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Characterisation of the dynamic gut microbiota of members of the *Anopheles gambiae* complex

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DECLARATION

I, Ashmika Singh, declare that this Thesis Report is my own unaided work. It is being submitted for the Doctor of Philosophy degree at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

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Ashmika Singh

17 day of October 2024

DEDICATION

I dedicate this thesis to my loving parents.

Thank you for always believing in me. You have been my unwavering support and inspiration, guiding me towards becoming the person I've always dreamed of being. This is for you.

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1. Singh A, Allam M, Chan WY, Ismail A, Oliver SV. Comparison of the effect of larval metal pollutants on the gut microbiota of wild and laboratory reared *Anopheles arabiensis* (Diptera: Culicidae). Submitted to MDPI Tropical Medicine.

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ABSTRACT

The gut microbiota of mosquitoes plays a pivotal role in their life history. This includes insecticide resistance and immune responses, which makes it a potential target for vector control interventions. Although a growing body of research characterises the gut microbiota of various mosquito species, there is limited information on most members of the *Anopheles gambiae* complex. This study characterised the gut microbiota of four laboratory reared strains, namely SENN (*Anopheles arabiensis*- an insecticide susceptible major vector), SENN DDT (*Anopheles arabiensis*- an insecticide resistant major vector), MAFUS (*Anopheles merus*- minor vector) and SANGWE (*Anopheles quadriannulatus*- non-vector). The gut microbiota of fourth instar larvae, three-day old, fifteen-day old non-blood fed and fifteen-day old blood fed females was characterised and compared, bacterial identification was performed using Next-Generation Sequencing (NGS) of the bacterial 16S ribosomal RNA (rRNA) gene. The larval environment plays a role in acquiring gut microbes. This study characterised the gut microbiota of laboratory reared and wild F_1 population *An. arabiensis* adult females and how changes in the larval environment affect the dynamics of the adult gut microbiota. Additionally, the effects of heavy metals on the gut microbiota of laboratory reared and F_1 population of *An. arabiensis* adult females post-exposure to heavy metals in the larval breeding site were assessed. Furthermore, the effect of changes in the salt concentration in the larval environment on the gut microbiota of *An. merus* larvae and adults were examined. All bacterial identification of the gut microbiota was performed using NGS of the bacterial 16S rRNA. The dynamic gut mycobiota of SENN, SENN DDT and fourth instar larvae, three-day old, fifteen-day old non-blood fed and fifteen-day old blood fed laboratory reared and wild F_1 population *An. arabiensis* females were characterised. Fungal identification was performed using NGS of the fungal internal (ITS) transcribed spacer of the rRNA cistron. Distinct differences were observed between the diversity and abundance of the gut microbiota of major malaria vectors and the minor and non-vectors, with the minor and non-vector harbouring more *Plasmodium*-protective bacterial genera. Pesticide-degrading bacteria were found in insecticide susceptible and insecticide resistant strains of *An. arabiensis*, suggesting that the gut microbiota does not contribute to insecticide resistance. However, differences between these strains indicate that the diversity of the gut microbiota is affected by the insecticide resistant phenotype. The F_1 population and wild population of *An. arabiensis* had a greater gut microbiota diversity than laboratory reared *An. arabiensis* strains. Exposure to heavy metals as larvae significantly changed the abundance of gut bacteria in adults. The F_1 population and SENN DDT adults had more heavy metal degrading bacterial genera present

than SENN. Changes in the salinity of the larval environment of *An. merus* significantly affected larval development time, adult longevity, fecundity and deltamethrin tolerance. Additionally, *An. merus* adults exposed to the highest salt concentration at the larval stage had more *Plasmodium*-protective bacterial genera than the lower concentrations. The insecticide resistant phenotype of laboratory reared *An. arabiensis* affected the diversity and the differential abundance of fungi observed in the gut mycobiota. Furthermore, the gut mycobiota of the F_1 population of *An. arabiensis* was similar to that of the laboratory reared insecticide resistant strain. In conclusion, these findings have implications for using gut bacteria as a vector control intervention in South Africa. The gut microbiota of the *An. gambiae* complex is a dynamic network consisting of what seems to be a core microbiota crucial to its function. The gut microbiota of the selected members of the *An. gambiae* complex corresponds to their capacity to transmit *Plasmodium*. Furthermore, the larval environment strongly influences the dynamics of the gut microbiota of members from the *An. gambiae* complex. Therefore, the impact of the larval environment on the adult microbiota must be considered for the development and implementation of future microbial-based strategies.

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LIST OF ABBREVIATIONS

ACE	Abundance-based coverage
ANOVA	Analysis of variance
ASV	Amplicon sequence variants
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic acid
dNTP's	Deoxynucleotide triphosphates
DVS	Dominant vector species
EDTA	Ethylenediaminetetraacetic acid
FBN	Fibronectin
gDNA	Genomic deoxyribonucleic acid
GST	Glutathione S-transferase
IGS	Intergenic Spacer
imd	Immune-deficiency
IRS	Indoor residual spraying
ITS	Internal transcribed spacer

kdr	*Knockdown resistance*
LLINs	Long-lasting insecticide-treated nets
M form	Mopti form
MATC	Maximum acceptable toxic concentration
$MgCl_2$	Magnesium chloride
NGS	Next-Generation Sequencing
NICD	National institute of communicable diseases
NOS	Nitric oxide synthase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PERMANOVA	Permutational multivariate analysis of variance
PGRP	Peptidoglycan recognition protein
pH	Potential of hydrogen
PNA	Peptide-nucleic acid oligonucleotides
rDNA	Ribosomal deoxyribonucleic acid
ROS	Reactive oxygen species

rRNA	Ribosomal ribonucleic acid
S form	Savannah form
TAE	Tris-acetate-EDTA
TEP	Thioester-containing protein
Tris-Cl	Tris(hydroxymethyl)methylamine hydrochloride
WHO	World Health Organisation

CHAPTER 1

General Introduction and Literature Review

1.1 Background

1.1.1 Malaria in Africa

Malaria is a significant burden on human populations in sub-Saharan Africa and is the single most devastating vector-borne disease in the world [1]. In terms of infectious diseases, it is the third leading cause of death after HIV/AIDS and tuberculosis [2]. In 2022, the World Health Organisation (WHO) estimated there were 249 million malaria cases globally, and the estimated number of deaths was 608 000 [3]. From the global estimations, Africa accounted for approximately 93.6% of cases (233 million) and 95.4% of deaths (580 000) [3].

Malaria elimination is defined as no locally transmitted disease cases, and South Africa is one of several countries where it is considered feasible to eliminate malaria within the next decade. South Africa aimed to have zero cases of local transmission by 2018 [4]. This deadline was subsequently moved to 2020, and South Africa missed the target of eliminating malaria. Combined with the fact that the COVID-19 pandemic resulted in an increase in malaria cases and mortality, it is evident that malaria is still a consistent problem both in South Africa and Africa at large [3]. As such, further research is required to improve the understanding of both the causative parasites and the vectors that transmit them.

1.1.2 *Plasmodium* species and their development in malaria vectors

The causative agent of malaria is a unicellular protozoan parasite belonging to the genus *Plasmodium* [5]. These parasites infect humans and other vertebrates. *Plasmodium* species infecting human hosts include *Plasmodium falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi* [5]. These species of *Plasmodium* are naturally transmitted exclusively by female mosquitoes belonging to the *Anopheles* genus [5]. The life cycles of the *Plasmodium* parasites in *Anopheles* are similar [6]. For malaria to be transmitted, a female *Anopheles* mosquito must ingest a blood meal from a human host that has already been infected with the *Plasmodium* parasite. Once ingested, the parasite's gametocytes infect the midgut lumen of the mosquito, where it develops into microgametes and macrogametes [7]. The gametes then combine to

form a zygote, which differentiates to form motile ookinetes [8]. The ookinetes penetrate the midgut wall, and the oocyst forms on the outside of the midgut [9]. In the oocyst, sporozoites are formed [10]. Once the oocyst matures, it ruptures, and the sporozoites are released into the haemocoel (body cavity) and circulate through the haemolymph to the salivary glands [11]. This makes the mosquito infectious. The mosquito can transfer the parasite to a human host during its next blood meal [6,11]. The female mosquito can only be infectious if the parasite can make it through two bottlenecks in the mosquito host: the gut and salivary glands of the mosquito. The transmission of the parasite from the *Anopheles* mosquito to the human host results in the disease known as malaria [12].

Developing an efficient vaccine has been challenging as *Plasmodium* parasites are complex microorganisms [13]. The parasite takes on intracellular and extracellular forms during the multiple stages of development, and targeting just one of these developmental stages of the parasite in a human host is insufficient for an efficient vaccine [14]. In 2019, the RTS S/AS01 vaccine (Mosquirix™) was deployed and implemented in malaria-endemic areas [15]. However, the efficacy of this vaccine decreased from 56% to 36% over four years [16]. Despite this, in 2021, the WHO approved this vaccine for widespread use. As reviewed by El-Moamly and El-Sweify [17], this vaccine cannot stand alone in effectively reducing malaria [17]. As such, multi-component vaccines are needed to eradicate malaria. Therefore, there is still a need for alternative strategies to eliminate malaria. One such preventative method includes limiting the contact between the vectors and humans, known as vector control [18]. Vector control methods remain at the forefront of controlling malaria transmission and understanding the vectors' biology is crucial for consistently efficient implementation and advancements.

1.1.3 Malaria vectors in Southern Africa and vector control strategies

Malaria control in sub-Saharan Africa is challenged by a complex vectorial system consisting of some of the world's most efficient vectors [19–21]. This includes members of the *An. gambiae* complex, a group of morphologically indistinguishable species that differ in behaviour and vectorial capacity [18]. *Anopheles gambiae* is one of the most efficient malaria vectors in the world. It is a dominant malaria vector species and the nominal member of this complex [22]. Other dominant vector species from the *An. gambiae* complex in Africa includes *An. arabiensis, An. coluzzii, An. melas, An. merus,* and *An. bwambae* [18,23,24].

The members of the *An. gambiae* complex present in South Africa are *An. arabiensis*, *An. merus*, and *An. quadriannulatus* [25]. However, it is worth noting that *An. quadriannulatus* is not considered a malaria vector [26]. Unlike *An. gambiae sensu stricto* (*s.s*) and *An. coluzzii*, which typically exhibit endophilic (indoor-resting) and endophagic (indoor-biting) behaviours, the members of the *An. gambiae* complex found in South Africa are predominantly exophilic (outdoor-resting) and exophagic (outdoor-biting) [18]. Although variable, *An. arabiensis*, *An. merus* and *An. quadriannulatus* primarily feed on animals (zoophagy) rather than humans [18].

