (Dong et al., 2006, 2009, 2011; Blumberg et al., 2013). The influence of the Anopheles microbiota on its vectorial competence to transmit pathogens is a growing field of investigation as demonstrated by the recent increase in studies, to cite a few (Favia et al., 2007; Briones et al., 2008; Dong et al., 2009; Rodrigues et al., 2010; Cirimotich et al., 2011; Blumberg et al., 2013; Gendrin and Christophides, 2013; Dennison et al., 2014; Buck et al., 2016). A previous report on the biodiversity of the abdomen microbiota of 100 wild Anopheles specimens (five species) from Dak Nong Province showed a high taxonomic diversity, including species reported as implicated in the mosquito resistance to Plasmodium infection (Ngo et al., 2015). By the same approach, we present here the bacterial diversity detected in 100 Anopheles specimens of eight species collected from Binh Phuoc, a Province characterized by high malaria endemicity. The Anopheles studied herein included two specimens naturally infected by Plasmodium vivax.

MATERIALS AND METHODS

Anopheles Samples: Collection and Identification

One hundred Anopheles (out of 486 specimens collected from 4 sites located in Bu Gia Map District, Binh Phuoc Province in central-southern Vietnam (Ngo et al., 2014), which belonged to eight Anopheles species, including Anopheles dirus, An. jeyporiensis, An. maculatus, An. minimus, An. pampanai, An. rampae, An. sawadwongporni, and An. scanloni, were randomly chosen for the present study. These Anopheles specimens were collected between November and December, 2011 (rainy-beginning of the dry season) during 11 consecutive nights (17:00–20:00 h using methods, such as light trap capture and human landing catches indoor and outdoor (Ngo et al., 2014). Anopheles mosquito identification was morphologically done in the field by sorting out each taxon. Specimens that belonged to the Dirus Complex or the Funestus and Maculatus Groups were individually identified to species level using the appropriate PCR-based assay as described by Walton et al. (1999, 2007) and Garros et al. (2005). Each individual was split into two pieces stored at −80°C until use: (1) head-thorax for species identification and detection of Plasmodium by molecular methods (Ngo et al., 2014); and (2) abdomen for bacterial analysis. One hundred abdomens of wild-caught Anopheles females were used for the subsequent bacterial study by both methods, culture and DNA fingerprint, as described below.

Bacterial Culture, DNA Extraction, and Strains Identification

Anopheles abdomens were surface rinsed twice in sterilized DNA-free water, and each abdomen was thoroughly disrupted using a sterilized tissue crusher device in 150 µl of sterile DNA-free water. Then, 10 µl of this suspension was spread on each prepared culture medium plate: blood sheep agar, R2A and water. These cultures were checked by DNA electrophoresis in 1.5% agarose gels containing ethidium bromide and visualized under ultraviolet light. Sterile DNA-free water used for mosquito preparations was used in negative control for each series of DNA extraction and PCR. The successfully amplified products were sequenced with primer 27f, on an ABI 3730xl sequencer (Cogenics, Meylan, France). Each sequencing chromatograph was visually inspected and corrected.

Independent-Culture Analysis by 16S PCR-Temporal Temperature Gel Electrophoresis (PCR-TTGE)

Whole DNA was extracted from 100 µl of mosquito abdomen suspension using the Master Pure Gram Positive DNA
Bacterial Diversity in the Abdomen of Anopheles Adults

Results

Anopheles Identification

Specific PCR assays identified eight Anopheles species among the 100 specimens collected in Binh Phuoc Province. Out of 100, 37 specimens belonged to the Dirus Complex including An. dirus (n = 33) and An. scanloni (n = 4), 43 to the Funestus Group with An. minimus (n = 34), An. jeyporiensis (n = 5), and An. pampalai (n = 4), and 20 to the Maculatus Group with An. sawadwongporni (n = 12), An. rampae (n = 6), and An. maculatus (n = 2; Figure 1A).

Bacterial Diversity in the Abdomen of Anopheles Adults

The abdomens of 100 Anopheles were analyzed using both bacterial culture and PCR-TTGE methods. Only a few negative controls presented weak amplification bands in agarose gel after nested-PCR but these bands were never observed after TTGE migration, permitting to validate the profiles for mosquitoes’ samples. The regular positive controls highlighted a sensitivity of detection comprised between 10^1 and 10^2 bacterial inclusions throughout the TTGE process (data not shown). Bacteria were cultured or detected in 98% of the Anopheles specimens, the two negative specimens belonged to species An. minimus and An. scanloni. A total of 63 bacterial strains from 22 specimens positive by culture and 441 TTGE bands from 98 specimens positive by PCR were obtained. Distributions of strains and TTGE bands according to Anopheles species are shown in Figures 1B,C, respectively.

