

Characterisation of bacterial symbionts of the *Anopheles funestus* group from KwaZulu-Natal Province, South Africa

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ABSTRACT

Anopheles funestus s.s. is the anthropophilic member of a group of mosquitoes that was the only member considered a malaria vector until recently. However, *Anopheles parensis* and *Anopheles vaneedeni* have recently been suspected to be implicated in disease transmission and may contribute to the residual malaria problem in South Africa. Unlike *An. funestus* s.s., the rest of the group would be more difficult to control due to their outdoor biting and resting behaviours. Thus, alternative vector control methods efficient in controlling both outdoor and indoor biting populations would be beneficial in controlling such secondary vectors. One of these alternative efforts is paratransgenesis, a technique that aims to inhibit *Plasmodium* infection through transgenesis by bacterial symbionts. Yet, bacterial symbionts of the *An. funestus* group are poorly studied. This study characterised the microbiota of the digestive system (salivary glands and midguts) of the *An. funestus* group by sequencing the V3-V4 hypervariable region of bacterial 16S rRNA genes in F1 *An. rivulorum*, *An. vaneedeni* and *An. parensis* sampled from KwaZulu-Natal Province, South Africa. Although alpha diversity did not differ significantly, beta diversity, differential abundance and relative abundance varied between the different members of the *An. funestus* group. Overall, there were shared bacterial genera between the different members of the *An. funestus* group, which were *Agromyces*, *Rahnella*, *Acinetobacter*, *Microbacterium* and *Asaia*. The study revealed a core microbiota in the digestive system of the *An. funestus* group which could be further explored for the use of paratransgenesis for the control of members of the *An. funestus* group.

1. Introduction

Anopheles funestus s.s. is the nominal member of the *An. funestus* group which is made up of an additional 11 sibling species: *Anopheles funestus*-like, *Anopheles parensis*, *Anopheles vaneedeni*, *Anopheles aruni*, *Anopheles confusus*, *Anopheles lesoni*, *Anopheles longipalpis*, *Anopheles rivulorum*, *Anopheles rivulorum*-like, *Anopheles brucei*, and *Anopheles fuscivenosus* (Coetzee, 2020). Within the *An. funestus* group, only *An. funestus* s.s. is considered to be a major malaria vector in Africa, due to its anthropophilic, indoor biting and indoor resting behaviour (Gillies and Coetzee, 1987; Sinka et al., 2012, 2010).

In South Africa, the population of *An. funestus* s.s. remained low after the introduction of insecticide-treated bed nets (ITNs) and long-lasting

insecticidal nets (LLINs). However, the resistance of *An. funestus* to pyrethroid insecticides emerged due to the discontinuation of DDT in the late 1990s. The invasion of resistant *An. funestus* from Mozambique subsequently caused the major malaria epidemic in 2000 (Coetzee et al., 2013; McCann et al., 2014; Okia et al., 2018). Cases of pyrethroid resistance of *An. funestus* in Africa continues to be reported (Cuamba et al., 2010; Djouaka et al., 2016; Menze et al., 2016; Morgan et al., 2010; Riveron et al., 2016).

The control of the *An. funestus* group is further hampered by the potential of other *An. funestus* group members to become secondary or minor vectors. *Anopheles rivulorum* has been implicated in malaria transmission in Tanzania and western Kenya (Kawada et al., 2012; Wilkes et al., 1996), while *An. vaneedeni* and *An. parensis* have been

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suspected to be implicated in the transmission of malaria in South Africa (Burke et al., 2019, 2017). Most recently, *An. lesoni* has been shown to transmit malaria in Cameroon (Kopya et al., 2021). Furthermore, unlike *An. funestus* s.s., other members of the *An. funestus* group, including the implicated secondary vectors, are outdoor biting and outdoor resting. Thus, ITNs and LLINs are not efficient in controlling these vectors. As such, these secondary vectors could contribute to the low-level outdoor transmission of malaria known as residual malaria (Carnevale and Manguin, 2021). Therefore, to eliminate malaria transmission in South Africa, novel supplementary vector control interventions which can target both the major and minor malaria vectors are needed.

Successful development and application of novel vector control interventions depend on reliable information on the biology and competence of the targeted vector species. Vector competence is affected in part by the interaction between native host microbiota, the immune system and vector behaviour (Dennison et al., 2014; Dong et al., 2009; Gabrieli et al., 2021; Villegas and Pimenta, 2014). The host and its community of associated microorganisms is termed the holobiont. The mosquito holobiont, specifically the mosquito vector and its microbial symbionts, plays a crucial role in its life history parameters (adult longevity, larval development, egg production and oviposition) as well as general metabolism (blood digestion, sugar digestion, vitamin and amino acids synthesis) (Coon et al., 2016, 2014; Guégan et al., 2018). Thus, by modulating the symbiotic microbiota of mosquitoes, in a technique known as paratransgenesis, it is possible to alter its vector competence and immunity thereby suppressing *Plasmodium* infection and eventually, malaria transmission (Boissière et al., 2012; Gabrieli et al., 2021; Gupta and Nair, 2020; Romoli and Gendrin, 2018).

Paratransgenesis often uses symbiotic bacteria that already exist within the core microbiota of the vector. For example, *Enterobacter Esp_Z* bacterium isolated from the midguts of wild *An. arabiensis* population from Zambia has been shown to elicit a 99 % refractoriness to *Plasmodium falciparum* infection in *An. stephensi* and *An. gambiae*. The refractoriness is achieved through the synthesis of reactive oxygen species (ROS) which inhibits *Plasmodium* oocyst formation (Cirimotich et al., 2011). *Serratia marcescens* is also efficient at compromising mosquito survival and inhibiting the sexual and asexual developmental stages of *Plasmodium* (Bahia et al., 2014). Similarly, *Serratia ureilytica* isolated from the midgut of wild *Anopheles sinensis* rendered mosquitoes resistant to infection by *P. falciparum* and *P. berghei* via the secretion of a 65-kDa lipase called Antimalaria Lipase (AmLip) that selectively killed the parasites at various stages (Gao et al., 2020).

Paratransgenesis can also use genetically modified bacterial symbionts to enhance the anti-*Plasmodium* properties of the symbionts. Recombinant *Pantoea agglomerans* (previously known as *Enterobacter agglomerans*), modified from the naturally occurring variant found in the mosquito midgut symbiont possess antimalarial effector molecules. These antimalarial effector molecules were shown to successfully suppress *P. falciparum* and *P. berghei* infection in both *An. gambiae* and *An. stephensi* (Wang et al., 2012). *Serratia* strains isolated from *An. stephensi* ovaries have also been genetically engineered to express multiple antimalarial effector molecules and have been successfully used in inhibiting *Plasmodium* oocyst loads by 92 to 93 % in *An. gambiae* (Wang et al., 2017).

The success of paratransgenesis requires prior knowledge of the microbiota because it relies on the identification of microbes that can readily infect the vector of interest to elicit responses such as strengthening the immune response, changing the vectorial capacity or the expression of anti-pathogen molecules. It is therefore important to assess the native bacteria present in vectors. The midgut microbiota of *Anopheles* mosquitoes has been reported to be dominated by *Asaia*, *Acinetobacter*, *Aeromonas*, *Pantoea*, *Pseudomonas*, *Enterobacter*, *Serratia* and *Elizabethkingia* (Silva et al., 2021; Gao et al., 2020; Minard et al., 2013a; Steven et al., 2021; Villegas and Pimenta, 2014). However, there is little knowledge of the core microbiota of the *An. funestus* group specifically, particularly of members that act as secondary vectors. We

have previously characterised the microbiota of three lab strains of *An. funestus* s.s (Chen et al., 2024), however, the characterisation of F1 populations (offspring of wild populations) of members of the *An. funestus* group would offer a more holistic view of the microbiota of the *An. funestus* group in South Africa. In order to gain a wider understanding of the microbiota of the *An. funestus* group, this study characterised the bacterial symbionts of the *An. funestus* group from the KwaZulu-Natal Province, South Africa.

2. Methods and materials

2.1. Insects

Wild specimens of *An. parensis*, *An. vaneedeni* and *An. rivulorum* were sampled from KwaZulu-Natal from November 2021 to April 2022. All specimens were sampled from Section 9 (S 27°23'50.5"; E 032° 12'20.1") in the KwaZulu-Natal Province, South Africa. The specimens were transported to the Botha de Meillon insectary in Sandringham, Johannesburg (National Institute for Communicable Diseases) where they were reared according to the methods described by Hunt et al., 2005. The F1 specimens (direct offspring of the wild specimens) were used in the study and represent the offspring of 30 families. Non blood-fed F1 adult females were harvested at the age of three days.