Anopheles arabiensis is a significant malaria vector in South Africa [25]. Unlike *An. gambiae s.s.* that predominantly feed and rest indoors, *An. arabiensis* can bite and rest both indoors and outdoors [27]. This versatility makes it a species of concern as it can contribute to the persistence of low-level malaria transmission, known as residual malaria [28]. This is especially true in areas where outdoor biting and resting are common [28]. The behavioural plasticity of both *An. arabiensis* and *An. merus* contributes to the challenges faced when implementing conventional vector control methods.

Conventional vector control methods include long-lasting insecticide-treated nets (LLINs) and indoor residual spraying of insecticides (IRS) [29], which are interventions that reduce malaria transmission by only targeting indoor biting and resting vectors [29]. Insecticide-based vector control methods are further hampered by increasing insecticide resistance in malaria vectors [30]. In particular, global resistance to pyrethroid and organochlorine insecticides has been recorded at 69.9 and 63.4% in at least one malaria vector in sites examined in 64 countries globally [30]. Although vector control is a vital tool for malaria control, the limited availability of new tools is a current challenge. This is particularly true for conventional chemical interventions as insecticide resistance of *Anopheles* vectors directly impacts vector control methods, reducing the efficacy of chemical-based methods. In parts of South Africa, *An. arabiensis* populations (80-97%) have low-level resistance to dichlorodiphenyltrichloroethane (DDT), permethrin, deltamethrin, and bendiocarb [31]. The incidence of resistance to insecticides and the behavioural adaptations of *An. arabiensis* in response to vector control interventions threatens South Africa's malaria elimination agenda. Consequently, alternate targets for vector control interventions are required.

One such target is the mosquito immune system, which has been recognised as a crucial factor that influences the susceptibility of the mosquito to *Plasmodium* infection [32]. This is

necessary to establish the parasite within the mosquito host and transmit it to a human host. Therefore, modulation of the mosquito immune system can alter vector competence, and the physiological ability to become infected and transmit a pathogen [33]. As reviewed by Beerntsen *et al*. [34], vector competence is a component of vectorial capacity, a complex multifactorial process dependent on the interplay between the environment, mosquito behaviour, fitness, and innate immunological factors that influence the efficiency of the vector to transmit a pathogen [34]. The mosquito gut microbiota can modulate the immune system of the mosquito [32].

1.1.4 The role of the gut microbiota in mosquito life history

The terms microbiome and microbiota are often used interchangeably but have different implications. The term "microbiome" is defined as the collection of microbial genomes in an environment [35,36]. The microbiome is comprised of distinct microbial communities, including archaea, bacteria, viruses, protozoans, and fungi (which will be discussed in detail in **CHAPTER 5**), as well as their genomes and the surrounding environmental conditions [35,37,38]. The term "microbiota" refers to "the characteristic microbial community in a reasonably well-defined habitat which has distinct physio-chemical properties" [38]. These microorganisms inhabit the mosquito gut, and together, they constitute the gut microbiota of the mosquito [39].

The larval gut microbiota is suggested to be acquired through the larval environment and maternal transmission pathways, as reviewed by Strand [40]. Larvae acquire their gut microbiota directly from their aquatic environment as it was observed that no gut bacteria was present in first instar larvae that hatched from surface-sterilised eggs [41]. Furthermore, mosquitoes contain gut bacteria found in their breeding site [42,43]. Strand [40] reviewed that larvae can also acquire gut bacteria through vertical transmission, meaning that gut bacteria in larvae is acquired from the mother [40]. The microbiota in the female reproductive tract can be transferred to the outer surface of eggs and consequently into the larval aquatic environment, where the larvae will ingest it [41,42,44–48].

The acquired gut microbiota plays a vital role in the life history traits of mosquito larvae [41,49]. One study demonstrated that *An. stephensi* larvae in antibiotic-treated water have stunted growth [49]. This could be reversed with the treatment of antibiotic-resistant bacteria [49]. This highlights the importance of the microbiota on larval growth and development.

Additionally, *An*. *gambiae* and *Ae*. *aegypti* larvae grown in sterile conditions resulted in the absence of gut microbiota and prevented the transition from pupae to adults [41]. This means that gut microbiota is essential in the development of larvae into adults.

After emerging as adults, mosquitoes are suggested to acquire their gut microbiota by ingesting the water from larval habitats during emergence [41,50,51]. Larvae may transstadially transmit a portion of their gut microbiota to adults during metamorphosis [41,50,51]. As the adult mosquito progresses through its lifespan, the composition of its gut microbiota continues to change. Additional microbes can be acquired from various sources such as water, nectar, and blood meals [50,52,53]. The adult gut microbiota has been implicated in many life history traits that are of epidemiological importance. These traits include digestion, nutrient absorption, reproduction, and adult longevity [39,54–58].

Mosquitoes require nutrients from sugar, which are obtained through carbohydrate sources such as floral and extrafloral nectar [59,60]. The mosquito requires the gut microbiota to aid in digesting sugar meals consumed. For example, male and female *Ae*. *albopictus* had increased activity of *Enterobacteriaceae* in their gut 24 hours after ingesting labelled fructose [61]. This suggested that bacteria belonging to the family *Enterobacteriaceae* contributed to the digestion of fructose in *Ae*. *albopictus* [61]. Furthermore, genes associated with carbohydrate metabolism in active bacteria were identified. This indicated that the mosquito uses fructose from a sugar meal, such as nectar, as an energy source to provide essential nutrients [61].

Female *Anopheles* are anautogenous, meaning they depend on blood meals from human or animal hosts to produce all their egg batches [62,63]. The gut microbiota is also involved in the digestion of blood meals. A study conducted on laboratory reared *Ae*. *aegypti* females demonstrated that antibiotic treatment affected the lysis of red blood cells, delayed the digestion of blood proteins, and decreased egg production [64]. Therefore, it has been suggested that gut bacteria release lytic enzymes that aid digestion [64]. As seen in *Ae*. *aegypti*, there is a selective pressure for haemolytic bacteria such as *Enterobacter* and *Serratia*, which are known to assist in the blood digestion process [64]. As reviewed by Gao *et al*. [65], nutrients absorbed and synthesised by the microbiota during blood meals are also essential for reproduction [65]. Therefore, the gut microbiota involved in digestion may affect reproduction in mosquitoes. Studies have demonstrated that *An*. *stephensi* adults had reduced female fertility and reproductive capacity when antibiotics were used to sterilise the gut [66].

Additionally, native gut bacteria in *Aedes* are needed for egg production [67]. Furthermore, a core microbiota consisting of endosymbiotic bacteria was observed in *An. coluzzii* and *An. gambiae* female and male reproductive tracts [44]. This emphasises the role of native gut microbiota in reproduction.

Studies have suggested that there is a correlation between the longevity of the adult mosquito and the gut microbiota [66,68–73]. It was noted that the life span of *An. stephensi* was shortened when the gut microbiota was sterilised with antibiotic treatment [66]. This was consistent with a similar study conducted on laboratory reared *An. coluzzii* and *An. gambiae*, where the lifespan of the adult decreased with antibiotic administration via blood meal [68]. The ingestion of a blood meal by wild *An. arabiensis* silenced genes involved in midgut homeostasis, resulting in a reduced lifespan [69]. This is because they could not cope with the drastic increases in bacteria ingested directly after a blood meal [69]. However, laboratory reared *An. gambiae* had an increased lifespan when a blood meal from malaria-infected people containing antibiotics was ingested [70]. Furthermore, native gut bacteria such as *Enterobacter* sp. *Zambiae* (*Esp_Z*), *Acinetobacter* sp., and *Serratia marcescens* administered through blood-feeding, significantly decreased the longevity of laboratory reared *An. gambiae* [71,72]. The lifespan of mosquitoes is a crucial aspect of vector biology. The vector must live long enough for the parasite to develop and be transmitted to a new host. As such, small changes in longevity significantly affect malaria transmission [73].

1.1.5 The role of the gut microbiota in mosquito immunity

In the gut, commensal bacteria are involved in host immunity and elicit a protective response towards pathogens [74]. These bacteria are recognised as essential regulators in the life history of various invertebrate organisms, such as mosquitoes [74]. The gut microbiota is implicated in mosquito immunity towards multiple pathogens, including viruses [75] and parasites [76]. The gut is the first bottleneck of *Plasmodium* development, making it a mandatory path to successful infection and transmission of *Plasmodium* in Anophelines [77].

The role of gut microbiota in parasite development makes it a potential target for manipulation. Therefore, the possible involvement of the gut microbiota in successful parasite development is an interesting target for examination. The gut microbiota interacts with the mosquito's immune system by modulating direct or indirect immune responses to pathogens, as reviewed by Romoli and Gendrin [77]. The microbiota has been implicated as a critical

determinant in the transmission of *Plasmodium* and is, therefore, one of the contributing factors to vector competence [41,78]. Microbiota-mediated interactions inhibit the development of pathogens, like *Plasmodium*, by eliciting numerous immune responses [65].

There are a range of direct immunological responses to *Plasmodium* induced by the gut microbiota [79–81]. The first is forming a physical barrier protecting the gut from direct contact with the blood meal [79]. Bacterial metabolites also mediate the immune system. These include reactive oxygen species (ROS) and nitric oxide (NO) [80]. Gram-negative bacteria participate in synthesising the peritrophic matrix, a layer consisting of chitin and other proteins that form rapidly in response to ingesting a blood meal [81]. This matrix protects the mosquito as a physical barrier to protect the gut from a blood meal [79]. This is because a blood meal is a heat shock for the mosquito and potentially has harmful host proteins [82]. A byproduct of the formation of the peritrophic matrix is that it vastly reduces the penetration of the parasite into the midgut wall [83]. This layer functions in mosquito immunity by exerting selective pressure on the *Plasmodium* parasite by creating a barrier in the midgut wall, making it harder, but not impossible, for *Plasmodium* ookinetes to penetrate [77].

Bacteria from the gut can disrupt *Plasmodium* infection in the mosquito by producing metabolites, enzymes, and antimicrobial molecules [72,80,84–86]. For example, *Esp_Z*, a Gram-negative bacterial isolate from the gut of Zambian *An. arabiensis* was co-fed with *P. falciparum* to *An. gambiae* resulted in stunted *Plasmodium* development as a reduction in ookinete, oocyst, and sporozoite loads was observed [80]. This was suggested to be due to the bacterially generated ROS [80]. Another metabolite suggested to be effective against the parasite is red pigment prodigiosin, this is produced by bacteria in the gut and interferes with *Plasmodium* development [84]. In addition, certain bacterial isolates from the midgut, such as some *S. marcescens* strains and *Chromobacterium* sp. *Panama* (*Csp_P*), can produce metabolites with anti-parasitic activity, which reduce *Plasmodium* infection in *An. gambiae* [72,85]. Lastly, the gut microbiota uses nitric oxide synthase (NOS) to catalyse the conversion of L-arginine to L-citrulline, releasing nitric oxide (NO) as a byproduct [86]. Nitric oxide is cytotoxic and limits the development of *Plasmodium* in the mosquito [86].