Both strains and 393 TTGE bands were further analyzed by 16S rRNA gene PCR and sequenced. The remaining 48 TTGE bands were identified by comparison with another co-migrating band on the same TTGE gel. Results were presented in Supplementary Tables. Strains and PCR-TTGE bands were affiliated to seven phyla, the most represented being Proteobacteria, Firmicutes, and Actinobacteria (Figure 2). Considering OTUs, 193 bacterial OTUs corresponding to 190 genera-level OTUs (including 85 OTUs for which identification was performed until species-level), one family-level and two phyla-level OTUs. The sequences obtained from 3 TTGE bands was performed as described in Ngo et al. (2015).

Sequence Analysis, Species Identification, and OTU Affiliation

The sequences were analyzed by comparison with Genbank1 and Ribosomal Databases Project 2 (RDPII)2 using Basic Local Alignment Search Tool (BLAST) and Seqmatch programs, respectively. The sequence with the highest percentage was used for OTU affiliation as previously described by Ngo et al. (2015). Briefly, a sequence was affiliated to a species-level OTU when the percent of sequence similarity with the species type strain was above 99.0% (Drancourt et al., 2000). Below 99.0%, the sequence was affiliated to the genus of the reference sequence with the highest percentage. When several species reference sequences matched equally, affiliation was done to the genus level or to a group of species if relevant. For example, sequence with 99.5% in similarity to both Aeromonas caviae and A. hydrophila was only assigned to the genus Aeromonas. The same rule was applied for the taxonomic level higher than the genus level if necessary. The 16S rRNA gene sequences from all cultured bacteria are available in GenBank (accession numbers KX449232–KX449311). Sequences from TTGE band are available by contacting corresponding author or by consulting the Supplementary Fasta File.

Diversity Index Calculation and Statistical Analysis

The microbiota diversity for each species of Anopheles was estimated by the calculation of crude diversity index (DI, number of different OTUs; Romano-Bertrand et al., 2012, 2014), Shannon–Wiener DI (H) and Simpson’s index (D; Gafan et al., 2005; Ledder et al., 2007; Romano-Bertrand et al., 2014). The 100 Anopheles specimens were classified into the Dirus Complex or into the Funestus and Maculatus Groups according to their species identification. The bacterial diversity scores (DI, H, and D) for the different Anopheles species, complex and groups were then calculated.

1http://www.ncbi.nlm.nih.gov/
2http://rdp.cme.msu.edu/
Only 32 from the 190 genera-level OTUs were retrieved in culture, mainly from Actinobacteria with 18 cultivable genera (56%). Out of these 32 cultivable OTUs, 15 (7.9%) were not retrieved by the molecular approach (genera underlined in Figure 3 and Supplementary Tables).

In this study, molecular approach allowed the detection of more bacteria than culture, although culture improved the description of bacterial richness in the Anopheles abdomen microbiota and should be considered as complementary to PCR-based methods. The Actinobacteria richness was particularly widen thanks to culture.

**Bacterial Richness and Diversity of the Abdomen Microbiota According to the Anopheles Species**

Bacterial diversity according to Anopheles species was displayed in Figure 4A. No obvious link was observed between Anopheles complex or groups and microbiota diversity. However, the number of specimens in several Anopheles species is too low for robust conclusions, only tendencies can be drawn. Amongst the three major phyla, Firmicutes and Proteobacteria were present in all Anopheles species, while Actinobacteria were absent from
An. jeyporiensis, An. scanloni, and An. rampae (Figure 4). An. minimus, considered as one of the primary vector of Plasmodium in Southeast Asia, including Vietnam, was the sole species colonized by members of the seven bacterial phyla. Two other malaria vectors well represented in the collection, An. dirus and An. sawadwongporni, were each colonized by five bacterial phyla (Figure 4A). Despite the low number of specimens (n = 6) in this species, An. rampae presented a particular diversity profile with the replacement of Actinobacteria by Bacteroidetes regarding other Anopheles species. Nevertheless, the phylum richness appeared more dependent to the number of specimens in a species of Anopheles than to the Anopheles complex and groups (Figures 4A,B). Indeed, if Anopheles complex or group were considered, the phyla distribution in percent was very similar among them (Figure 4B).

However, at the OTU level the distribution varied according Anopheles complex and groups (Figure 4C). Among the 193 OTUs, 113, 109, and 57 OTUs were identified within the Dirus Complex, the Funestus and Maculatus Groups, respectively (Figure 4C). Out of them, 24 OTUs were shared by the three Anopheles taxa, 20 OTUs were shared by the Dirus Complex and the Funestus Group, and nine OTUs were commonly identified either between the Funestus and Maculatus Groups, or the Dirus Complex and Funestus Group (Figure 4C).