2.2. Tissue dissections and DNA extraction

The F1 specimens of *An. parensis*, *An. vaneedeni* and *An. rivulorum* were freeze-killed at -70 °C. The midguts and salivary glands of frozen specimens were dissected following aseptic procedures. A total of 25 midguts (5 midguts per replicate for five replicates) were dissected. A total of 30 salivary glands (10 salivary glands per replicate for three replicates) were dissected. The dissected tissues were pooled straight into buffer ATL and proteinase K, provided by the DNeasy Blood & Tissue Kit (Qiagen, Germany). Total genomic DNA was extracted using the DNeasy® Blood & Tissue kit (Qiagen, Germany). DNA extraction of total genomic DNA was carried out following an overnight incubation as recommended in the user's manual. A buffer-only negative control was subjected to the same DNA extraction procedure.

2.3. DNA amplification and sequencing

The V3-V4 hypervariable region of the bacterial 16S rRNA gene was amplified from total genomic DNA extracted from the midguts and salivary glands of *An. parensis*, *An. vaneedeni* and *An. rivulorum*. PCR amplification was performed using KAPA HiFi HotStart Ready mix (Roche Diagnostics Ltd, West Sussex) and sequencing libraries were prepared using the 16S Metagenomic Sequencing Library Preparation protocol (Illumina TM, San Diego, CA, USA). The primer sequences were as follows: Forward primer: 5' GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C 3' and reverse primer 5' TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG 3' (Integrated DNA Technologies, USA).

Multiplexed paired-end libraries (2 × 300 bp) were sequenced on the Illumina NextSeq 2000 platform at the Sequencing Core Facility, National Institute for Communicable Diseases, Johannesburg, South Africa. A buffer-only negative control, a non-template control and a positive control containing known bacterial species (ZymoBIOMICS® Microbial Community Standard Cat: D6300, Zymo Research, USA) were also amplified and sequenced.

2.4. Bioinformatics

The Nextera adapter sequences were trimmed from the raw sequences as pair-end sequences using trimGalore (v0.6.5–1). All the FastQC files of the trimmed sequences were then visualised in MultiQC (v1.6) to ensure clean data were used for downstream analysis. The

downstream analyses, including bacterial species classification, abundance estimations, statistical analysis, and visualisations were performed in R studio (v4.2.1). Clean reads were pre-processed using the Dada2 package (v1.24.0), including quality inspection, filtering, trimming, dereplication, sample inference, merging paired-end reads and removal of chimeric sequences (Callahan et al., 2016). Taxonomy was assigned to the obtained amplicon sequence variants (ASVs) and the ASV abundance estimates were determined using training sequence sets based on the SILVA reference database (v138; <https://zenodo.org/record/4587955#.Y9JGXnZBxPY>). Dada2 outputs were then constructed into phyloseq objects using the phyloseq package (v1.40.0) (McMurdie and Holmes, 2013). The phyloseq objects were then used for further analyses. For alpha diversity, species richness was determined using the Chao1 index while relative abundance (with more emphasis on species evenness) was measured using the Simpson index (Kim et al., 2017). The Wilcoxon rank-sum tests were used for pair-wise comparison of the alpha diversity either between the tissues (midguts and salivary glands) or between species of the *An. funestus* group. For beta diversity, ordination plots were constructed using the Non-metric Multidimensional Scaling (NMDS) method. The data clustering between the three different tissues for the NMDS plot was statistically assessed using a PERMANOVA (permutation test with pseudo-F ratios) as implemented by the adonis function in the vegan package (<https://github.com/vegandevs/vegan>). Bar plots showing the top 5 most abundant genera ranked across the different tissues were also constructed. Differential abundance analysis between sample groups was performed using DESeq2 (v1.24.0) (Love et al., 2014). All plots were constructed using ggplot2 (v3.4.0) (Wickham, 2011).

3. Results

3.1. The top five most abundant bacterial genera are the same across members of the *An. funestus* group

The top five most abundant bacterial genera across the three members of the *An. funestus* group (*An. parensis*, *An. vaneedeni*, *An. rivulorum*) were *Acinetobacter*, *Agromyces*, *Asaia*, *Microbacterium* and *Rahnella* (Fig. 1). The full list of microbiota found across the three members of the *An. funestus* group as well as the top 100 most abundant taxa can be found in Supplementary Tables S1 and S2, respectively.

3.2. Alpha diversity between species and tissues of the *An. funestus* group are not significantly different

Both the Chao1 (species richness) and Simpson (diversity) indices were not significantly different when looking at the alpha diversity of the microbiota of either salivary glands or midguts of each of the *An. funestus* group members (Supplementary Figures S1 and S2). Thus, the alpha diversity of both tissues was combined to give an overall view of the alpha diversity of the microbiota from members of the *An. funestus* group from KwaZulu-Natal Province (Fig. 2). However, both Chao1 and Simpson indices still indicated no significant differences in the alpha diversity between *An. parensis*, *An. vaneedeni* and *An. rivulorum*.

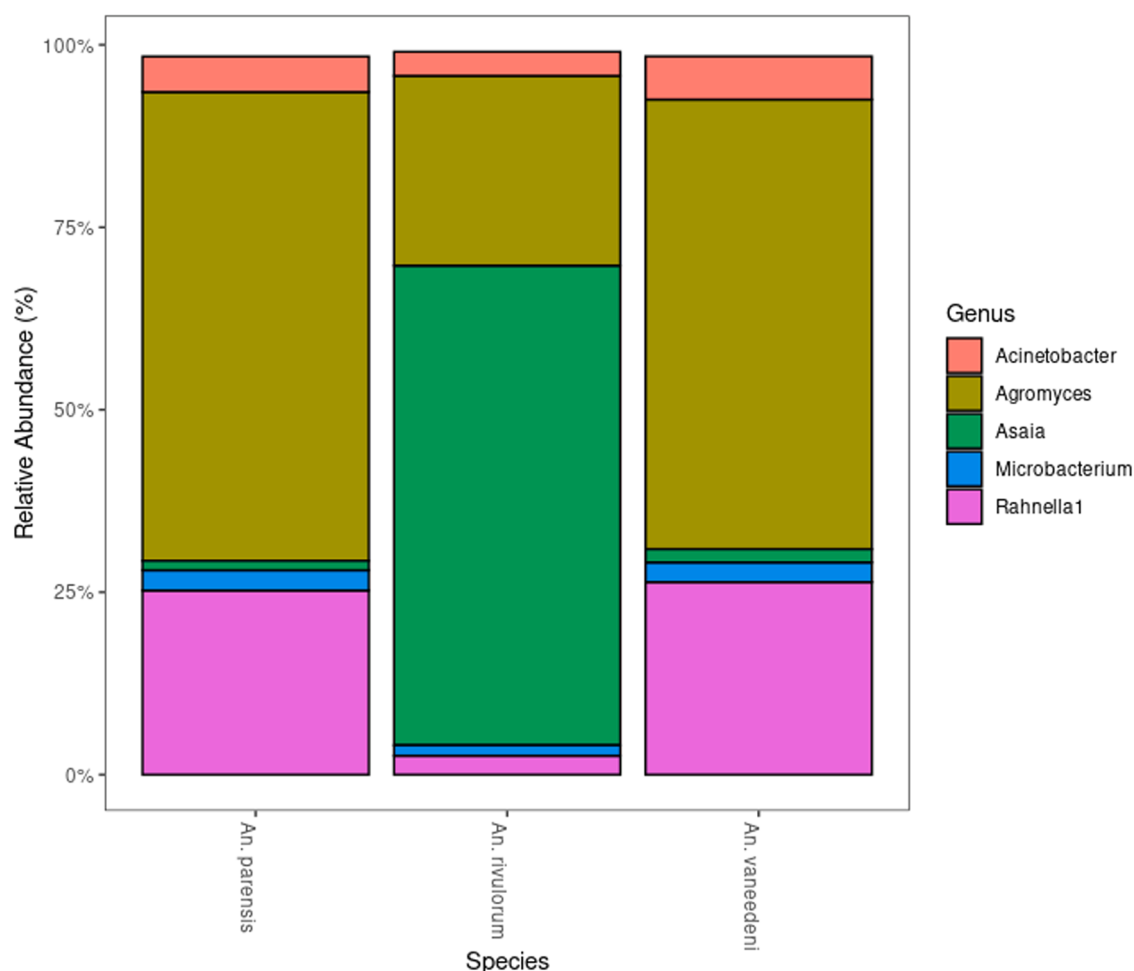


Fig. 1. Relative abundance of the top five ranked bacterial genera in members of the *An. funestus* group from KwaZulu-Natal.