The immune response to the *Plasmodium* parasite includes activating multiple immune pathways, including the immune-deficiency (*imd*), Toll, and JAK/STAT pathways [41,70,78,87]. The bacteria in the gut can activate the NF-κB dependent *imd* pathway to fight

Plasmodium infection [88,89]. This pathway is induced when peptidoglycan in the bacterial cell wall is recognised by its specific receptor, peptidoglycan recognition protein (PGRP) LC [89]. The *imd* pathway is involved in anti-bacterial protection against invading Gram-negative and Gram-positive bacteria in *An*. *gambiae* [88–90]. As reviewed by Dennison *et al*. [91], the gut microbiota is responsible for the PGRP-LC-mediated anti-*Plasmodium* immune response [91]. This results in an increased expression of immune-associated genes, such as thioester-containing protein (*TEP*) 1, that participate in the microbiota-dependent control of *Plasmodium* [92]. Bacteria such as *Serratia marcescens* Y1, an isolate from the midgut of wild *An*. *sinensis* inhibited *Plasmodium* development by modulating genes such as *TEP*1 and fibronectin (FBN) 9 [93]. The *imd* pathway is the most significant immunological pathway in the defence against *P*. *falciparum* [94].

1.1.6 The role of the gut microbiota in insecticide resistance of the mosquito

Insecticide resistance is a phenotype constructed by a range of factors. The first of these is behavioural avoidance of the pesticide due to the mosquitoes' feeding and resting behaviours [95]. The two classical methods of constructing the phenotype are target site resistance and metabolic detoxification tolerance [96–98]. Target site resistance is the modification of molecules targeted by the insecticide such that the xenobiotic no longer exerts the neurotoxic effect. A typical example of a target site mutation is the knockdown resistance (*kdr*) mutation [98,99]. This mutation in the voltage-gated sodium channel prevents DDT and pyrethroids from resulting in a continuous influx of sodium into the cell, preventing continuous firing of the action potential [98,99]. As reviewed by Hemingway and Ranson [100], metabolic detoxification is the increased activity of detoxification enzymes that modify, hydrolyse or sequester xenobiotics [100]. These actions are most commonly catalysed by members of the Cytochrome P450, general esterase and Glutathione S-transferase (GST) families. This phenotype has a range of pleiotropic effects, from fitness costs to increased stress tolerance [96,97].

Despite a general understanding of the construction of the resistance phenotype, ongoing discoveries reveal new mechanisms contributing to the expression of insecticide resistance. One of the latest contributors is the microbiota [101]. There is evidence that the microbiota may play a role in the insecticide resistance phenotype [102]. Pesticide-degrading bacteria have been observed in organophosphate resistant *An*. *stephensi* when compared to their susceptible counterparts [102]. Furthermore, pyrethroid exposure has also been shown to alter

the cuticle surface and internal bacterial communities of *An. Stephensi* [103]. Despite this, when examining the effects of commensal bacteria on life history, the insecticide resistance phenotype is not often considered. Insecticide resistance affects how mosquitoes respond to starvation, oxidative stress, blood meals and thermal stress [104–107]. Therefore, it is crucial to include this factor when examining the role of gut microbiota in the life history of vector mosquitoes.

1.1.7 Factors influencing the gut microbiota in mosquitoes

If the gut microbiota affects insecticide resistance, other factors influencing the mosquito gut microbiota need to be considered. For example, the aqueous environment of the larvae, the transition into adulthood, and the food sources of female mosquitoes affect the gut microbiota [65]. These factors are important to consider as they potentially affect mosquito fitness and vector competence.

The gut microbiota of larvae is essential throughout the mosquito's life span [41,87,108]. It is crucial for their development into adulthood, as several studies have demonstrated that gut-sterilised mosquitoes cannot pupate [41,87,108]. Larvae eat and drink what is available in the surrounding water. It has been suggested that the diet they obtain from the environment is essential for the survival and replacement of new bacteria in their gut [78,109,110]. Members of the *An. gambiae* complex are known to breed in clear, sunlit temporary pools [27]. However, there have been reports of members of the *An. gambiae* complex larvae breeding in polluted water [111–114]. *Anopheles arabiensis* is associated with two sectors that are significant sources of environmental pollution, namely, agricultural activities and urbanisation [115,116]. The adaptation of larvae to breed in such polluted waters has several potential biological effects on mosquitoes. The impact of environmental stressors, including heavy metal pollutants and osmotic stress, on the gut microbiota is yet to be investigated.

During development, the gut microbiota of larvae is replaced with a different set of microbes as it transitions into an adult [117]. This coincides with a change from an aquatic to aerial lifestyle [108]. Therefore, a comprehensive study of both the larval and adult stages is essential for a holistic view of the dynamics of the gut microbiota. Furthermore, the diet of the adult female mosquito needs to be considered. There is a change in the microbiota when the feeding habits of the female mosquito shift from only a sugar source, which includes nectar from flowers [118], to include both sugar meals and blood meals. As an example, when

female *An. gambiae* ingests a blood meal, there is a decrease in the bacterial diversity and an increased presence of *Enterobacteriaceae* in the gut microbiota [108]. Such shifts in the gut microbiota may affect mosquito fitness.

1.1.8 The gut microbiota of mosquitoes as a target for novel vector control strategies

The gut is one of the critical bottlenecks for parasite development, and parasite escape at this site is a hallmark of vector competence [119]. Therefore, modulating and exploiting the gut microbiota is a potential target site for establishing novel vector control strategies for efficient transmission blocking of *Plasmodium*. Consequently, microbes colonising the mosquito are regarded as possible tools to curb the spread of malaria. A microbial-based strategy for vector control is advantageous as it can target several species of mosquitoes and *Plasmodium* at the same time [40].

A potential transmission-blocking strategy is paratransgenesis. This is a strategy whereby unmodified native symbiotic microbes or genetically modified versions are introduced into the mosquito host. Unmodified symbionts would need to be directly associated with protection against *Plasmodium* [120]. These symbionts then express effector molecules to act against the parasite, making the mosquito resistant to infection by pathogens. For example, *Esp_Z* is an unmodified isolate from the gut of Zambian *An. arabiensis* and is involved in the inhibition of *Plasmodium* [71]. *Enterobacter* sp. was isolated from the gut of Zambian *An. gambiae*, this bacterium directly impacted *Plasmodium* by producing ROS [80]. Furthermore, *Microsporidia MB* is a spore-forming unicellular parasite related to fungi, it is native to *An. arabiensis* and has protective properties against *Plasmodium* [121]. These examples of candidates for paratransgenesis are ideal as they are unmodified native symbionts from *Anopheles* species. Therefore, it is unlikely that other native symbionts would interfere with the colonisation of the mosquito.

Paratransgenesis as a novel vector control strategy for malaria has already been tested in mosquito species such as *An. gambiae* and *An. stephensi* [122]. Bacterial genera in the mosquito gut have been identified as prospective microbial agents for paratransgenesis as they have been suggested to decrease vector competence by either affecting longevity, eliciting an immune response, or directly impacting the *Plasmodium* parasite by producing anti-*Plasmodium* effector molecules [120,122]. This strategy is appealing as microbes are readily attainable from the environment, and it bypasses additional ethical and regulatory approvals

associated with transgenic mosquito releases. *Wolbachia* is an intracellular bacterium commonly found in insects. As reviewed by Wilke and Marelli [120], *Wolbachia* is an example of a well-studied endosymbiont that can inhibit the transmission of human pathogens by mosquitoes [120]. In laboratory reared *An. stephensi*, *Wolbachia* provided the mosquito with increased protection against *P. falciparum* infection [123]. However, while this bacterium has been observed in the reproductive tissues of West African populations of *An. gambiae* and *An. coluzzi*, where it is vertically transmissible, *Wolbachia* infections in *An. stephensi* and *An. gambiae* were only accomplished when the gut microbiota was sterilised with antibiotics [46,123,124]. Additionally, bacterial competition between *Asaia* and *Wolbachia* is suggested to inhibit the vertical transmission and colonisation of *Wolbachia* in *Anopheles* mosquitoes [125]. Therefore, it is necessary to have clear catalogues of the microbiota and symbiotic bacteria in *Anopheles* species.

A comprehensive catalogue of the microbiota and symbiotic bacteria present in local vector populations is necessary to explore potential paratransgenesis candidates. However, such a record is currently absent for South African vector species. Although mosquito microbiome studies are advancing, the studies tend to be limited in the species studied, typically focussing on *An. stephensi*, *An. albimanus* and *An. gambiae* [52,126,127]. Studies of the microbiome of *An. arabiensis* and other members of the *An. gambiae* complex is notably lacking. This project intended to provide additional information on potential candidates for paratransgenesis by characterising the dynamic gut microbiota of dominant vector species from the *An. gambiae* complex and to determine the effect the larval environment has on the microbiota. This would provide better insight and more knowledge surrounding the mosquito gut microbiota as a strategy to develop novel vector control interventions.

1.2 Aims

This study aims to characterise the dynamic gut microbiota of select members of the *Anopheles gambiae* complex and the gut mycobiota of *An. arabiensis*. Furthermore, this study aims to provide an understanding of the role of the environment in shaping the microbial community of the mosquito gut and to characterise the subsequent effects on the mosquito's life history.

1.3 Objectives

1. To characterise the dynamic gut microbiota of laboratory reared *An. merus*, *An. quadriannulatus* and *An. arabiensis* at different life stages, including fourth instar larvae, three-day old adults, fifteen-day old non-blood fed adults, and fifteen-day old blood fed adults, by identifying bacteria in the midgut with Next-Generation Sequencing (NGS) of the bacterial 16S ribosomal RNA (rRNA) gene.

2. To characterise and compare the dynamic gut microbiota of three-day old laboratory reared, a first filial (F_1) population, and a wild population (larvae collected from the field) of *An. arabiensis* by identifying bacteria in the midgut with NGS of the bacterial 16S rRNA gene.

3. To investigate the effect of larval exposure to heavy metals on the gut microbiota of three-day old laboratory reared and the F_1 population of *An. arabiensis* by identifying bacteria in the midgut with NGS of the bacterial 16S rRNA gene.

4. To characterise the dynamic gut mycobiota (yeasts and moulds) of laboratory reared and the F_1 population of *An. arabiensis* at different life stages, including fourth instar larvae, three-day old adults, fifteen-day old non-blood fed adults, and fifteen-day old blood fed adults, by identifying yeasts and moulds in the midgut with NGS of the fungal internal transcribed spacer 1 (ITS1) region of the rRNA cistron.

5. To investigate the effects of varying saltwater concentrations on larval development, adult longevity, fertility, fecundity, insecticide tolerance and gut microbiota of laboratory reared *An. merus*.