Anopheles abdomen microbiota diversity quantified by the mean index and scores was presented in Table 1. The average DI per specimen reached 4.843. An. maculatus (DI = 7.0) and An. jeyporiensis (DI = 6.0) microbiota were the most diverse, whereas An. minimus displayed lowest diversity (DI = 4.429) (Table 1). Considering both bacterial richness and equitability of the OTUs within Anopheles species, the average Shannon–Wiener score (H) for all species was 5.569. An. minimus had the higher H score (H = 6.213) indicating that the numbers of OTUs are evenly distributed between all specimens (Table 1). The Simpson index (D) takes into account both bacterial species richness and an evenness of abundance among the bacterial species present, and is inversely proportional to the diversity (DI). For all Anopheles species, except An. sawadwongporni (D = 0.308), the D-values were low ranging between 0 and 0.082, suggesting high diversities (Table 1). However, these results should be taken with caution because of the low number of specimens for An. scanloni (n = 3), An. jeyporiensis (n = 5), An. pampanai (n = 4), An. maculatus (n = 2), and An. rampae (n = 6).
The Core Microbiota of Vietnam Anopheles Is Limited

Three Firmicutes genera, Staphylococcus, Bacillus, and Clostridium, were the most prevalent in the abdomen microbiota of Binh Phuoc Anopheles, respectively, identified in 29.6, 23.5, and 21.4% of the specimens. Besides, Propionibacterium, Peptostreptococcus, Acinetobacter, Burkholderia, Comamonas, Delftia, Lysobacter, and Sphingomonas were detected in more than 10% of the specimens, with frequencies ranging from 10.2 to 18.4%. The Family Enterobacteriaceae (Enterobacter, Klebsiella, Pantoea, and Proteus) was detected in 15.3% of the specimens. The other 90 genera were found at frequencies lower than 10%, including 81 genera detected in only 1–4 samples (<5%), certainly due to the important bacterial diversity in the mosquito microbiota (Supplementary Table 1).

The most represented genera in Binh Phuoc Anopheles microbiota were Staphylococcus and Sphingomonas (Supplementary Table 1 and Figure 3). Six other genera...
retrieved at lower frequencies were newly identified in our study: Agroccocus, Anoxybacillus, Peptostreptococcus, Lysobacter, Paracoccus, and Sphingopyxis (Supplementary Table 1 and Figure 3). Finally, a total of 21 genera were detected in more than 4 of 8 Anopheles species but these genera were present in <30% of the mosquito specimens confirming that the high bacterial diversity in Binh Phuoc Anopheles impaired the definition a core microbiota. Nevertheless, the presence of some frequent genera in Binh Phuoc Anopheles microbiota that were not yet described in Anopheles pan-microbiota, suggested the existence of specific microbiota according to the geographic region probably due to a strong influence of local environmental conditions.

Concerning the pan-microbiota of the whole genus Anopheles, meta-analysis of recent studies (Gendrin and Christophides, 2013; Manguin et al., 2013; Minard et al., 2013; Sharma et al., 2014; Villegas and Pimenta, 2014; Ngo et al., 2015) and bibliography databases survey for older publications, indicated that 51 bacterial genera (48.6%) were probably detected herein for the first time in Anopheles (1 in Figure 3 and Supplementary Table 1). These newly described genera could be slightly overestimated due to the meta-analysis approach and the comparison with non-fully exhaustive data but it clearly suggested that the overall richness of Anopheles microbiome is far from being fully explored. This is particularly true in Southeast Asia where very little work has been done on this topic (Gendrin and Christophides, 2013; Ngo et al., 2015).

In comparison with a previous study on the microbiota diversity in specimens from Dak Nong Province, Vietnam (Ngo et al., 2015), 17 genera (in bold in Figure 3) were common in Anopheles species from both provinces. Most of these genera were found at frequencies below 10% in the Anopheles specimens from Binh Phuoc Province, except four genera: Sphingomonas (13.3%), Acinetobacter (18.4%), Bacillus (23.5%), and Staphylococcus (29.6%). The genus Acinetobacter was detected in more than half Anopheles species in both Binh Phuoc and Dak Nong Anopheles microbiota. It could be considered as the sole genus forming the core microbiota of Vietnam Anopheles.