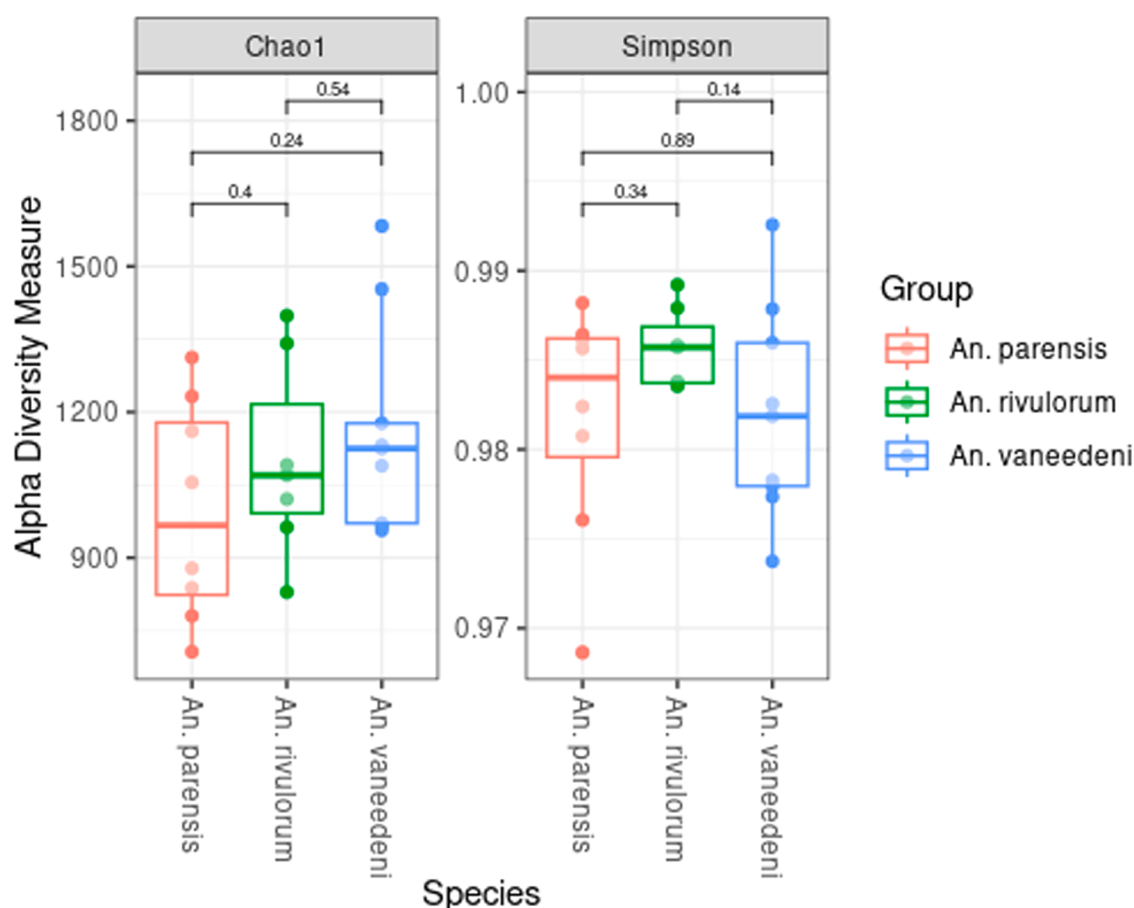


Fig. 2. Alpha diversity (Chao1 and Simpson index) of the microbiota from members of the *An. funestus* group (*An. parensis*, *An. vaneedeni* and *An. rivulorum*) from KwaZulu-Natal. Adults from the F1 generation were used in the study. The P-values were determined by the Wilcoxon rank-sum test for pair-wise comparisons of the alpha diversity between the different species.

3.3. Beta diversity of bacteria in the members of *An. funestus* group differ in on the species-level and tissue-level

Unlike alpha diversity, beta diversity of the microbiota of *An. parensis*, *An. vaneedeni* and *An. rivulorum* were significantly different with $P = 0.001$ (Fig. 3). There was also a significant difference in beta diversity between midguts and salivary glands ($P = 0.031$). However, there was no significant difference in the interaction between species and tissues ($P = 0.186$) (Fig. 3).

3.4. Members of the *An. funestus* group harbour shared bacteria

For the analysis of shared bacteria, bacterial sequences from both tissues (midguts and salivary glands) were combined. The three members of the *An. funestus* group in the present study shared a total of 55 bacterial families, 66 genera, and 89 species (Fig. 4). Furthermore, *An. rivulorum* appeared to harbour the most distinct bacterial families (33), genera (66) and species (108) compared to *An. parensis* and *An. vaneedeni* (Fig. 4). The full lists of shared bacterial families, genera and species between the three members of the *An. funestus* group can be found in Supplementary Tables S3–5.

3.5. *An. rivulorum* had less differentially abundant bacterial genera than *An. parensis* and *An. vaneedeni*

For the analysis of differentially abundant bacteria, bacterial sequences from both tissues (midguts and salivary glands) were combined. There were less differentially abundant bacteria in *An. rivulorum* when

compared with *An. parensis* or *An. vaneedeni* (Fig. 5). When comparing *An. parensis* with *An. vaneedeni*, minimal differences in differentially abundant bacterial genera were observed (Fig. 5B). The exact Log2fold changes between each bacteria can be found in Supplementary Tables S6–8.

4. Discussion

We have previously characterised the core microbiota of laboratory strains of *An. funestus* s.s., one of the dominant vectors of malaria in Africa (Chen et al., 2024). However, laboratory strains tend to have lower bacterial richness and diversity compared to wild *An. funestus* and so may not fully represent the microbiota of *An. funestus* s.s. (Akorli et al., 2019). Furthermore, since *An. funestus* s.s. has not been recorded in the KwaZulu-Natal Province since the 2000s, the present study sought to better understand the overall bacterial landscape harboured by members of the *An. funestus* group that are found in the KwaZulu-Natal Province by characterising the microbiota of F1 *An. funestus* s.l. (*An. parensis*, *An. vaneedeni* and *An. rivulorum*) from KwaZulu-Natal.

The bacterial genera *Acinetobacter*, *Agromyces*, *Asaia*, *Microbacterium* and *Rahnella* were the top five most abundant bacterial genera across *An. parensis*, *An. vaneedeni* and *An. rivulorum*. *Acinetobacter*, *Asaia* and *Rahnella* have been previously recorded in *An. funestus* s.s laboratory strains (Chen et al., 2024; E Silva et al., 2021) as well as in other *Anopheles* mosquitoes (Akintola and Hwang, 2024; Cirimotich et al., 2011; Ngo et al., 2015; Tchioffo et al., 2016; Yadav et al., 2015). Thus, these bacteria likely form part of the core microbiota of the *An. funestus* group.