CHAPTER 2

General Materials and Methods

2.1 Mosquito Collection and Preparation

2.1.1 Laboratory reared *Anopheles* species/strains and their phenotypes

In this study, four strains representing three mosquito species were used. An insecticide susceptible strain of *An. arabiensis*, known as SENN and originating from Sennar, Sudan, has been used and maintained in a colony since 1980 [128]. Secondly, an insecticide resistant strain of *An. arabiensis*, referred to as SENN DDT, was also utilised. This strain has been continuously exposed to the insecticide dichlorodiphenyltrichloroethane (DDT) since 1995 and is resistant to other insecticides including permethrin, deltamethrin, λ-cyhalothrin and malathion [104,107,129]. This strain carries the knockdown resistance (*kdr*) L1014S mutation [130] and exhibits elevated cytochrome P450, general esterase, and glutathione S-transferase (GST) activity, contributing to resistance against various insecticides [104,107]. Additionally, the study included an insecticide-susceptible strain of *An. quadriannulatus*, known as SANGWE, originating from Zimbabwe and has been maintained in a colony since 1998 [131]. Lastly, an insecticide-tolerant strain of *An. merus*, referred to as MAFUS, was used. This strain was colonised from Limpopo, South Africa, in 2012 and has been continuously reared in 50% seawater (17.5g of sodium chloride per litre of water) [132,133].

2.1.2 Insectary conditions for laboratory reared *Anopheles* mosquitoes

All laboratory reared *Anopheles* mosquitoes were housed in the Botha de Meillon insectary of the National Institute of Communicable Disease (NICD), Johannesburg, South Africa. All larvae were reared in reverse osmosis water at 25˚C (±2˚C) and 80% relative humidity (±5%) with a 12-hour light/dark cycle and a 30-minute dusk/dawn cycle[134]. Larvae were fed a mixture of powdered Beano^{TM} dog biscuits and Brewer's yeast (3:1). Adult mosquitoes were placed in cages with *ad libitum* access to a 10% (w/v) sucrose solution.

2.1.3 Sample details of A*nopheles* specimens and blood feeding protocol

Various life stages of *Anopheles* were sampled, including fourth instar larvae, three-day old non-blood fed adults, fifteen-day old non-blood fed adults and fifteen-day old blood fed

adults. Only female *Anopheles* adults were used in this study because they are the only ones capable of transmitting the *Plasmodium* parasite, as they require a blood meal to lay eggs [62]. Therefore, female mosquitoes are of epidemiological significance in this study. Blood feeding took place at three, seven and eleven days old. Blood was supplied by a single human volunteer (SVO). The ethical clearance waiver approving blood meals was provided by the University of the Witwatersrand (**Appendix 1: Ethics Waiver**).

2.1.4 Midgut dissection and DNA extraction

All equipment, surfaces, and specimens were sterilised with 70% (v/v) ethanol and 3% (v/v) hydrogen peroxide (H_2O_2). The midguts from fourth instar larvae and female adult mosquitoes were dissected using a dissection microscope (Olympus, SZ2-ILST) with a magnification of 40×. The midguts were then suspended in 200 µL of 0.1M phosphate buffered saline (PBS) (pH 7.2). Depending on specimen availability as many replicates as possible were made. Each replicate consisted of three to five midguts, depending on the extraction kit used. Total genomic DNA (gDNA) from the mosquito's midgut was extracted using the DNeasy PowerSoil® Kit (QIAGEN: 12888-50) or the Qiagen DNeasy Blood & Tissue kit (Cat no. 69506). ZymoBIOMICS^{TM} Microbial Community Standard (Cat no. D3300) was used as a positive control, and PBS served as a no template control.

2.2 Next-Generation Sequencing of the gut microbiota

Bacteria from the mosquito gut were identified using Next-Generation Sequencing (NGS) of the bacterial 16S ribosomal RNA (rRNA). This technique is a gold standard in bacterial identification as it can identify bacteria that cannot be identified using culturomics [135,136]. The 16S rRNA genes are essential and highly conserved across bacterial species. This gene occurs at least once in the genome of prokaryotes, making this a good candidate for taxonomic classification [136]. The 16S rRNA gene contains nine hypervariable regions that are 1600 base pairs (bp) long [135]. The full length of the 16S rRNA gene cannot be sequenced on the Illumina platform, as it only allows for single-end and paired-end reads (2x300 bp reads). Therefore, an insert size of approximately 550 bp or smaller is recommended [135]. Therefore, the V3-V4 hypervariable region, approximately 443 bp long, was selected to sequence on the Illumina platform [135]. Unique sequences in the V3-V4 region can be used to identify different bacterial species [135,136].

2.2.1 Preparation of the 16S rDNA library

The 16S Metagenomic Sequencing Library Preparation (Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq® System) [137] and the NICD Sequencing Core Facility Laboratory Manual (NIC 1094v6) [138] workflows were followed to obtain the 16S rDNA library.

The V3-V4 hypervariable region of the bacterial 16S rRNA gene was amplified by Polymerase Chain Reaction (PCR) from the extracted DNA samples. Each PCR reaction set-up contained the forward primer (1 μM), the reverse primer (1 μM), Roche KAPA HiFi HotStart ReadyMix (2X)(Cat no. KK2601) and extracted gDNA (5 ng/μL). To ensure no contamination of the kit, a no template control reaction was set up, and PBS was used as a negative control. The ZymoBIOMICS™ Microbial Community DNA Standard (Cat no. D6306) was used as a positive PCR control. The PCR reaction was set up using the following reagents: Primers specific to the V3-V4 region of the 16S rRNA gene (4 nmol Ultramer® DNA Oligo 55 bases; supplied by Integrated DNA Technologies, Coralville, IA, USA) [139] were used: 1 μM Amplicon PCR Forward Primer (5' GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C 3')(Cat no. 74775360) and 1 μM Amplicon PCR Reverse Primer (5' TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG 3')(Cat no. 74775361)[139]. Roche 2x KAPA HiFi HotStart ReadyMix contained KAPA HiFi HotStart DNA polymerase (0.5 U per 25 μL reaction) in a propriety reaction buffer, which contained magnesium chloride ($MgCl_2$)(2.5 mM at 1X), and deoxynucleotide triphosphates (dNTP's)(0.3 mM of each dNTP at 1X). To this, 2.5 μL of 5 ng/μL of extracted gDNA was added.

The bacterial 16S rRNA gene was then amplified using the T100™ thermal cycler (Bio-rad) under the following cycling conditions: One cycle of 95 ºC for three minutes, followed by 25 cycles of 95 ºC for 30 seconds, 55 ºC for 30 seconds and 72 ºC for 30 seconds and finally one cycle of 72 ºC for five minutes. The size of the PCR amplicon was then visualised by agarose gel electrophoresis. A 2.5% (w/v) Tris-acetate-EDTA (TAE) agarose gel was electrophoresed at 120V for 90 minutes. The amplicon size was visualised using the molecular imager® ChemiDoc™ XRS+ with Image Lab™ software. Amplicons were only selected for further downstream analysis if a band of approximately 550 bp was observed.

2.2.2 PCR amplicon clean-up and PCR indexing

Beckman Coulter Agencourt® AMPure® XP beads (Cat no. A63882) were used to purify the amplicons. Per the manufacturer's instructions, AMPure XP beads were used at a ratio of 0.75:1 for the first PCR clean-up. This was done to purify the 16S rRNA V3-V4 amplicons and remove excess solution, free primers, nucleotides, salts, enzymes, primer dimers, and protein contaminants [138].

The amplicons within each replicate were labelled with a unique index. This ensured that each amplicon was identified after the samples were pooled. Amplicons were labelled with the Illumina Nextera® DNA Flex Index Primer Set (Cat no. 20018705)(**Appendix 3: Nextera® Index Adapter Sequences**). Each PCR reaction set-up contained PCR grade water, KAPA HiFi HotStart Ready Mix (2X), Illumina Nextera® DNA Flex Index Primer Set and the purified PCR amplicon [137]. The PCR indexing was performed using the following cycling conditions: One cycle of 95ºC for three minutes, followed by twelve cycles of 95ºC for thirty seconds, 55ºC for thirty seconds and 72ºC for 30 seconds and finally, one cycle of 72ºC for five minutes. Free index primers were removed with a second PCR clean-up. As previously described, the Agencourt® AMPure® XP PCR purification method was used to remove excess index primers[138].

After purification, library validation of the concentration and size of the indexed amplicons were assessed. The DNA concentrations of the samples were measured with a Qubit® Fluorometer 2.0 (Invitrogen) and the Invitrogen Qubit dsDNA HS Assay Kit (Cat no. Q32854) as per manufacturer's instructions described in the NICD Sequencing Core Facility Laboratory Manual [138]. The size of the index amplicons, which were approximately 630 bp after indexing, were visualised using Agilent Technologies D5000 Assay reagents (Cat no. 5067-5588) and the Bioanalyser 4200 TapeStation (Agilent Technologies) as described in the NICD Sequencing Core Facility Laboratory Manual [138].

2.2.3 Sample multiplexing (DNA library normalisation, pooling, denaturation, and dilution)

The purified DNA library samples were normalised to a concentration of 2 nM using dilution buffer (10 mM Tris-Cl, pH 8.5, containing 0.1% Tween 20 (v/v)). This buffer reduced the adherence of DNA to plastic surfaces. The calculated amount of dilution buffer added to each

sample for normalisation depended on the concentration reading collected from the Qubit library validation step. All normalised DNA samples were then pooled based on sequencing requirements. The instructions for pooled samples were constructed and given by the head of the NICD Core Sequencing Facility. A pooled DNA library was created from the individual normalised DNA samples by adding each sample to a single microcentrifuge tube.

Following normalisation, the DNA quality control was prepared. This included the gBlocks® Gene Fragment library and 10nM PhiX Control v3 library. These were added to the final sample pool, which was ready for loading into the Reagent Cartridge. Before the final dilution, DNA quality controls were prepared. This included gBlocks® Gene Fragment library (4 ng/µL) diluted to a 2 nM solution and 20 pM PhiX. The pooled DNA library was then denatured with a 0.2 N NaOH working solution (1:1). The solution was left to incubate for five minutes at room temperature to allow the double-stranded pooled DNA to denature into single-stranded DNA. The HT1 Hybridisation buffer was added, resulting in a 20 pM denatured DNA library.

The denatured DNA library was then further diluted with a hybridisation buffer, resulting in a 6 pM denatured library. The final library reaction included a 5% PhiX control spike-in (Illumina) before the library was run on the MiSeq® sequencing platform, as it served as an internal control for low-diversity libraries obtained during 16S metagenomic studies. Illumina recommends using MiSeq® v3 reagent kits for improved run metrics. The total diluted denatured DNA library was loaded into the Illumina MiSeq® Reagent Kit v3 (Catalogue no. 15043895) per the manufacturer's instructions. Samples were sequenced at the NICD Core Sequencing Facility using the Illumina MiSeq® Instrument (Illumina) with 2 x 300 bp read chemistry.