Characterization of the Abdomen Microbiota of Plasmodium Naturally Infected Anopheles Specimens

Two specimens were naturally infected by P. vivax, out of the 486 Anopheles specimens collected in Binh Phuoc Province (Ngo et al., 2014). They belonged to the species An. dirus and An. pampanaï. The abdominal bacterial microbiota of these two specimens was described by PCR-TTGE only, because cultured isolates were not obtained from these two infected mosquitoes. The sequence analysis of seven TTGE bands showed the presence of six bacterial taxa: Acinetobacter sp., Geobacillus sp., Luteimonas sp., Methylbacterium sp., Rubellimicrobium sp., and Sphingomonas sp., among which five (83.3%) belonged to Proteobacteria and only one, Geobacillus sp., to Firmicutes. The genus Acinetobacter, Geobacillus, Methylbacterium, and Sphingomonas belonged to the core50% microbiota and were also retrieved from other non-infected specimens of Anopheles, whereas Luteimonas was only retrieved from the infected An. pampanaï specimen, and Rubellimicrobium from both the infected An. dirus and one specimen of An. rampae (Supplementary Table 1).

DISCUSSION

The description of microbiota in vector arthropods presented increasing interest because of potential role in vector competence. Protective role of Anopheles bacterial microbiota against Plasmodium infections has been demonstrated because clearing midgut microbiota by antibiotic treatment resulted in enhanced Plasmodium infections rate (Beier et al., 1994; Favia et al., 2007; Dong et al., 2009; Rodrigues et al., 2010). Similarly but in semi-natural settings, antibiotics in ingested blood enhanced the susceptibility of An. gambiae to Plasmodium infection and increasing mosquito fitness through higher survival and fecundity. Moreover, the presence of antibiotics in the blood of malaria-infected people was identified as a risk for increased disease transmission (Gendrin et al., 2015). In addition, the experimental co-infections of bacteria with Plasmodium have showed the reduction of the number of developing oocysts in the mosquito midgut, both in laboratory and field conditions (Pumpuni et al., 1993; Lowenberger et al., 1999; Gonzalez-Ceron et al., 2003; Dong et al., 2009; Meister et al., 2009; Cirimotich et al., 2011). More recently, it was observed that expansion of midgut microbiota by negative regulation of An. gambiae immune response increased susceptibility to Plasmodium as observed before for microbiota depletion (Dennison et al., 2015). Finally, it’s probably the microbiota equilibrium that confers resistance to Plasmodium infection. Then, the development of new approaches in malaria control needs the description of the microbiota of wild Anopheles species and its variations (Rani et al., 2009; Boissière et al., 2012; Chavshin et al., 2012; Osei-Poku et al., 2012; Gendrin and Christophides, 2013; Manguin et al., 2013; Ngo et al., 2015).

As previously done (Ngo et al., 2015), we present herein a combined approach associating molecular detection and culture that improve the performance of each separate method. About one third of the bacterial genera detected in this study were obtained by culture, thus providing a valuable collection of isolates for further experiments such as experimental challenges (Ramirez et al., 2014) and in paratransgenesis projects (Chavshin et al., 2014). Due to the low resolution of TTGE fingerprints when the number of band is high, PCR-TTGE is only suitable for the description of microbiota with relative low richness as demonstrated for human neonatal microbiota (Jacquot et al., 2011) and surgical microbiota (Romano-Bertrand et al., 2014), but also for the description of mosquito microbiota (Manguin et al., 2013; Ngo et al., 2015). By this mean, we previously described most of the known repertory of bacterial taxa found in Asian Anopheles as shown by Villegas and Pimenta (2014). TTGE fingerprinting gave a good description of majority bacterial populations in mosquito microbiota, with a rather good sensitivity, as demonstrated by the extent of the taxonomic richness described herein compared to other studies even those which used Next Generation Sequencing (NGS; Villegas and
Moreover, the use of PCR-TTGE in this study allowed a comparison with previous studies in Vietnam (Ngo et al., 2015), but also in Thailand (Manguin et al., 2013). NGS is now becoming a reference method for microbiota description and knowledge of mosquito-associated bacteria would likely benefit from studies by NGS. Because NGS provide huge amounts of data sometimes partially exploited, preliminary and comparative studies by TTGE or other community fingerprinting approaches allow asking scientific questions to be further in-depth explored by NGS.

The Anopheles collection of 100 wild specimens collected in Binh Phuoc Province was colonized by a great diversity of bacteria with, to our knowledge, 51 genera not yet described in anophelines. Thereby, this study widens the description of the Anopheles pan-microbiota. The huge diversity of Anopheles associated bacteria contrasts the low richness observed per specimen. This result is similar to that shown by Ossei-Poku et al. (2012) in different mosquito genera, including Anopheles, in which bacterial richness per specimen is very low, nearly always dominated by a small number of taxa. This low richness could explain that only 78% of the specimens are positive in culture. Other reasons could be the trapping of some bacteria in the Anopheles tissues despite the preparative crushing step, the presence of viable but non-cultivable bacteria and the limited panel of culture medium used in the study. Culturomics approaches (Tandina et al., 2016) with the use of large panel of culture media and conditions could enhance the culture yield in next studies.