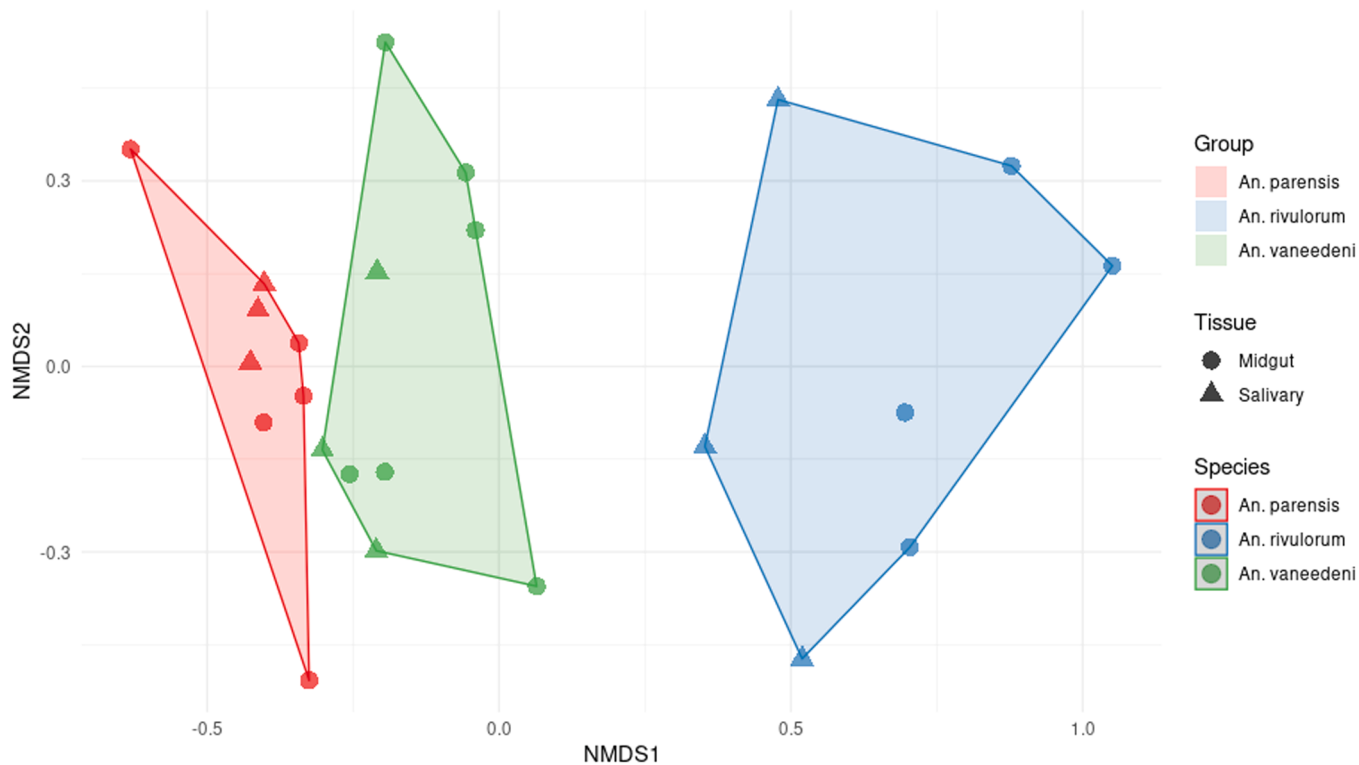


Fig. 3. Beta diversity of the microbiota of the members of *An. funestus* group from KwaZulu-Natal plotted as an ordination plot using the Nonmetric Multidimensional Scaling (NMDS) method based on the Bray-Curtis distance measurement. P values were determined by PERMANOVA: $P = 0.001$ for species ($R^2 = 0.030$), $P = 0.031$ for tissue ($R^2 = 0.058$), $P = 0.186$ for species: tissue ($R^2 = 0.758$). Stress value = 0.12.

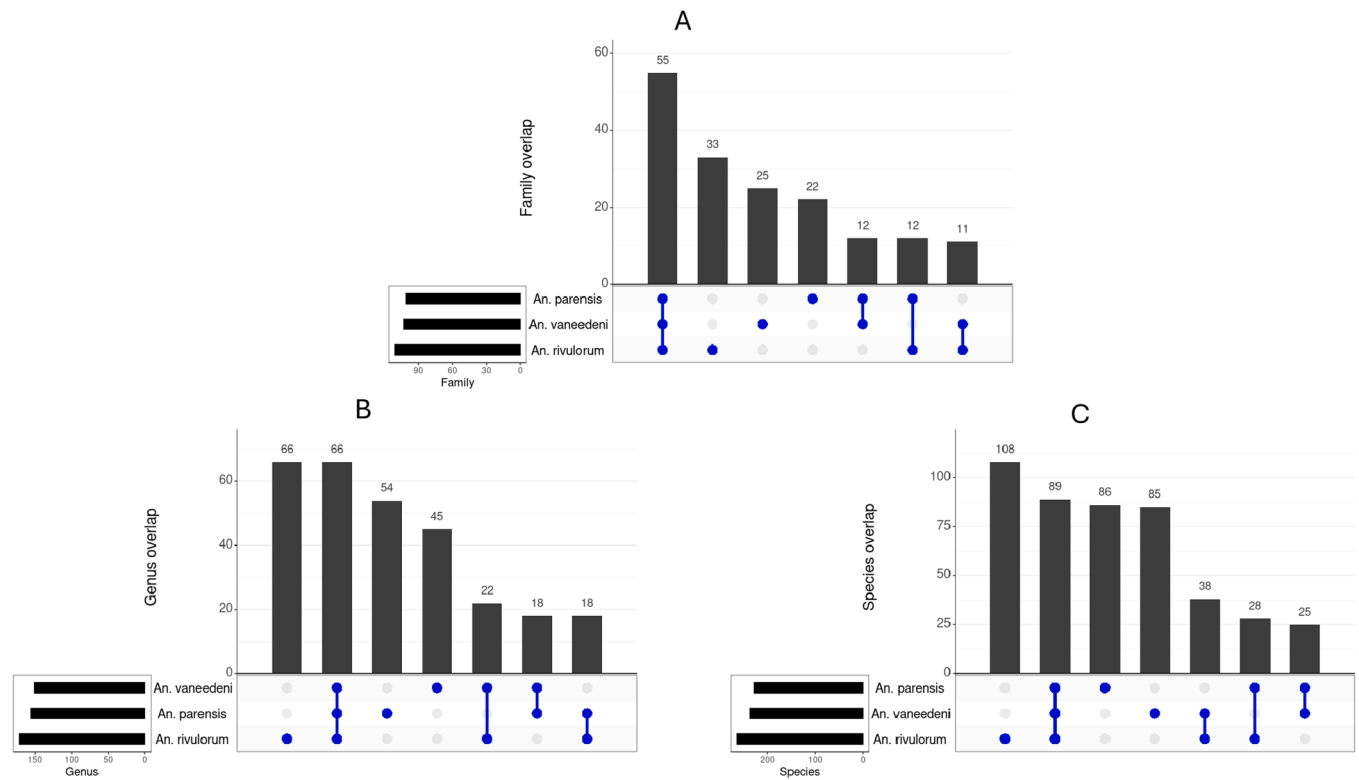


Fig. 4. Shared bacteria between *An. parensis*, *An. vaneedeni* and *An. rivulorum* from KwaZulu- Natal. **A:** Shared bacterial families between the three members of the *An. funestus* group, **B:** Shared bacterial genera between the three members of the *An. funestus* group, **C:** Shared bacterial species between the three members of the *An. funestus* group.

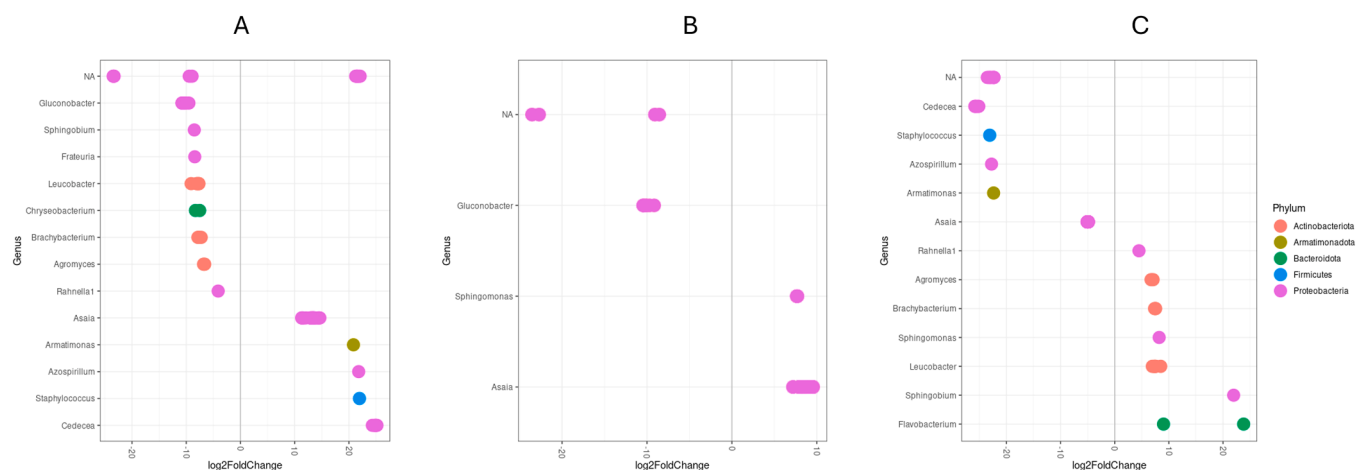


Fig. 5. Differential abundance of bacterial genera in members of the *An. funestus* group from KwaZulu-Natal. A: Differential abundance between *An. parensis* (left) and *An. rivulorum* (right), B: Differential abundance between *An. parensis* (left) and *An. vaneedeni* (right), C: Differential abundance between *An. rivulorum* (left) and *An. vaneedeni* (right). Numbers on either side of the 0 represent a log2fold change of bacterial genera that are significantly different at an alpha value of 0.01. The greater the log2fold change, the further away an OTU dot would be from the zero. Each dot represents a single OTU. Where there are more than one dot, more than one OTU is present. N/A represents OTUs that could not be identified.