2.2.4 Bioinformatic analysis

The paired-end MiSeq® Illumina reads (2 x 300 bp) were pre-processed using the DADA2 package (v1.12.1) [140], including quality inspection, trimming, dereplication, merging paired-end reads and removal of chimeric sequences. The quality profiles of the forward reads, followed by the reverse reads, were assessed. The sequences were then filtered and trimmed, where the forward reads were truncated by ten nucleotides from position 240 and reverse reads were truncated at position 160. The error rates were then assessed, followed by sample inference of the filtered and trimmed sequence data. Complete denoised sequences

were then obtained by merging the paired reads. The amplicon sequence variant (ASV) table was then constructed, and chimaeras were removed. Taxonomy was assigned to the obtained ASVs. The ASV abundance estimates were determined using training sequence sets based on the SILVA reference database (version 138.1; https://zenodo.org/record/1172783#.XvCmtkUzY2w). A phyloseq object was then prepared for further downstream analysis of the data. Bacterial diversity analysis was done using raw reads, which were quality controlled and filtered (Q>20 and length >50 bp) using fastqc (v0.11.8) and trimGalore (v0.6.4 dev; https://github.com/FelixKrueger/TrimGalore), respectively [141,142]. In addition, trimGalore was used to remove the adapter.

All the downstream analyses were performed in RStudio (v3.6.1) [143], including classification, abundance estimations, statistical analysis, and visualisation. Ordinations for beta diversity, abundance bar plots, alpha diversity and richness estimates, and heatmaps were generated using the phyloseq package (v1.28.0) [144], ggplot2 (v3.2.1) [145] and AmpVis2 package (v2.6.4) [146]. To determine the α-diversity (within-sample diversity) [147], the Chao1 and Abundance-based Coverage Estimator (ACE) [148–152], Shannon-Weiner, and Simpson's dominance indices [153–156] were assessed. Kruskal-Wallis rank-sum tests were then used to compare the α-diversity between groups [157]. To determine the β-diversity (between-sample diversity) [147], data clustering in a nonmetric multidimensional scaling (NMDS) [158] plot was assessed using permutational analysis of variance (PERMANOVA) [159] (permutation test with pseudo F ratios) as implemented in the adonis function in the vegan package (https://github.com/vegandevs/vegan). Venn diagrams were generated using VennDiagram (v1.6.20) and UpsetR (v1.4.0) [160]. Differential abundance analysis between sample groups was performed using DESeq2 (v1.24.0) [161].

CHAPTER 3

Characterisation of the dynamic gut microbiota of members of the *Anopheles gambiae* complex

The data for this chapter was published at https://doi.org/10.1038/s41598-022-05437-y. This manuscript can be found in **Appendix 2: Manuscripts.**

3.1 Introduction

The *Anopheles gambiae* complex consists of several members. Initially, this included six members: *An. gambiae sensu stricto* (*s.s.*) Giles, *An. arabiensis* Patton, *An. bwambae* White, *An. melas* Theobald, *An. quadriannulatus* Theobald, and *An. merus* Dönitz [23,162–165]. *Anopheles gambiae s.s.* was classified into two molecular forms: Savannah (S) and Mopti (M) [166]. In 2013, the M form of *An. gambiae* was formally given the name *An. coluzzii*, while the S form kept the name *An. gambiae* Giles [167]. Two additional species, *An. comorensis* and *An. quadriannulatus* species B were later added to the *An. gambiae* complex [168,169]. However, *An. quadriannulatus* species B has been formally assigned with the new name *An. amharicus* [167]. In the *An. gambiae* complex *An. gambiae*, *An. coluzzii* and *An. arabiensis* are dominant vector species (DVS) and are considered major malaria vectors [18,23,24]. *Anopheles melas*, *An. merus*, and *An. bwambae* are also dominant vector species. However, they are less efficient in transmitting malaria than the other dominant vector species and are considered minor vectors [18,23]. *Anopheles quadriannulatus* and *An. amharicus* are considered non-vector species [18,23,24,167,169].

Anopheles quadriannulatus is a non-vector species [170]. They are predominantly zoophilic (prefer feeding on animals) and exophilic (outdoor resting) [170]. However, adult populations have been observed resting indoors [171] and have shown the potential to feed on humans [172]. This species has not yet been implicated in malaria transmission but has been demonstrated only to be susceptible to *Plasmodium falciparum* when force-fed infected blood in a laboratory setting [173]. Additionally, *An. quadriannulatus* in KwaZulu Natal, South Africa, has shown some resistance to the insecticide dichlorodiphenyltrichloroethane (DDT); this is suggested to be from selected pressure during the larval stage [174].

Anopheles merus has not yet been implicated in malaria transmission in South Africa [18,24]. As reviewed by Bartilol [175], this species has been implicated in the localised transmission of malaria along the Eastern and Southern coasts of Africa and is, therefore, considered a minor vector species [175]. It has been recorded that this species feeds on humans and cattle and rests indoors and outdoors [176]. This species is of concern as there have been reports of increased geographical range and population densities throughout the Eastern and Southern African coasts [177–180]. Due to this, there is an increase in their potential to play a more significant role in malaria transmission [177–180]. Furthermore, *An. merus* in Mpumalanga, South Africa, has exhibited a 97% mortality rate to the insecticide DDT, indicating the presence of resistance within the population [181].

Anopheles arabiensis is considered a major malaria vector and a DVS responsible for widespread malaria transmission in sub-Saharan Africa [18]. These mosquitoes feed on humans but have a higher affinity for feeding on animals [182,183]. This species is highly opportunistic in its feeding and resting behaviours. They exhibit a high degree of behavioural plasticity, with reports indicating adaptations in behaviour to suit the host's location [18,182,184,185]. This highly adaptable species is also a major driver of the increasing levels of urban malaria in sub-Saharan Africa [18,184]. The efficacy of conventional vector controls for controlling the DVS population and reducing malaria transmission is highly dependent on the feeding and resting behaviour of the mosquito. Furthermore, insecticide resistance in *An. arabiensis* is rapidly increasing throughout Africa, thereby decreasing the effectiveness of insecticide-based control interventions used to control the population [31,186–189]. Conventional vector control methods such as indoor residual spraying (IRS) and long-lasting insecticidal nets (LLINs) are implemented indoors. Hence, only insecticide susceptible vector species that feed and rest indoors are efficiently targeted [29]. Due to the insecticide resistance and behavioural flexibility of *An. arabiensis* populations, which feed and rest both indoors and outdoors, these populations are difficult to control [29]. As such, novel vector control strategies targeting these outdoor biting and resting populations are needed. Furthermore, with insecticide resistance being as prevalent as it is, these strategies must account for increasing levels of insecticide resistance and ideally be a non-insecticidal intervention.

The immune system is a crucial determinant of vector competence, making it an attractive target for alternative strategies [34]. Altering the immune system would block transmission, increasing the population's refractoriness to the parasite. This can be examined by comparing

vectors' immune systems to those of non-vectors to gain insights into these dynamics. This is possible with members of the *An. gambiae* complex which includes major malaria vectors, minor malaria vectors, and non-vectors. For example, the prophenoloxidase system is crucial in the defence of *P. falciparum*, as it is initiated at a lower stimulating threshold for non-vector *An. quadriannulatus* than the major malaria vector *An. gambiae* [190]. This demonstrates the immune response of the non-vector, *An. quadriannulatus* is more efficient than that of the major malaria vector species. As such, specific aspects of the immunological response could be exploited for transmission.

The mosquito immune system can be affected by insecticide resistance as there is a potential interplay between insecticide resistance and the mosquito immune system [191,192]. However, it is unclear whether insecticide resistance increases or decreases vector competence. As reviewed by Juache-Villagrana *et al*. [193] and Suh *et al*. [194], it has been suggested that insecticide exposure possibly contributes to a potential increase in vector competence [193,194]. Although there is contradicting evidence, more studies show that insecticide resistant *Anopheles* are more susceptible to *Plasmodium* [195–197]. This is highly variable by the type of effect measure (parasite prevalence vs parasite intensity), and the effect differs depending on the insecticide resistance mechanism [194].

The gut microbiota of Anophelines has a vital role in the immune response to the *Plasmodium* parasite, thereby influencing their vector competence, as reviewed by Gabrieli *et al*. [54]. As such, this study included members of the *An. gambiae* complex with varying vector competencies. The gut microbiota of insecticide susceptible and resistant populations in the wild have been shown to differ [64,102]. This has been demonstrated in both *An. albimanus* and *An. gambiae* [102,103,198]. Notably, fenitrothion resistant populations of *An. albimanus* had a reduced bacterial diversity, with selection for pesticide-degrading bacterial species [102]. Similarly, insecticide resistant *An. gambiae* had an increased abundance of permethrin-degrading taxa [198]. Furthermore, exposure to insecticides can alter internal and external microbial communities [103]. As such, this study included insecticide susceptible and resistant strains of *An. arabiensis*. These strains have already been demonstrated to be differentially responsive to bacterial supplementation as ingestion of bacteria altered the resistant phenotype [199]. Given the gut microbiota's role in modulating immunity and its interplay with insecticide resistance, the impact of insecticide resistance is a crucial aspect to consider when examining the gut microbiota for paratransgenesis purposes.

For this study, the gut microbiota of three members of the *An. gambiae* complex prevalent in South Africa was assessed. This included laboratory reared *An. arabiensis*, *An. merus* and *An. quadriannulatus*. These species were chosen based on their vector competence as a direct comparison of the gut microbiota of a major, a minor, and a non-malaria vector species has not yet been reported. Additionally, the gut microbiota of an insecticide susceptible and resistant laboratory reared strains of *An. arabiensis* was compared to evaluate the effect of the insecticide resistance phenotype on the gut microbiota.

3.2 Materials and Methods

The characterisation of the gut microbiota between selected members of the *An. gambiae* complex was conducted as described in **CHAPTER 2 General Materials and Methods**. The members included an insecticide susceptible strain of *An. arabiensis* (SENN), an insecticide resistant strain of *An. arabiensis* (SENN DDT), an insecticide tolerant strain of *An. merus* (MAFUS), and an insecticide susceptible *An. quadriannulatus* (SANGWE) details on each strain are provided in **2.1.1 Laboratory reared *Anopheles* species/strains and their phenotypes**. These mosquitoes were collected and maintained as described in **2.1.2 Insectary conditions for laboratory reared *Anopheles* mosquitoes**. The gut microbiota was characterised at different life stages, including fourth instar larvae, three-day old adults, fifteen-day old non-blood fed adults, and fifteen-day old blood-fed adults. Blood feeding was conducted as described in **2.1.3 Sample details of *Anopheles* specimens and blood feeding protocol**. Three replicates consisting of five guts for each life stage and strain were prepared. The guts from the respective samples were dissected, and the total genomic DNA from the mosquito's midgut was extracted using the DNeasy PowerSoil® Kit (QIAGEN: 12888-50) as described in **2.1.4 Midgut dissection and DNA extraction**. The gut microbiota of the samples were then identified as described in **2.2 Next-Generation Sequencing of the gut microbiota**.