Within the bacteria detected in Anopheles specimens from Binh Phuoc, approximately half of the genera have previously been identified in the intra-abdominal microbiota of Anopheles populations from Thailand (Manguin et al., 2013), Vietnam (Manguin et al., 2013; Ngo et al., 2015), Cameroon (Boissière et al., 2012), Iran (Chavshin et al., 2012), India (Rani et al., 2009), or in reviews (Gendrin and Christophides, 2013; Minard et al., 2013; Hughes et al., 2014; Villegas and Pimenta, 2014).

Our results also showed that there is no link between bacterial diversity and host species or group of host species collected in Binh Phuoc Province. This result is in accordance with previous reports that rather showed the influence of the local environment of the sampling site, environment that gives a specific bacterial profile to mosquito specimens (Boissière et al., 2012; Manguin et al., 2013; Buck et al., 2016). The microbiota diversity of host species will also be significantly influenced by the origin of specimens like wild versus laboratory reared Anopheles mosquitoes (Chavshin et al., 2012). For instance, the selective pressure of laboratory conditions may limit bacteria acquisition at both larval and adult stages resulting in great reduction or nil cultivable bacteria in mosquito midgut (Riehle and Jacobs-Lorena, 2005; Favia et al., 2007; Gusmão et al., 2010). Studies on microbiota of An. gambiae larvae and pupae showed that only subset of bacteria from the aquatic habitat were able to inhabit the mosquito gut (Wang et al., 2011). The same influence of environment on Anopheles microbiota is likely to occur during the terrestrial life of adults. Considering the repertory of bacterial genera presented in Figure 3, the presence of soil- and plant-associated bacteria in Anopheles microbiota is probable. For instance, Agrococcus, Janibacter, Streptomyces, Bacillus (and related genera), and Lysobacter are classical soil inhabitants, whereas Asiaia, Burkholderia, Comamonas, Delftia, and Sphingomonas and Methylobacterium are frequently associated with plants. In addition, several prevalent bacterial genera are associated with animal or human microbiota, mainly to cutaneous microbiota: Propionibacterium, Staphylococcus, Peptostreptococcus, Clostridium, and Acinetobacter. These results suggest that bacteria from diverse origins could colonize adult Anopheles.

Interestingly, the Anopheles species from Binh Phuoc Province contained members of the genus Enterobacter sp. recently isolated from wild An. gambiae from Zambia. Enterobacter sp. received lot of attention due its anti-Plasmodium effect through the production of reactive oxygen species that directly target Plasmodium parasites in the midgut of An. gambiae (Cirimotich et al., 2011). Moreover, another bacteria, Chromobacterium Csp. P, has been described as responsible of vectors refractoriness to P. falciparum and dengue virus (Ramirez et al., 2014). Then, further studies on mosquito microbiota should provide new insights on pathogens transmission and might open new ways to prevent vector-borne diseases.

Recently published studies have described new bacterial species, first isolated from Anopheles mosquitoes, such as E. anophelis (Kampfer et al., 2011), Thorsellia anophelis (Kampfer et al., 2006a), and Janibacter anophelis (Kampfer et al., 2006b). The latter species was accurately identified herein after bacterial culture and the species f. terrae described in soil was also found in Binh Phuoc Anopheles. Bacterial identification to the species level or below, defining variants within a bacterial species (genovar, genomovar, biovar, etc.), is needed to determine whether strictly identical bacterial populations are shared between environment, host and vector, or if Anopheles-associated bacteria are specific.

As a tendency to be confirmed, we also described herein the microbiota of two Anopheles (An. dirus and An. pampai) naturally infected by P. vivax. The genus Acinetobacter solely was common to the two infected specimens but it was also one of the most prevalent bacterial species forming Anopheles microbiota. Indeed, it was frequently identified in the microbiota of both Asian and African anopheles species (Cameroon, Iran, India, Kenya, Mali, and Vietnam; Straif et al., 1998; Rani et al., 2009; Chavshin et al., 2012). Nevertheless, there is no available report on the implication of this genus for the development of Plasmodium in Anopheles and the interaction between this bacterial genus and malarial parasites in Anopheles might be studied.

The huge diversity of the microbial world even within a single species urges to overpass genus or species level for microbiota descriptions. Indeed, it is likely that the informative taxonomic level for host and microbes interactions is the subspecies level. Such a precise characterization needs availability of bacterial strains, highlighting again the interest of culture in microbiota studies. Moreover, the phenotype of bacterial strains could be of great interest to understand mechanisms involved in bacteria/Plasmodium/Anopheles interactions. This study provides 51 strains available for further experiments. Moreover, to the niche level, functional metagenomic and
metabolic approaches should be undertaken to determine microbiota conditions that favor or not vector competence.