Acinetobacter was an expected core microbe of the *An. funestus* group as it may play an important role in the biology of the mosquitoes. Martinson and Strand (2021), demonstrated that monoxenic (experimental mosquitoes consisting of a single bacteria of interest) *Aedes aegypti* mosquitoes that fed on diets supplemented with *Acinetobacter* could successfully develop to adulthood. Furthermore, *Ae. aegypti* inoculated with *Acinetobacter* alone resulted in larval developmental time that was not significantly different from larvae fed a mixture of 7 bacterial species (ALL7). The study also showed that the earlier *Acinetobacter* is added in with *Rahnella*, *Escherichia*, or *Sphingobacterium*, the faster the larval developmental time became, while eliminating *Acinetobacter* with kanamycin antibiotic caused longer larval development (Martinson and Strand, 2021). Evidently, *Acinetobacter* plays a crucial role in the development of *Ae. aegypti* larvae, and likely *An. funestus* s.l. as well. Furthermore, *Acinetobacter* plays a crucial role in blood digestion and nectar assimilation in *Ae. Albopictus* and could play a similar role in *An. funestus* s.l., making it an important core microbe of the *An. funestus* group (Minard et al., 2013b).

Interestingly, *Acinetobacter* showed in vitro *Plasmodium*-blocking activity in *An. gambiae*. The *Plasmodium*-blocking mechanism was theorised to be due to *Acinetobacter* triggering the immune deficiency (IMD) signalling pathway which is the pathway primarily involved in the clearance of the *Plasmodium* parasite as well (Bahia et al., 2014). The fact that *Acinetobacter* exhibits *Plasmodium*-blocking activity in *An. gambiae* also warrants an in-depth look at the potential of *Acinetobacter* for paratransgenesis in the *An. funestus* group.

In addition to *Acinetobacter*, Martinson and Strand, 2021, showed that diets supplemented solely with *Rahnella* also resulted in the full development of *Ae. aegypti* larvae to adulthood although time to pupation was significantly slower than that of *Acinetobacter*-inoculated larvae as well as the ALL7-inoculated larvae (Martinson and Strand, 2021). Thus, like *Acinetobacter*, *Rahnella* is a vital microorganism for the development of mosquitoes. This is further evident by the fact that *Rahnella* has been found in *An. arabiensis* (Singh et al., 2022a) and *Anopheles merus* (Singh et al., 2022b). In the pine forest bark beetle, *Dendroctonus*, *Rahnella* is a dominant member of the gut bacterial core of species where it plays a crucial role in nutritional and detoxification processes degrading substrates such as esters and lipids (Morales-Jiménez et al., 2012; Pineda-Mendoza et al., 2022). The role of *Rahnella* could be similar in members of the *An. funestus* group and explain why it was found to be a core microbe in the present study.

Less studies are focused on *Agromyces* and *Microbacterium* in terms of

potential for paratransgenesis. However, *Agromyces* has been found in *Ae. aegypti* and *Ae. albopictus* midguts (Ranasinghe et al., 2021). Interestingly, *Agromyces* was found to be unique in African samples of mosquitoes (Villegas and Pimenta, 2014) and could explain why the present study harboured such high levels of it. *Microbacterium* has been found in *Anopheles quadrimaculatus*, *An. merus*, *An. gambiae* and *Ae. albopictus* (Dong et al., 2009; Singh et al., 2022a; Tchioffo et al., 2016; Tuanudom et al., 2021). Moreover, *Microbacterium* load decreases after a blood meal in female mosquitoes (Tchioffo et al., 2016). *Microbacterium* therefore likely plays a role other than blood digestion in members of the *An. funestus* group.

Occurrence of *Asaia* as a core microbe of *An. funestus* s.l. in this study was also expected as *Asaia* has been found as part of the core microbiota in several tissues (midgut, testes, ovaries and salivary glands) of most mosquito species including *Aedes* sp., *Anopheles* sp., and *Culex* sp. (Crotti et al., 2009; Damiani et al., 2010, 2008; De Freece et al., 2014; Dong et al., 2009; Favia et al., 2007; Ramos-Nino et al., 2020; Singh et al., 2022b). *Asaia* plays an important metabolic role in mosquitoes. For example, the inhibition of *Asaia* in *An. stephensi* impacted the survival and the metabolism of the mosquitoes (Chouaia et al., 2012; Mancini et al., 2020). *Asaia* therefore likely plays a crucial role in the physiology of mosquitoes and can be expected to form part of the core microbiota of *Anopheles* mosquitoes.

The present study found no significant differences in alpha diversity at the species level or when comparing tissues (midguts and salivary glands). However, beta diversity was significantly different at the species and tissue level. Alpha diversity measures species richness (number of species) and/or evenness (distribution of individuals among species) within a sample. If mosquito samples harbour a similar number of microbial taxa and these taxa are evenly distributed, the alpha diversity might not show significant differences. However, this does not mean the specific microbial composition is identical across samples. The observed significant beta diversity differences suggest that even though all samples may harbour a similar overall diversity which reflects a core microbiota (as measured by alpha diversity), the specific microbial species or their proportions vary significantly between species and tissues. This difference in microbial composition was seen in the shared bacteria and in the differential abundance.

An. rivulorum harboured the most unique bacterial genera compared to *An. parensis* and *An. vaneedeni*. This was further seen when looking at differential abundance where not much was found to be differentially abundant between *An. parensis* and *An. vaneedeni*. *Asaia* in particular

appeared to be more relatively abundant in *An. rivulorum* compared to the other top 5 most abundant bacterial genera. Similarly, *Asaia* was more differentially abundant in *An. rivulorum* when compared with *An. parensis* or *An. vaneedeni*. Thus, while *Asaia* is important for the life history of *An. funestus* s.l in general, it could play a more important role in the vectorial capacity of *An. rivulorum*. *Asaia* has been reported to prime the immune response of *An. stephensi* and *An. gambiae* thereby preventing the development of the *Plasmodium* parasite (Capone et al., 2013; Cappelli et al., 2019). It is plausible that the elevated levels of *Asaia* in *An. rivulorum* better primed the immune system of *An. rivulorum* thereby enabling it to fight off the *Plasmodium* parasite (Capone et al., 2013; Cappelli et al., 2019). It is worth noting that it has been postulated that the microbiota does not affect the transcriptome of *Ae. aegypti* in a way that alter immune genes significantly (Hyde et al., 2020; Vogel et al., 2017). However, other literature do link the critical role of the microbiota to immune response and vector competence of mosquito vectors (Gabrieli et al., 2021). This may explain why *An. rivulorum* is not yet implicated in malaria transmission in South Africa. Another possible reason is due to the evolutionary distance. Phylogenetic analyses based on three genetic markers showed that *An. rivulorum* clustered separately from *An. parensis*, *An. vaneedeni* and *An. funestus* s.s (Garros et al., 2005). Thus, it is possible that *An. rivulorum* harbours unique bacteria due to inherent genetic and evolutionary differences.

However, despite the differences in microbial composition, the members of the *An. funestus* group in the present study do appear to share 66 bacterial genera and 89 bacterial species. The shared bacteria enables research into potential paratransgenesis candidates that would work for the whole *An. funestus* group. Thus, future research should identify the shared microbiota observed in the study down to species and strain so that potential bacteria that influence *Plasmodium* transmission can be explored for the use of paratransgenesis for the control of the *An. funestus* group. For example, the study revealed two bacterial genera (*Acinetobacter* and *Asaia*) that have immense potential for paratransgenesis in South Africa due to their anti-*Plasmodium* properties. In characterising the core microbiota of F1 *An. funestus* s.l., the study expanded on what is currently known about the microbiota of members of the *An. funestus* group and laid the groundwork for future paratransgenesis studies for mosquito vectors.