3.3 Results

Comparisons of the gut microbiota between selected members of the *An. gambiae* complex at different life stages

3.3.1 α-diversity of the gut microbiota

The α-diversity (within-sample diversity) [147] was assessed for each laboratory reared strain from the *An. gambiae* complex for all life stages. Boxplots were used to visualise and compare differences in the α-diversity between the insecticide susceptible strain of *An. arabiensis* (SENN), insecticide resistant strain of *An. arabiensis* (SENN DDT), *An. merus* (MAFUS), and *An. quadriannulatus* (SANGWE). Significant differences between the indices were assessed using a Kruskal-Wallis rank-sum test [157]. The Chao1 and Abundance-based Coverage Estimator (ACE) diversity indices were used as estimators of species richness by considering the number of bacterial species in the gut [148–152]. Shannon-Weiner and Simpson's dominance indices were used as estimators of species richness and evenness by considering the number of different bacterial species and the relative abundance of each species [153–156].

The Chao1 index was significantly higher in the insecticide susceptible strain SENN compared to the insecticide resistant strain SENN DDT ($p = 0.01$), SANGWE ($p = 0.01$) and MAFUS ($p < 0.01$) (**Figure 3.1 A**). There were no significant differences in the Chao1 indices between SENN DDT and MAFUS ($p = 0.67$), SENN DDT and SANGWE ($p = 0.93$) or between MAFUS and SANGWE ($p = 0.07$) (**Figure 3.1 A**).

Similarly, the ACE index was significantly higher in SENN than in SENN DDT ($p = 0.02$) and SANGWE ($p = 0.02$) (**Figure 3.1 B**). However, no significant differences were observed in the ACE indices between SENN and SANGWE ($p = 0.05$). Additionally, there were no significant differences in the ACE indices between SENN DDT and MAFUS ($p = 0.80$), SENN DDT and SANGWE ($p = 0.14$), or between MAFUS and SANGWE ($p = 0.13$) (**Figure 3.1 B**).

The Shannon-Weiner index was significantly higher in both SANGWE ($p = 0.03$) and MAFUS ($p = 0.02$) compared to SENN (**Figure 3.1 C**). There were no significant differences between SENN and SENN DDT ($p = 0.44$), SENN DDT and MAFUS ($p = 0.76$), SENN DDT and SANGWE ($p = 0.71$), or between SANGWE and MAFUS ($p = 0.44$) (**Figure 3.1 C**).

The Simpson's dominance index was significantly higher in MAFUS compared to SENN ($p < 0.01$) (**Figure 3.1 D**). There were no significant differences between SENN and SENN DDT ($p = 0.51$), SENN and SANGWE ($p = 0.06$), SENN DDT and MAFUS ($p = 0.38$), SENN DDT and SANGWE ($p = 0.80$), or between SANGWE and MAFUS ($p = 0.51$) (**Figure 3.1 D**).

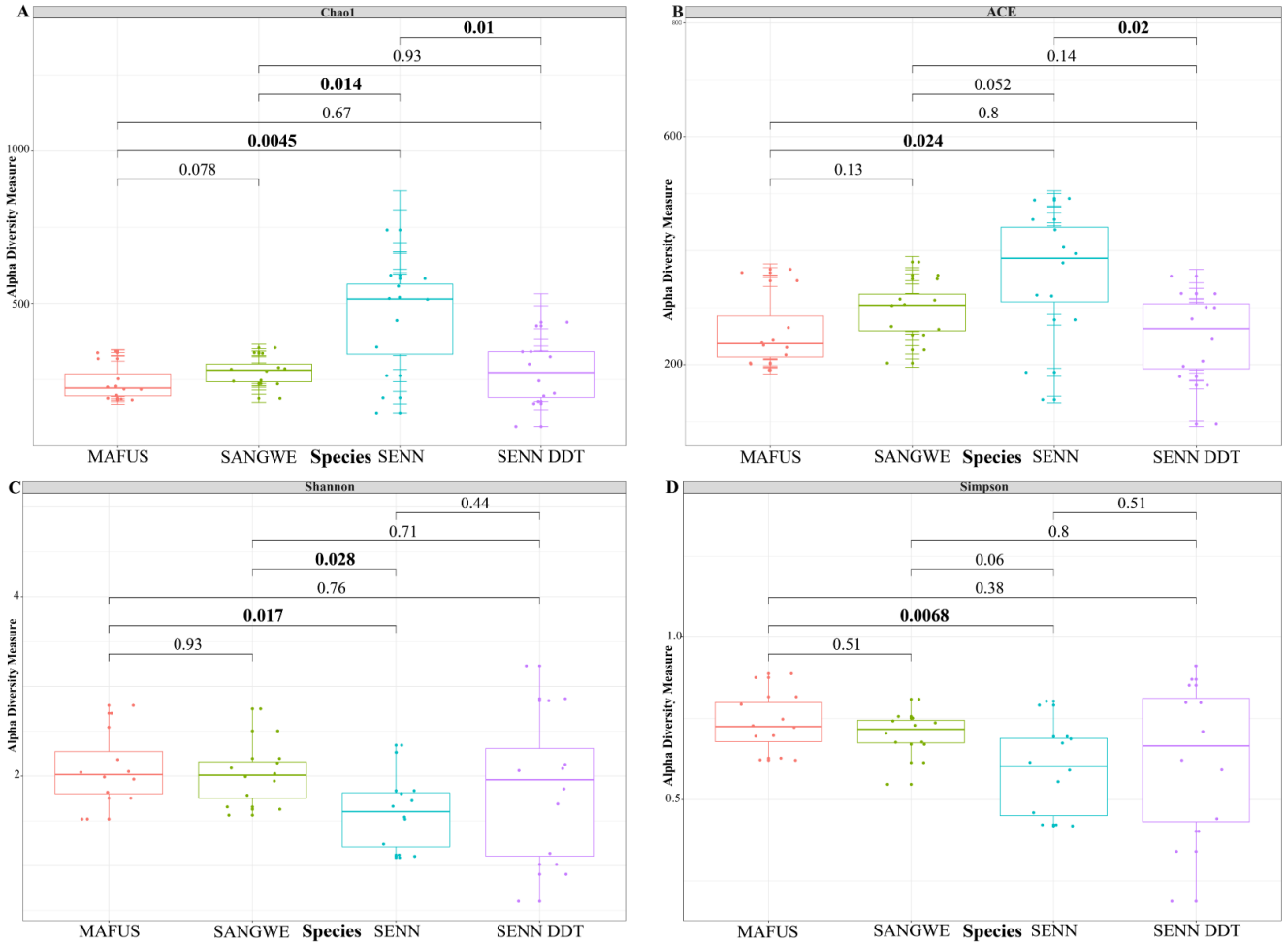

Figure 3.1 Comparison of the α-diversity of gut microbiota between selected zoophilic members of the *Anopheles gambiae* complex for all life stages.

(A) Chao1 diversity indices. **(B)** ACE diversity indices. **(C)** Shannon-Weiner diversity indices. **(D)** Simpson's dominance indices. Comparisons of the diversity indices between the laboratory reared strains were assessed using a Kruskal-Wallis rank-sum test. The dots represent the number of samples used for each strain. Significant differences in the diversity indices between samples are indicated by bold print.

The α-diversity was assessed for each life stage. Boxplots were used to visualise and compare differences in the α-diversity between the fourth instar larvae, three-day old adults, fifteen-day old non-blood fed adults, and fifteen-day old blood fed adults. Significant differences between the indices were assessed using a Kruskal-Wallis rank-sum test.

The Chao1 index of fourth instar larvae was significantly higher than that of the fifteen-day old non-blood ($p < 0.01$) and blood fed adults ($p = 0.02$) (**Figure 3.2 A**). No significant differences in the Chao1 indices were observed between the fourth instar larvae and three-day old adults ($p = 0.06$), three-day old adults and fifteen-day old non-blood fed ($p = 0.71$) and blood fed adults ($p = 0.84$), and fifteen-day old non-blood fed and blood fed adults ($p = 0.71$) (**Figure 3.2 A**).

The ACE index of fourth instar larvae was significantly higher than that of the three-day old adults ($p = 0.02$), fifteen-day old non-blood ($p < 0.01$), and blood fed adults ($p < 0.01$) (**Figure 3.2 A**). There were no significant differences in the ACE indices between three-day old adults and fifteen-day old non-blood fed ($p = 0.71$) and blood fed adults ($p = 0.89$), and fifteen-day old non-blood fed and blood fed adults ($p = 0.63$) (**Figure 3.2 A**).

The Shannon-Weiner index of fourth instar larvae was significantly higher than that of the three-day old adults ($p < 0.01$), fifteen-day old non-blood ($p < 0.01$), and blood fed adults ($p < 0.01$) (**Figure 3.2 A**). There were no significant differences in the Shannon-Weiner indices between three-day old adults and fifteen-day old non-blood fed ($p = 0.71$) and blood fed adults ($p = 0.67$), and fifteen-day old non-blood fed and blood fed adults ($p = 0.41$) (**Figure 3.2 A**).

The Simpson's dominance index of fourth instar larvae was significantly higher than that of the three-day old adults ($p < 0.01$), fifteen-day old non-blood ($p < 0.01$), and blood fed adults ($p < 0.01$) (**Figure 3.2 A**). There were no significant differences in the Simpson's dominance indices between three-day old adults and fifteen-day old non-blood fed ($p = 0.59$) and blood fed adults ($p = 0.10$), and fifteen-day old non-blood fed and blood fed adults ($p = 0.93$) (**Figure 3.2 A**).