**AUTHOR CONTRIBUTIONS**

CN made the bacterial analyses (bacterial cultures and molecular analyses), and results’ interpretation. SR-B helped for DNA sequencing, bacterial identification, and results’ interpretation. SM contributed to the study design, the specimens collection and the results’ interpretation. EJ-B supervised the study design, the bacterial analyses, and the results’ interpretation. All the authors contributed to the manuscript redaction.

**REFERENCES**

Abe, T., Honda, S., Nakazawa, S., Tuong, T. D., Thieu, N. Q., Hung, L. X., et al. (2009). Risk factors for malaria infection among ethnic minorities in Binh Phuoc, Vietnam. *Southeast Asian J. Trop. Med. Public Health* 40, 18–29.

Beier, M. S., Pampuni, C. B., Beier, J. C., and Davis, J. R. (1994). Effects of para-aminobenzoic acid, insulin, and gentamicin on Plasmodium falciparum development in anopheline mosquitoes (Diptera: Culicidae). *J. Med. Entomol.* 31, 561–565. doi: 10.1093/jmedent/31.4.561

Blumberg, B. J., Trop, S., Das, S., and Dimopoulos, G. (2013). Bacteria- and bacterial analyses, and the results' interpretation. All the authors contributed to the manuscript redaction.

**ACKNOWLEDGMENTS**

This study was made possible through a PhD allowance provided to the CN by the Institute of Research for Development (IRD), France. This work was also supported by the association ADEREMPHA, Sauzet, France.

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.02095/full#supplementary-material

Dennison, N. J., Jupatanakul, N., and Dimopoulos, G. (2014). The mosquito microbiota influences vector competence for human pathogens. *Curr. Opin. Insect Sci.* 3, 6–13. doi: 10.1016/j.cois.2014.07.004

Dong, Y., Aguilar, R., Xi, Z., Watt, E., Mongin, E., and Dimopoulos, G. (2006). Anopheles gambiae immune responses to human and rodent Plasmodium parasite species. *PLoS Pathog* 2:e52. doi: 10.1371/journal.ppat.0020052

Dong, Y., Das, S., Cirimotich, C., Souza-Neto, J. A., McLean, K. J., and Dimopoulos, G. (2011). Engineered anopheles immunity to Plasmodium infection. *PLoS Pathog* 7:e1002458. doi: 10.1371/journal.ppat.1002458

Dong, Y., Manfredini, F., and Dimopoulos, G. (2009). Implication of the mosquito midgut microbiota in the defense against malaria parasites. *PLoS Pathog* 5:e1000423. doi: 10.1371/journal.ppat.1000423

Drancourt, M., Bollet, C., Carloto, A., Martelin, R., Gayral, J. P., and Raoult, D. (2000). 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. *J. Clin. Microbiol.* 38, 3623–3630.

Favia, G., Ricci, I., Damiani, C., Raddadi, N., Crotti, E., Marzorati, M., et al. (2007). Bacteria of the genus Asia stably associate with Anopheles stephensi, an Asian malarial mosquito vector. *Proc. Natl. Acad. Sci. U.S.A.* 104, 9047–9051. doi: 10.1073/pnas.0610451104

Gafan, G. P., Lucas, V. S., Roberts, G. J., Petrie, A., Wilson, M., and Spratt, D. A. (2005). Statistical analyses of complex denaturing gradient gel electrophoresis profiles. *J. Clin. Microbiol.* 43, 3971–3978. doi: 10.1128/JCM.43.8.3971-3978.2005

Garros, C., Harbach, R. E., and Manguin, S. (2005). Morphological assessment and molecular phylogenetics of the Funestus and Minimus groups of Anopholes (Cellia). *J. Med. Entomol.* 42, 522–536. doi: 10.1603/0022-25852005040522-MAAMPO\[2.0.CO;2

Gendrin, M., and Christophides, G. K. (2013). “The Anopholes mosquitoes microbiota and their impact on pathogen transmission,” in *Anopholes mosquitoes - New insights into malaria vectors*, ed. S. Manguin (Rijeka: InTech Open Access), 525–548.