While the present study explored and expanded on the microbiota of members of the *An. funestus* group, further research will be required. The microbiota of F1 *Anopheles leesoni* and *An. funestus* s.s would give a more well-rounded perspective on the microbiota of the *An. funestus* group. Although *An. funestus* s.s. is unlikely to be found in South Africa, particularly in the numbers to allow for robust research, the microbiota of F1 *An. funestus* s.s. would have offered a better perspective of the *An. funestus* group in South Africa. Furthermore, the study was limited to wild specimens and the investigation of the microbiota of *Anopheles leesoni* would have also strengthened the study.

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CRediT authorship contribution statement

Chia-Yu Chen: Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis. **AshleyM Burke:** Writing – review & editing, Resources. **Givemore Munhenga:** Writing – review & editing, Resources. **Arshad Ismail:** Writing – review & editing, Resources. **Shūnē V Oliver:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2025.107653.

Data availability

Data will be made available on request.

References

- Akintola, A.A., Hwang, U.W., 2024. Microbiome profile of South Korean vector mosquitoes. *Acta Trop* 255, 107213. <https://doi.org/10.1016/j.actatropica.2024.107213>.
- Akorli, J., Namaali, P.A., Ametsi, G.W., Egyirifa, R.K., Pels, N.A.P., 2019. Generational conservation of composition and diversity of field-acquired midgut microbiota in *Anopheles gambiae* (sensu lato) during colonization in the laboratory. *Parasit. Vectors*. 12, 27. <https://doi.org/10.1186/s13071-019-3287-0>.
- Bahia, A.C., Dong, Y., Blumberg, B.J., Mlambo, G., Tripathi, A., BenMarzouk-Hidalgo, O. J., Chandra, R., Dimopoulos, G., 2014. Exploring *Anopheles* gut bacteria for *Plasmodium* blocking activity. *Environ. Microbiol.* 16, 2980–2994. <https://doi.org/10.1111/1462-2920.12381>.
- Boissière, A., Tchioffo, M.T., Bachar, D., Abate, L., Marie, A., Nsango, S.E., Shahbazkia, H.R., Awono-Ambene, P.H., Levashina, E.A., Christen, R., Morlais, I., 2012. Midgut microbiota of the malaria mosquito vector *Anopheles gambiae* and interactions with *Plasmodium falciparum* infection. *PLoS Pathog* 8, e1002742. <https://doi.org/10.1371/journal.ppat.1002742>.
- Burke, A., Dahan-Moss, Y., Duncan, F., Qwabe, B., Coetzee, M., Koekemoer, L., Brooke, B., 2019. *Anopheles parensis* contributes to residual malaria transmission in South Africa. *Malar. J.* 18, 257. <https://doi.org/10.1186/s12936-019-2889-5>.
- Burke, A., Dandolo, L., Munhenga, G., Dahan-Moss, Y., Mbokazi, F., Ngxongo, S., Coetzee, M., Koekemoer, L., Brooke, B., 2017. A new malaria vector mosquito in South Africa. *Sci. Rep.* 7, 43779. <https://doi.org/10.1038/srep43779>.
- Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: high-resolution sample inference from Illumina amplicon data. *Nat. Methods*. 13, 581–583. <https://doi.org/10.1038/nmeth.3869>.
- Capone, A., Ricci, I., Damiani, C., Mosca, M., Rossi, P., Scuppa, P., Crotti, E., Epis, S., Angeletti, M., Valzano, M., Sacchi, L., Bandi, C., Daffonchio, D., Mandrioli, M., Favia, G., 2013. Interactions between *Asaia*, *Plasmodium* and *Anopheles*: new insights into mosquito symbiosis and implications in malaria symbiotic control. *Parasit. Vectors*. 6, 182. <https://doi.org/10.1186/1756-3305-6-182>.
- Cappelli, A., Damiani, C., Mancini, M.V., Valzano, M., Rossi, P., Serrao, A., Ricci, I., Favia, G., 2019. *Asaia* activates immune genes in mosquito eliciting an anti-*Plasmodium* response: implications in malaria control. *Front. Genet.* 10, 836. <https://doi.org/10.3389/fgene.2019.00836>.
- Carnevale, P., Manguin, S., 2021. Review of issues on residual malaria transmission. *J. Infect. Dis.* 223, S61–S80. <https://doi.org/10.1093/infdis/jiab084>.
- Chen, C.-Y., Chan, W.-Y., Ismail, A., Oliver, S.V., 2024. Characterization of the tissue and strain-specific microbiota of *Anopheles funestus* Giles (Diptera: culicidae). *J. Trop. Med.* 9, 84. <https://doi.org/10.3390/tropicalmed9040084>.
- Chouaia, B., Rossi, P., Epis, S., Mosca, M., Ricci, I., Damiani, C., Ulissi, U., Crotti, E., Daffonchio, D., Bandi, C., Favia, G., 2012. Delayed larval development in *Anopheles* mosquitoes deprived of *Asaia* bacterial symbionts. *BMC Microbiol* 12, S2. <https://doi.org/10.1186/1471-2180-12-S1-S2>.
- Cirimotich, C.M., Dong, Y., Clayton, A.M., Sandiford, S.L., Souza-Neto, J.A., Mulenga, M., Dimopoulos, G., 2011. Natural microbe-mediated refractoriness to