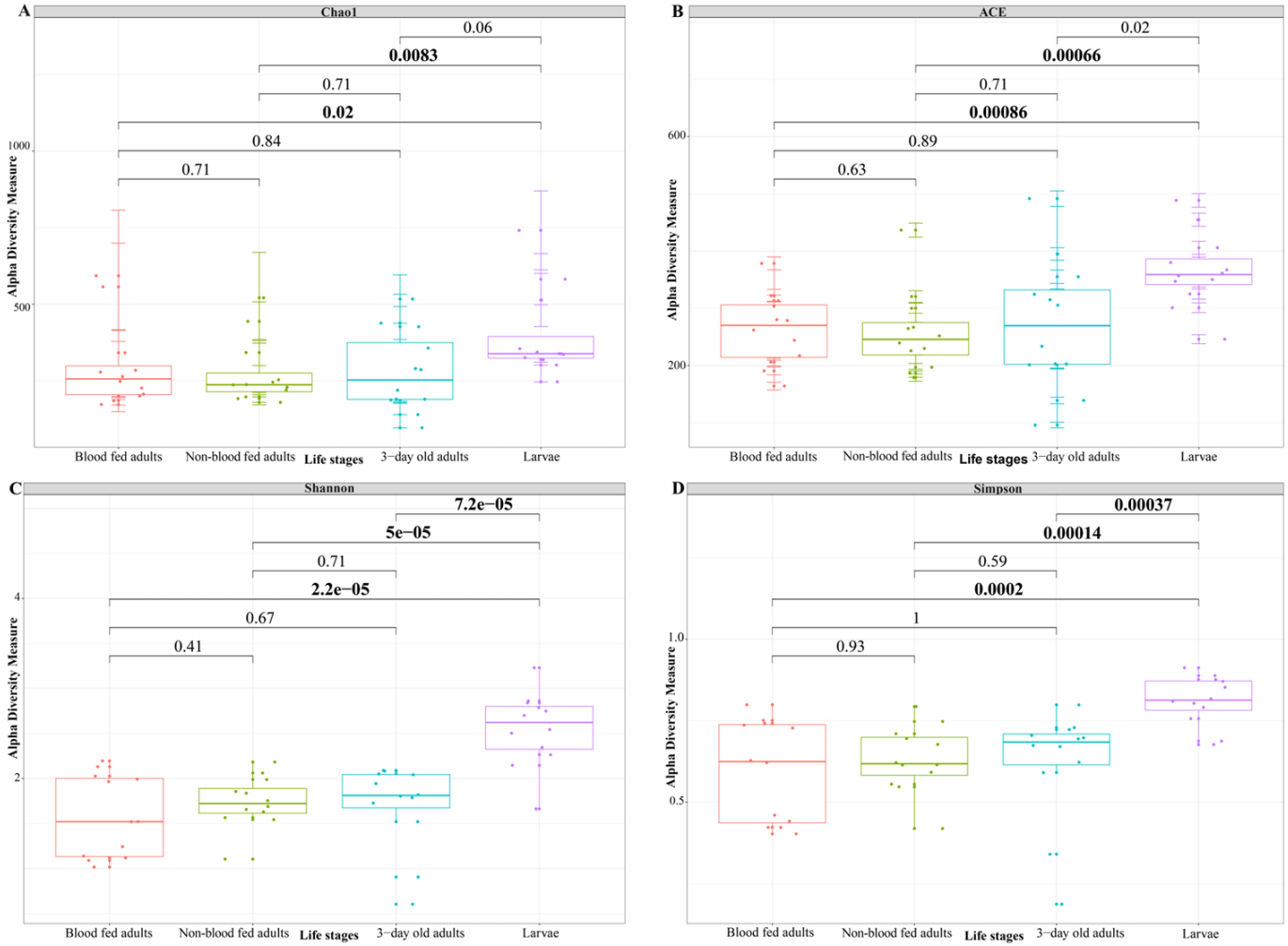

Figure 3.2 Comparison of the α-diversity of gut microbiota between zoophilic members of the *Anopheles gambiae* complex at different life stages.

(A) Chao1 diversity indices. **(B)** ACE diversity indices. **(C)** Shannon-Weiner diversity indices. **(D)** Simpson's dominance indices. Comparisons of the diversity indices between the life stages were assessed using a Kruskal-Wallis rank-sum test. The dots represent the number of samples used for each life stage. Significant differences in the diversity indices between samples are indicated by bold print.

3.3.2 β-diversity of the gut microbiota

The β-diversity (between-sample diversity) [147] of gut bacteria between the different strains, SENN, SENN DDT, MAFUS, and SANGWE, was visualised using non-metric dimensional scaling (NMDS) ordination for each life stage. The differences in the bacterial communities of the gut were assessed using the Bray-Curtis dissimilarity distances. Permutational multivariate analysis (PEMANOVA) was used to determine if there were any significant differences in the bacterial diversity between the selected members of the *An. gambiae* complex.

The diversity of gut microbiota of MAFUS and SANGWE, the minor and non-malaria vector species respectively, overlapped and clustered closely. Similarly, the insecticide resistant and susceptible strains of the major malaria vector *An. arabiensis* overlapped and clustered closely. Bray-Curtis dissimilarity measure showed that the bacterial diversity of the minor and non-malaria vectors (MAFUS and SANGWE) was significantly different from the insecticide susceptible and resistant major malaria vectors (SENN and SENN DDT) (PERMANOVA; p = 0.01, F = 6.59, stress value = 0.13) (**Figure 3.3** Differences in the diversity of gut microbiota between selected members of the Anopheles gambiae complex and their respective life stages.).

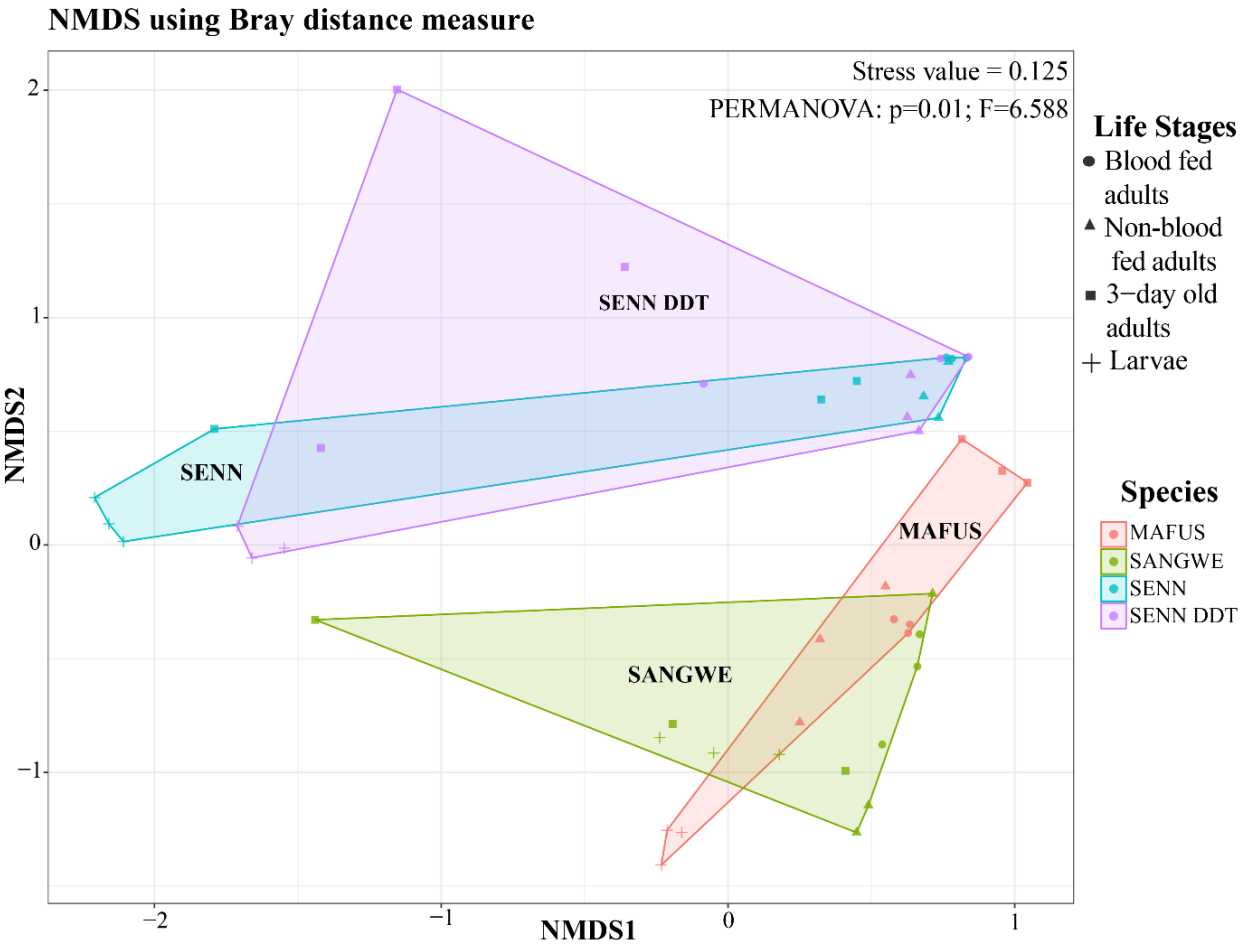

Figure 3.3 Differences in the diversity of gut microbiota between selected members of the *Anopheles gambiae* complex and their respective life stages.

A visual representation of the β-diversity analysis of the gut bacteria between laboratory reared strains of selected members of the *An. gambiae* complex. The different colours represent the different laboratory reared members of the *An. gambiae* complex, insecticide susceptible strain of *An. arabiensis* (SENN) as blue, insecticide resistant strain of *An. arabiensis* (SENN DDT) as purple, *An. merus* (MAFUS) as red, and *An. quadriannulatus* (SANGWE) as green. The various shapes indicate the different life stages: crosses represent larvae, squares represent three-day-old adults, triangles represent fifteen-day old non-blood fed adults, and circles represent fifteen-day old blood fed adults. A PERMANOVA (p = 0.01) assessed significant differences between strains.

3.3.3 Overlapping bacterial communities

The shared and unique bacteria between the selected members of the *An. gambiae* complex was represented numerically on UpSet plots. The number of shared and unique bacterial families are shown in **Figure 3.4 A**, genera in **Figure 3.4 B**, and species in **Figure 3.4 C**. Bacterial communities that were identified until family, genus, and species level are listed below. Note that not all bacterial communities were identified to the respective taxonomic levels, as this is a limitation of 16S rRNA gene sequencing [200–202]. Therefore, the identified bacterial communities may not correspond to the numbers below.

The gut microbiota of all members of the *An. gambiae* complex had seventeen bacterial families in common: *Enterobacteriaceae, Weeksellaceae*, *Sphingobacteriaceae, Sphingomonadaceae*, *Rhizobiaceae, Microbacteriaceae*, *Aeromonadaceae*, *Acetobacteraceae*, *Burkholderiaceae, Chitinophagaceae, Moraxellaceae*, *Kaistiaceae*, *Xanthobacteraceae*, *Beijerinckiaceae,* and *Mycobacteriaceae*. The insecticide susceptible and resistant *An. arabiensis* strains shared six bacterial families: *Chromobacteriaceae*, *Nocardiaceae, Micrococcaceae, Bdellovibrionaceae,* and *Caulobacteraceae*. The minor and non-vector species MAFUS and SANGWE had *Staphylococcaceae* in common (**Figure 3.4 A**).