Gendrin, M., Rodgers, F. H., Yerbaanga, R. S., Ouedraogo, J. B., Basanez, M. G., Cohuet, A., et al. (2015). Antibiotics in ingested human blood affect the mosquito microbiota and capacity to transmit malaria. *Nat. Commun.* 6, 5921. doi: 10.1038/ncomms6921

Gonzalez-Ceron, L., Santillan, F., Rodriguez, M. H., Mendez, D., and Hernandez-Avila, J. E. (2003). Bacteria in midguts of field-collected Anopheles albimanus block Plasmodium vivax sporogonic development. *J. Med. Entomol.* 40, 371–374. doi: 10.1603/0022-2585–40.3.371

Gusmão, D. S., Santos, A. V., Marini, D. C., Bacci, M. Jr., Berbert-Molina, M. A., and Lemos, F. J. (2010). Culture-dependent and culture-independent characterization of microorganisms associated with Aedes aegypti (Diptera: Culicidae) (L) and dynamics of bacterial colonization in the midgut. *Acta Trop.* 115, 275–281. doi: 10.1016/j.actatropica.2010.04.011

Hughes, G. L., Dodson, B. L., Johnson, R. M., Murdock, C. C., Tsujimoto, H., Suzuki, Y., et al. (2014). Native microbiome impedes vertical transmission of Wolbachia in Anopheles mosquitoes. *Proc. Natl. Acad. Sci. U.S.A.* 111, 12498–12503. doi: 10.1073/pnas.1408888111

Huang, N. M., Hewitt, S., Davis, T. M., Dao, L. D., Toan, T. Q., Kim, T. B., et al. (2001). Resistance of Plasmodium falciparum to antimalarial drugs in
a highly endemic area of southern Viet Nam: a study in vivo and in vitro. Trans. R. Soc. Trop. Med. Hyg. 95, 325–329. doi: 10.1016/S0035-9203(01) 90254-8

Jacquot, A., Neveu, D., Aujoulat, F., Mercier, G., Marchandin, H., Jumas-Bilak, E., et al. (2011). Dynamics and clinical evolution of bacterial gut microbiota in extremely premature patients. J. Pediatr. 158, 390–396. doi: 10.1016/j.jpeds.2010.09.007

Kabula, B., Tungu, P., Malima, R., Rowland, M., Minja, J., Wililo, R., et al. (2013). Distribution and spread of pyrethroid and DDT resistance among the Anopheles gambiae complex in Tanzania. Med. Vet. Entomol. 28, 244–252. doi: 10.1111/mve.12036

Kampfer, P., Lindh, J. M., Terenius, O., Haghdoost, S., Falsen, E., Busse, H. J., et al. (2006a). Thorsellia anophelis gen. nov., sp. nov., a new member of the Gammaproteobacteria. Int. J. Syst. Evol. Microbiol. 56, 335–338. doi: 10.1099/ ijs.0.63999-0

Kampfer, P., Terenius, O., Lindh, J. M., and Faye, I. (2006b). Janibacter anophelis nov., isolated from the midgut of Anopheles arabiensis. Int. J. Syst. Evol. Microbiol. 56, 389–392. doi: 10.1099/ijs.0.63905-0

Kampfer, P., Matthews, H., Glasier, S. P., Martin, K., Lodders, N., and Faye, I. (2011). Elizaebethkingia anophelis sp. nov., isolated from the midgut of the mosquito Anopheles gambiae. Int. J. Syst. Evol. Microbiol. 61, 2670–2675. doi: 10.1099/ijs.0.026393-0

Ledder, R. G., Gilbert, P., Huws, S. A., Arons, L., Ashley, M. P., Hull, P. S., et al. (2007). Molecular analysis of the subgingival microbiota in health and disease. Appl. Environ. Microbiol. 73, 516–523. doi: 10.1128/AEM.01419-06

Lovenberger, C. A., Kamal, S., Chiles, J., Paskewitz, S., Bulet, P., Hoffmann, J. A., et al. (1999). Mosquito-Microbial interactions in response to immune activation of the vector. Exp. Parasitol. 91, 59–69. doi: 10.1006/expo.1999.4350

Manguin, S., Ngo, C. T., Tainchum, K., Juntaarunmong, W., Charoenviriyaphap, T., Michon, A. L., et al. (2013). “Bacterial biodiversity in midguts of Anopheles mosquitoes, malaria vectors in Southeast Asia,” in Front. Microbiol. 379323

Meister, S., Agianian, B., Turlure, F., Relogio, A., Morlais, I., Kafatos, F. C., Hoffmann, J. A., et al. (1999). Mosquito-Microbial interactions in response to immune activation of the vector. Exp. Parasitol. 91, 59–69. doi: 10.1006/expo.1999.4350

Manguin, S., Ngo, C. T., Tainchum, K., Juntaarunmong, W., Charoenviriyaphap, T., Michon, A. L., et al. (2013). “Bacterial biodiversity in midguts of Anopheles mosquitoes, malaria vectors in Southeast Asia,” in Front. Microbiol. 379323

Minard, G., Mavingui, P., and Moro, C. V. (2013). Diversity and function of bacterial microbiota in the mosquito holobiont. Parasit Vectors 6, 146. doi: 10.1186/1756-3305-6-146