- Plasmodium* infection in *Anopheles gambiae*. Science 332, 855–858. <https://doi.org/10.1126/science.1201618>.
- Coetzee, M., 2020. Key to the females of Afrotropical *Anopheles* mosquitoes (Diptera: culicidae). Malar. J. 19, 1–20. <https://doi.org/10.1186/s12936-020-3144-9>.
- Coetzee, M., Kruger, P., Kruger, P., Hunt, R.H., Hunt, R.H., Durrheim, D.N., Durrheim, D. N., Urbach, J., Urbach, J., Hansford, C.F., Hansford, C.F., 2013. Malaria in South Africa: 110 years of learning to control the disease. S. Afr. Med. J. 103, 770–778.
- Coon, K.L., Brown, M.R., Strand, M.R., 2016. Mosquitoes host communities of bacteria that are essential for development but vary greatly between local habitats. Mol. Ecol. 25, 5806–5826. <https://doi.org/10.1111/mec.13877>.
- Coon, K.L., Vogel, K.J., Brown, M.R., Strand, M.R., 2014. Mosquitoes rely on their gut microbiota for development. Mol. Ecol. 23, 2727–2739. <https://doi.org/10.1111/mec.12771>.
- Crotti, E., Damiani, C., Pajoro, M., Gonella, E., Rizzi, A., Ricci, I., Negri, I., Scuppa, P., Rossi, P., Ballarini, P., Raddadi, N., Marzorati, M., Sacchi, L., Clementi, E., Genchi, M., Mandrioli, M., Bandi, C., Favia, G., Alma, A., Daffonchio, D., 2009. *Asaia*, a versatile acetic acid bacterial symbiont, capable of cross-colonizing insects of phylogenetically distant genera and orders. Environ. Microbiol. 11, 3252–3264. <https://doi.org/10.1111/j.1462-2920.2009.02048.x>.
- Cuamba, N., Morgan, J.C., Irving, H., Steven, A., Wondji, C.S., 2010. High level of pyrethroid resistance in an *Anopheles funestus* population of the Chokwe district in Mozambique. PLoS one 5, e11010. <https://doi.org/10.1371/journal.pone.0011010>.
- Damiani, C., Ricci, I., Crotti, E., Rossi, P., Rizzi, A., Scuppa, P., Capone, A., Ulissi, U., Epis, S., Genchi, M., Sagnon, N., Faye, I., Kang, A., Chouaia, B., Whitehorn, C., Moussa, G.W., Mandrioli, M., Esposito, F., Sacchi, L., Bandi, C., Daffonchio, D., Favia, G., 2010. Mosquito-bacteria symbiosis: the case of *Anopheles gambiae* and *Asaia*. Microb. Ecol. 60, 644–654. <https://doi.org/10.1007/s00248-010-9704-8>.
- Damiani, C., Ricci, I., Crotti, E., Rossi, P., Rizzi, A., Scuppa, P., Esposito, F., Bandi, C., Daffonchio, D., Favia, G., 2008. Paternal transmission of symbiotic bacteria in malaria vectors. Curr. Biol. 18, R1087–R1088. <https://doi.org/10.1016/j.cub.2008.10.040>.
- De Freece, C., Damiani, C., Valzano, M., D'amelio, S., Cappelli, A., Ricci, I., Favia, G., 2014. Detection and isolation of the α -proteobacterium *Asaia* in *Culex* mosquitoes. Med. Vet. Entomol. 28, 438–442. <https://doi.org/10.1111/mve.12045>.
- Dennison, N.J., Jupatanakul, N., Dimopoulos, G., 2014. The mosquito microbiota influences vector competence for human pathogens. Curr. Opin. Insect Sci. 3, 6–13. <https://doi.org/10.1016/j.cois.2014.07.004>.
- Djouaka, R., Riveron, J.M., Yessoufou, A., Tchigossou, G., Akoton, R., Irving, H., Djegbe, I., Moutairou, K., Adeoti, R., Tamò, M., Manyong, V., Wondji, C.S., 2016. Multiple insecticide resistance in an infected population of the malaria vector *Anopheles funestus* in Benin. Parasit. Vectors. 9, 453. <https://doi.org/10.1186/s13071-016-1723-y>.
- Dong, Y., Manfredini, F., Dimopoulos, G., 2009. Implication of the mosquito midgut microbiota in the defense against malaria parasites. PLoS Pathog 5, e1000423. <https://doi.org/10.1371/journal.ppat.1000423>.
- E Silva, B., Matsena Zingoni, Z., Koekemoer, L.L., Dahan-Moss, Y.L., 2021. Microbiota identified from preserved *Anopheles*. Malar. J. 20, 230. <https://doi.org/10.1186/s12936-021-03754-7>.
- Favia, G., Ricci, I., Damiani, C., Raddadi, N., Crotti, E., Marzorati, M., Rizzi, A., Urso, R., Brusetti, L., Borin, S., Mora, D., Scuppa, P., Pasqualini, L., Clementi, E., Genchi, M., Corona, S., Negri, I., Grandi, G., Alma, A., Kramer, L., Esposito, F., Bandi, C., Sacchi, L., Daffonchio, D., 2007. Bacteria of the genus *Asaia* stably associate with *Anopheles stephensi*, an Asian malarial mosquito vector. Proc. Natl. Acad. Sci. 104, 9047–9051. <https://doi.org/10.1073/pnas.0610451104>.
- Gabrieli, P., Caccia, S., Varotto-Boccali, I., Arnoldi, I., Barbieri, G., Comandatore, F., Epis, S., 2021. Mosquito trilogy: microbiota, immunity and pathogens, and their implications for the control of disease transmission. Front. Microbiol. 12. <https://doi.org/10.3389/fmicb.2021.630438>.
- Gao, H., Cui, C., Wang, L., Jacobs-Lorena, M., Wang, S., 2020. Mosquito microbiota and implications for disease control. Trends Parasitol 36, 98–111. <https://doi.org/10.1016/j.pt.2019.12.001>.
- Garros, C., Harbach, R.E., Manguin, S., 2005. Systematics and biogeographical implications of the phylogenetic relationships between members of the *Funestus* and *Minimus* groups of *Anopheles* (Diptera: culicidae). J. Med. Entomol. 42, 7–18. <https://doi.org/10.1093/jmedent/42.1.7>.
- Gillies, M.T., Coetzee, M., 1987. A supplement to the anophelinae of Africa South of the Sahara. The South African Institute for Medical Research.
- Guégan, M., Zouache, K., Démichel, C., Minard, G., Tran Van, V., Potier, P., Mavingui, P., Valiente Moro, C., 2018. The mosquito holobiont: fresh insight into mosquito-microbiota interactions. Microbiome 6, 49. <https://doi.org/10.1186/s40168-018-0435-2>.
- Gupta, A., Nair, S., 2020. Dynamics of insect-microbiome interaction influence host and microbial symbiont. Front. Microbiol. 11, 1357. <https://doi.org/10.3389/fmicb.2020.101357>.
- Hunt, R.H., Brooke, B.D., Pillay, C., Koekemoer, L.L., Coetzee, M., 2005. Laboratory selection for and characteristics of pyrethroid resistance in the malaria vector *Anopheles funestus*. Med. Vet. Entomol. 19, 271–275. <https://doi.org/10.1111/j.1365-2915.2005.00574.x>.
- Hyde, J., Correa, M.A., Hughes, G.L., Steven, B., Brackney, D.E., 2020. Limited influence of the microbiome on the transcriptional profile of female *Aedes aegypti* mosquitoes. Sci. Rep. 10, 10880. <https://doi.org/10.1038/s41598-020-67811-y>.
- Kawada, H., Dida, G.O., Sonye, G., Njenga, S.M., Mwandawiro, C., Minakawa, N., 2012. Reconsideration of *Anopheles rivulorum* as a vector of *Plasmodium falciparum* in western Kenya: some evidence from biting time, blood preference, sporozoite positive rate, and pyrethroid resistance. Parasit. Vectors. 5, 230. <https://doi.org/10.1186/1756-3305-5-230>.
- Kim, B.-R., Shin, J., Guevarra, R.B., Lee, J.H., Kim, D.W., Seol, K.-H., Lee, J.-H., Kim, H. B., Isaacson, R.E., 2017. Deciphering diversity indices for a better understanding of microbial communities. J. Microbiol. Biotechnol. 27, 2089–2093. <https://doi.org/10.4014/jmb.1709.09027>.
- Kopya, E., Ndo, C., Djamouko-Djonkam, L., Nkahe, L., Awono-Ambene, P., Njikou, F., Wondji, C.S., Antonio-Nkondjio, C., 2021. *Anopheles lesoni* Evans 1931, a member of the *Anopheles funestus* group, is a potential malaria vector in Cameroon. Adv. Entomol. 10, 99–109. <https://doi.org/10.4236/ae.2022.101008>.
- Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 15, 550. <https://doi.org/10.1186/s13059-014-0550-8>.
- Mancini, M.V., Damiani, C., Short, S.M., Cappelli, A., Ulissi, U., Capone, A., Serrao, A., Rossi, P., Amici, A., Kalogris, C., Dimopoulos, G., Ricci, I., Favia, G., 2020. Inhibition of *Asaia* in adult mosquitoes causes male-specific mortality and diverse transcriptome changes. Pathogens 9, 380. <https://doi.org/10.3390/pathogens9050380>.
- Martinson, V.G., Strand, M.R., 2021. Diet-microbiota interactions alter mosquito development. Front. Microbiol. 12. <https://doi.org/10.3389/fmicb.2021.650743>.
- McCann, R.S., Ochomo, E., Bayoh, M.N., Vulule, J.M., Hamel, M.J., Gimnig, J.E., Hawley, W.A., Walker, E.D., 2014. Reemergence of *Anopheles funestus* as a vector of *Plasmodium falciparum* in Western Kenya after long-term implementation of insecticide-treated bed nets. Am. J. Trop. Med. Hyg. 90, 597–604. <https://doi.org/10.4269/ajtmh.13-0614>.
- McMurdie, P.J., Holmes, S., 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 8, e61217. <https://doi.org/10.1371/journal.pone.0061217>.
- Menze, B.D., Riveron, J.M., Ibrahim, S.S., Irving, H., Antonio-Nkondjio, C., Awono-Ambene, P.H., Wondji, C.S., 2016. Multiple insecticide resistance in the malaria vector *Anopheles funestus* from Northern Cameroon is mediated by metabolic resistance alongside potential target site insensitivity mutations. PLoS One 11, e0163261. <https://doi.org/10.1371/journal.pone.0163261>.
- Minard, G., Mavingui, P., Moro, C.V., 2013a. Diversity and function of bacterial microbiota in the mosquito holobiont. Parasit. Vectors. 6, 146. <https://doi.org/10.1186/1756-3305-6-146>.
- Minard, G., Tran, F.H., Raharimalala, F.N., Hellard, E., Ravelonandro, P., Mavingui, P., Valiente Moro, C., 2013b. Prevalence, genomic and metabolic profiles of *Acinetobacter* and *Asaia* associated with field-caught *Aedes albopictus* from Madagascar. FEMS Microbiol. Ecol. 83, 63–73. <https://doi.org/10.1111/j.1574-6941.2012.01455.x>.
- Morales-Jiménez, J., Zúñiga, G., Ramírez-Saad, H.C., Hernández-Rodríguez, C., 2012. Gut-associated bacteria throughout the life cycle of the bark beetle *Dendroctonus rhizophagus* Thomas and Bright (Curculionidae: scolytinae) and their cellulolytic activities. Microb. Ecol. 64, 268–278. <https://doi.org/10.1007/s00248-011-9999-0>.
- Morgan, J.C., Irving, H., Okedi, L.M., Steven, A., Wondji, C.S., 2010. Pyrethroid resistance in an *Anopheles funestus* population from Uganda. PLoS One 5, e11872. <https://doi.org/10.1371/journal.pone.0011872>.
- Ngo, C.T., Aujoulat, F., Veas, F., Jumas-Bilak, E., Manguin, S., 2015. Bacterial diversity associated with wild caught *Anopheles* mosquitoes from Dak Nong province, Vietnam using culture and DNA fingerprint. PLoS One 10, e0118634. <https://doi.org/10.1371/journal.pone.0118634>.
- Oki, M., Hoel, D.F., Kirunda, J., Rwakimari, J.B., Mpeka, B., Ambayo, D., Price, A., Ogutu, D.W., Okui, A.P., Govere, J., 2018. Insecticide resistance status of the malaria mosquitoes: *anopheles gambiae* and *Anopheles funestus* in eastern and northern Uganda. Malar. J. 17, 157. <https://doi.org/10.1186/s12936-018-2293-6>.
- Pineda-Mendoza, R.M., Zúñiga, G., López, M.F., Hidalgo-Lara, M.E., Santiago-Hernández, A., López-López, A., Orduña, F.N.R., Cano-Ramírez, C., 2022. *Rahnella* sp., a dominant symbiont of the core gut bacteriome of *Dendroctonus* species, has metabolic capacity to degrade xylan by bifunctional xylanase-ferulic acid esterase. Front. Microbiol. 13, 911269. <https://doi.org/10.3389/fmicb.2022.911269>.
- Ramos-Nino, M.E., Fitzpatrick, D.M., Eckstrom, K.M., Tighe, S., Hattaway, L.M., Hsueh, A.N., Stone, D.M., Dragon, J.A., Cheetham, S., 2020. Metagenomic analysis of *Aedes aegypti* and *Culex quinquefasciatus* mosquitoes from Grenada, West Indies. PLoS one 15, e0231047. <https://doi.org/10.1371/journal.pone.0231047>.
- Ranasinghe, K., Gunathilaka, N., Amarasinghe, D., Rodrigo, W., Udayanga, L., 2021. Diversity of midgut bacteria in larvae and females of *Aedes aegypti* and *Aedes albopictus* from Gampaha District, Sri Lanka. Parasit. Vectors. 14, 433. <https://doi.org/10.1186/s13071-021-04900-5>.
- Riveron, J.M., Osae, M., Egyir-Yawson, A., Irving, H., Ibrahim, S.S., Wondji, C.S., 2016. Multiple insecticide resistance in the major malaria vector *Anopheles funestus* in southern Ghana: implications for malaria control. Parasit. Vectors. 9, 504. <https://doi.org/10.1186/s13071-016-1787-8>.
- Romoli, O., Gendrin, M., 2018. The tripartite interactions between the mosquito, its microbiota and *Plasmodium*. Parasit. Vectors. 11, 200. <https://doi.org/10.1186/s13071-018-2784-x>.
- Singh, A., Allam, M., Kwenda, S., Khumalo, Z.T.H., Ismail, A., Oliver, S.V., 2022a. The dynamic gut microbiota of zoophilic members of the *Anopheles gambiae* complex (Diptera: culicidae). Sci Rep 12, 1495. <https://doi.org/10.1038/s41598-022-05437-y>.
- Singh, A., Patel, N.F., Allam, M., Chan, W.-Y., Mohale, T., Ismail, A., Oliver, S.V., 2022b. Marked effects of larval salt exposure on the life history and gut microbiota of the malaria vector *Anopheles merus* (Diptera: culicidae). Insects 13, 1165. <https://doi.org/10.3390/insects13121165>.
- Sinka, M.E., Bangs, M.J., Manguin, S., Coetzee, M., Mbogo, C.M., Hemingway, J., Patil, A.P., Temperley, W.H., Gething, P.W., Kabaria, C.W., Okara, R.M., Van Boeckel, T., Godfray, H.C.J., Harbach, R.E., Hay, S.I., 2010. The dominant *Anopheles* vectors of human malaria in Africa, Europe and the Middle East: occurrence data,