Ngo, C. T., Aujoulat, F., Veas, F., Jumas-Bilak, E., and Manguin, S. (2015). Bacterial diversity associated with wild caught Anopheles mosquitoes from Dak Nong Province, Vietnam using culture and DNA fingerprint. PLoS ONE 10:e0118653. doi: 10.1371/journal.pone.0118653

Ng, C. T., DuBois, G., Sinou, V., Parry, D., Le, H. Q., Harbach, R. E., et al. (2014). Diversity of Anopheles mosquitoes in Binh Phuoc and Dak Nong Provinces of Vietnam and their relation to disease. Parasit Vectors 7, 316. doi: 10.1186/1756-3305-7-316

Nguyen, M. H., Davis, T. M., Cox-Singh, J., Hewitt, S., Tran, Q. T., Tran, B. K., et al. (2014). Treatment of uncomplicated falciparum malaria in southern Vietnam: can chloroquine or sulfadoxine-pyrimethamine be reintroduced in Vietnam and their relation to disease. Parasit Vectors 7, 316. doi: 10.1186/1756-3305-7-316

Nguyen, M. H., Davis, T. M., Cox-Singh, J., Hewitt, S., Tran, Q. T., Tran, B. K., et al. (2014). Treatment of uncomplicated falciparum malaria in southern Vietnam: can chloroquine or sulfadoxine-pyrimethamine be reintroduced in Vietnam and their relation to disease. Parasit Vectors 7, 316. doi: 10.1186/1756-3305-7-316

Nguyen, M. H., Davis, T. M., Cox-Singh, J., Hewitt, S., Tran, Q. T., Tran, B. K., et al. (2013). Alternative Treatments for Indoor Residual Spraying for Malaria Control in a Village with Pyrethroid- and DDT-Resistant Vectors in The Gambia. PLoS ONE 8:e74351. doi: 10.1371/journal.pone.0074351

Thanh, N. V., Toan, T. Q., Cowman, A. F., Casey, G. J., Phuc, B. Q., Tien, N. T., et al. (2010). Monitoring for Plasmodium falciparum drug resistance to artemisinin and artesunate in Binh Phuoc Province, Vietnam: 1998-2009. Malar. J. 9, 181. doi: 10.1186/1475-2875-9-181

Tran, T. H., Nguyen, T. T., Nguyen, H. P., Boni, M. F., Ngo, V. T., Nguyen, T. N., et al. (2012). In vivo susceptibility of Plasmodium falciparum to artesunate in Binh Phuoc Province, Vietnam. Malar. J. 11, 355. doi: 10.1186/1475-2875-11-355

Verheagen, K., Van Bortel, W., Trung, H. D., Sochantha, T., and Coosemans, M. (2009). Absence of knockdown resistance suggests metabolic resistance in the main malaria vectors of the Mekong region. Malar. J. 8, 84. doi: 10.1186/1475-2875-8-84

Verheagen, K., Van Bortel, W., Trung, H. D., Sochantha, T., Keokenchanh, K., and Coosemans, M. (2010). Knockdown resistance in Anophelles vagus, An. sinensis, An. paraliae and An. peditaeniatus populations of the Mekong region. Parasit. Vectors 3, 59. doi: 10.1186/1756-3305-3-59

Villegas, L. M., and Pimenta, P. F. (2014). Meta-genomics, paratransgenesis and the Anophelles microbiome: a portrait of the geographical distribution of the anopheles microbiota based on a meta-analysis of reported taxa. Mem. Inst. Oswaldo Cruz 109, 672–684. doi: 10.1590/0070-27640194

Walton, C., Handley, J. M., Kuvangakdikol, C., Collins, F. H., Harbach, R. E., Buium, V., et al. (1999). Identification of five species of the Anopheles dirus complex from Thailand, using allele-specific polymerase chain reaction. Med. Vet. Entomol. 13, 24–32. doi: 10.1046/j.1365-2915.1999.00142.x
Walton, C., Somboon, P., O’Loughlin, S. M., Zhang, S., Harbach, R. E., Linton, Y. M., et al. (2007). Genetic diversity and molecular identification of mosquito species in the Anopheles maculatus group using the ITS2 region of rDNA. Infect. Genet. Evol. 7, 93–102. doi: 10.1016/j.meegid.2006.05.001

Wang, Y., Gilbreath, T. M. III, Kukutla, P., Yan, G., and Xu, J. (2011). Dynamic gut microbiome across life history of the malaria mosquito Anopheles gambiae in Kenya. PLoS ONE 6:e24767. doi: 10.1371/journal.pone.0024767

WHO (2012). World Malaria Report. Geneva: WHO Press.

WHO (2015). World Malaria Report. Geneva: World Health Organization.

**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2016 Ngo, Romano-Bertrand, Manguin and Jumas-Bilak. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.