- distribution maps and bionomic précis. *Parasit. Vectors.* 3, 117. <https://doi.org/10.1186/1756-3305-3-117>.
- Sinka, M.E., Bangs, M.J., Manguin, S., Rubio-Palis, Y., Chareonviriyaphap, T., Coetzee, M., Mbogo, C.M., Hemingway, J., Patil, A.P., Temperley, W.H., Gething, P. W., Kabaria, C.W., Burkot, T.R., Harbach, R.E., Hay, S.I., 2012. A global map of dominant malaria vectors. *Parasit. Vectors.* 5, 69. <https://doi.org/10.1186/1756-3305-5-69>.
- Steven, B., Hyde, J., LaReau, J.C., Brackney, D.E., 2021. The axenic and gnotobiotic mosquito: emerging models for microbiome host interactions. *Front. Microbiol.* 12, 714222. <https://doi.org/10.3389/fmicb.2021.714222>.
- Tchioffo, M.T., Boissière, A., Abate, L., Nsango, S.E., Bayibéki, A.N., Awono-Ambéné, P. H., Christen, R., Gimonneau, G., Morlais, L., 2016. Dynamics of bacterial community composition in the malaria mosquito's epithelia. *Front. Microbiol.* 6, 1500. <https://doi.org/10.3389/fmicb.2015.01500>.
- Tuanudom, R., Yurayart, N., Rodkhum, C., Tiawsirisup, S., 2021. Diversity of midgut microbiota in laboratory-colonized and field-collected *Aedes albopictus* (Diptera: culicidae): a preliminary study. *Heliyon* 7. <https://doi.org/10.1016/j.heliyon.2021.e08259>.
- Villegas, L.M., Pimenta, P.F.P., 2014. Metagenomics, paratransgenesis and the *Anopheles* microbiome: a portrait of the geographical distribution of the anopheline microbiota based on a meta-analysis of reported taxa. *Mem. Inst. Oswaldo Cruz.* 109, 672–684. <https://doi.org/10.1590/0074-0276140194>.
- Vogel, K.J., Valzania, L., Coon, K.L., Brown, M.R., Strand, M.R., 2017. Transcriptome sequencing reveals large-scale changes in axenic *Aedes aegypti* larvae. *PLoS Negl. Trop. Dis.* 11, e0005273. <https://doi.org/10.1371/journal.pntd.0005273>.
- Wang, S., Dos-Santos, A.L.A., Huang, W., Liu, K.C., Oshaghi, M., Wei, G., Agre, P., Jacobs-Lorena, M., 2017. Driving mosquito refractoriness to *Plasmodium falciparum* with engineered symbiotic bacteria. *Science* 357, 1399–1402. <https://doi.org/10.1126/science.aan5478>.
- Wang, S., Ghosh, A.K., Bongio, N., Stebbings, K.A., Lampe, D.J., Jacobs-Lorena, M., 2012. Fighting malaria with engineered symbiotic bacteria from vector mosquitoes. *Proc. Natl. Acad. Sci. U.S.A.* 109, 12734–12739. <https://doi.org/10.1073/pnas.1204158109>.
- Wickham, H., 2011. ggplot2. *WIREs. Comput. Stat.* 3, 180–185. <https://doi.org/10.1002/wics.147>.
- Wilkes, T.J., Matola, Y.G., Charlwood, J.D., 1996. *Anopheles rivulorum*, a vector of human malaria in Africa. *Med. Vet. Entomol.* 10, 108–110. <https://doi.org/10.1111/j.1365-2915.1996.tb00092.x>.
- Yadav, K.K., Bora, A., Datta, S., Chandel, K., Gogoi, H.K., Prasad, G.B.K.S., Veer, V., 2015. Molecular characterization of midgut microbiota of *Aedes albopictus* and *Aedes aegypti* from Arunachal Pradesh, India. *Parasit. Vectors.* 8, 641. <https://doi.org/10.1186/s13071-015-1252-0>.