Examination of the bacterial genera between the four strains of mosquitoes showed that there were thirty-three genera in common: *Rahnella, Enterobacter, Elizabethkingia*, *Klebsiella*, *Sphingobacterium*, *Cedecea*, *Pantoea*, *Microbacterium*, *Aeromonas*, *Asaia*, *Comamonas*, *Leucobacter*, *Serratia*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Kluyvera*, *Acinetobacter*, *Hafnia-Obesumbacterium*, *Kaistia Pedobacter*, *Citrus maxima*, *Gluconobacter*, *Raoultella*, *Salmonella*, *Shimwellia*, *Delftia*, *Yersinia*, *Bosea*, *Morganella*, *Ancylobacter*, *Mycobacterium*, two uncultured bacterium and ambiguous taxa (**Figure 3.4 B**).

The insecticide susceptible and resistant *An. arabiensis* (SENN and SENN DDT) had fourteen shared bacterial genera, including *Aquitalea, Rhodococcus*, *Flectobacillus*, *Zoogloea*, *Phreatobacter, Rhodoferax*, *Arthrobacter*, *Bdellovibrio*, *Caulobacter*, *Cloacibacterium*, *Xanthobacter, Mucilaginibacter,* and *Asinibacterium*. Four genera were shared between MAFUS and SANGWE: *Sphingopyxis, Mesorhizobium*, *Escherichia-Shigella,* and *Staphylococcus* (**Figure 3.4 B**).

Each of the four mosquito strains had unique bacterial genera identified in their gut. SENN had *Terrimicrobium,* SENN DDT had *Asticcacaulis*, *Azospirillum*, *Bradyrhizobium*, and *Roseomonas*. MAFUS had *Achromobacter*, *Taibaiella*, *Leifsonia, and Acetobacter*. SANGWE had *Calothrix* PCC-6303 and *Paenibacillus* (**Figure 3.4 C**).

Thirty-four bacterial species were shared between all four mosquito strains; this included *Rahnella aquatilis*, *Klebsiella pneumoniae*, *Microbacterium laevaniformans*, *uncultured Comamonas* sp., *Enterobacter* sp., *Rahnella* sp. SYC84, *Hafnia alvei*, *Rahnella* sp. SYC7, uncultured *Klebsiella* sp., bacterium 37(2011), uncultured *Klebsiella* sp., *Salmonella enterica*, uncultured *Rahnella* sp., *Nubsella* sp. EsD18, bacterium BM0238, *Citrus maxima*, *Gluconobacter cerinus*, uncultured *Raoultella* sp., *Rahnella* sp. UYFA288, uncultured gamma *proteobacterium*, *Yersinia pestis* 2944, *Enterobacter lignolyticus*, uncultured bacterium, *Rhizobium* sp., *Mycobacterium aubagnense*, *Serratia* sp. mNW20, and *Aeromonas hydrophila* subsp. *hydrophila* (**Figure 3.4 C**)

There were eleven shared bacterial species between SENN and SENN DDT: *Rhodococcus fascians*, *Albidiferax* sp. HME6627, *Flectobacillus* sp. BAB-3569, *Rhodococcus* sp. H-CA8f, *Sphingobacterium*-like sp. PC1.9, *Asaia krungthepensis*, and *Rhodococcus* sp. SA4. Seventeen bacterial species were shared between MAFUS and SANGWE: *Pseudomonas* sp., uncultured Rhizobiales *bacterium*, *Serratia marcescens* WW4, *Pseudomonas* sp. SB15-Ch27-6, *Pseudomonas veronii*, *Enterobacter asburiae*, *Pseudomonas fluorescens*, uncultured *Pseudomonas* sp., *Pseudomonas* sp. KOPRI80188, *Pseudomonas marginalis*, *Enterobacter* sp. BR3362, *Enterobacter asburiae*, *Escherichia coli*, *Pseudomonas* sp. HHS29, uncultured organism, *Enterobacter cloacae* subsp. *cloacae* ATCC 13047, and bacterium L194WBS.90 (**Figure 3.4 C**).

The strains SENN DDT, MAFUS and SANGWE had unique bacterial species identified in their gut. Ten unique gut bacteria species were identified in MAFUS: *Achromobacter* sp., *Leifsonia shinshuensis*, bacterium N155G.102, *Stenotrophomonas rhizophila*, *Leucobacter chironomi*, *Leclercia adecarboxylata*, *Enterobacter cloacae*, *Enterobacter* sp. P4562, and *Klebsiella aerogenes*. Three unique bacterial species were found in the gut of SENN DDT: *Asaia* sp., Perchlorate-reducing bacteria enrichment culture clone yan09, and *Azospirillum* sp. B506. Four unique bacterial species found in SANGWE: Calothrix sp. NQAIF310, *Paenibacillus cavernae*, methanotrophic bacterium MG1, and bacterium 10RO2 (**Figure 3.4 C**).

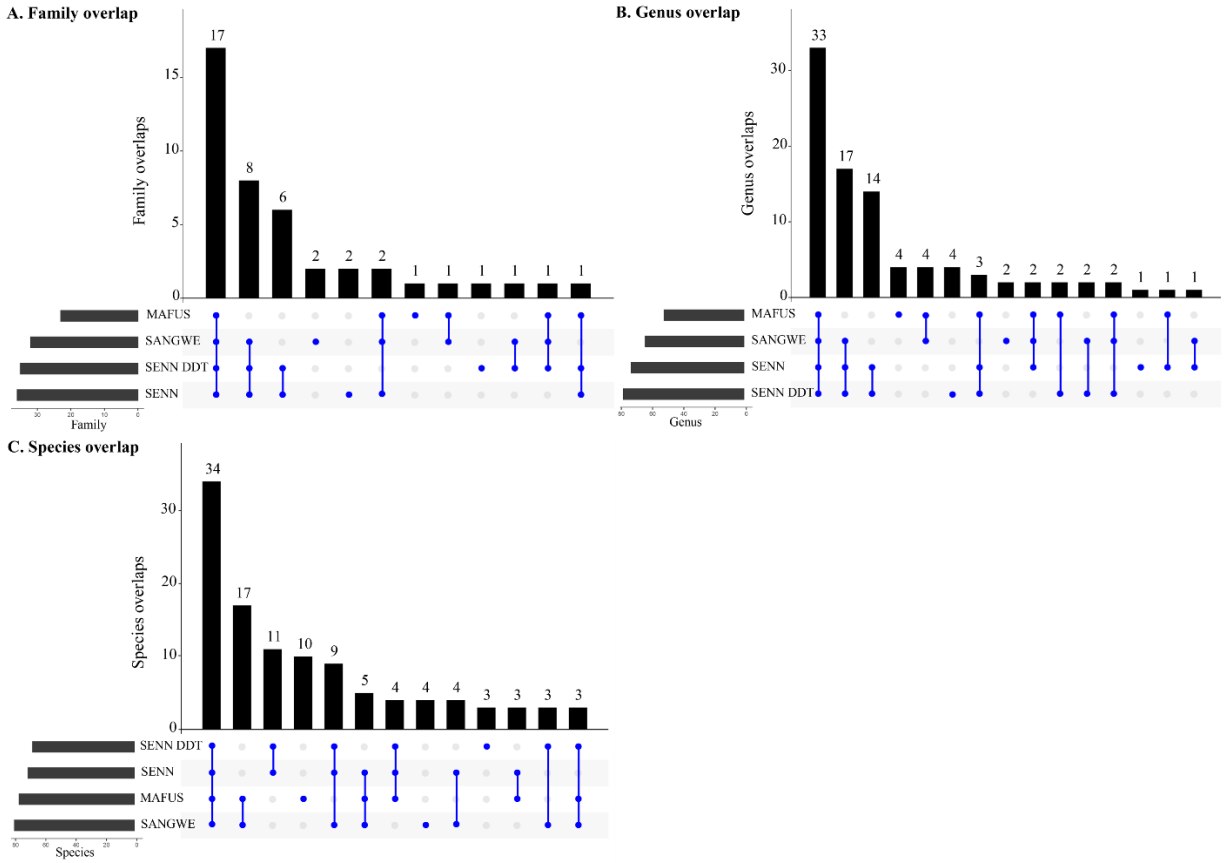

Figure 3.4 Comparison of overlapping gut bacteria between selected members of the *Anopheles gambiae* complex for all life stages.

(A) Comparison of shared bacterial families between SENN, SENN DDT, MAFUS, and SANGWE. **(B)** Comparison of shared bacterial genera between SENN, SENN DDT, MAFUS, and SANGWE. **(C)** Comparison of shared bacterial species between SENN, SENN DDT, MAFUS, and SANGWE. The blue dots with a joined line represent the overlaps of bacteria between the selected members of the *An. gambiae* complex, with single dots indicating the unique bacteria per strain. Each numbered column represents the number of bacterial families **(A)**, genera **(B)**, or species **(C)** that overlap.

3.3.4 Differential abundance of bacterial genera

Differential abundance of the gut microbiota was assessed between members of the *An. gambiae* complex for all life stages. Differentially abundant genera were considered significant for each pairwise comparison using the adjusted *p*-values < 0.01 of the log2 Fold Change values.

Bacterial genera *Aeromonas*, *Aquitalea*, *Flectobacillus*, *Arthrobacter*, *Rhodococcus*, *Elizabethkingia*, *Allorhizobium-neorhizobium-pararhizobium-rhizobium,* and *Rahnella* were significantly abundant in SENN and SENN DDT when compared to SANGWE (**Figure 3.5 A**-**B**). Additionally, *Pseudomonas*, *Klebsiella*, *Serratia*, *Kluyvera*, *Enterobacter*, *Microbacterium*, *Sphingobacterium*, *Sphingopyxis*, and *Mesorhizobium* were significantly abundant in SANGWE when compared to SENN (**Figure 3.5 A**). *Pseudomonas*, *Kluyvera*, *Klebsiella*, *Sphingobacterium*, *Sphingopyxis*, *Microbacterium,* and *Mesorhizobium* were significantly abundant in SANGWE when compared to SENN DDT (**Figure 3.5 B**).

Bacterial genera *Serratia*, *Aquitalea*, *Polynucleobacter*, *Leucobacter*, *Mycobacterium*, *Undibacterium*, *Aeromonas*, *Flectobacillus*, *Arthrobacter*, *Rhodococcus*, and *Allorhizobium-neorhizobium-pararhizobium-rhizobium* were significantly abundant in SENN and SENN DDT when compared to MAFUS (**Figure 3.5 C**-**D**). *Pseudomonas*, *Pantoea*, *Kluyvera*, *Enterobacter*, *Sphingobacterium*, *Achromobacter*, *Microbacterium*, *Mesorhizobium,* and *Sphingopyxis* were significantly abundant in MAFUS compared to both SENN and SENN DDT (**Figure 3.5 C**-**D**). *Achromobacter*, *Sphingobacterium*, *Elizabethkingia*, and *Pantoea* were significantly abundant in MAFUS. *Rahnella*, *Klebsiella*, *Serratia*, *Undibacterium*, *Polynucleobacter*, *Leucobacter*, and *Mycobacterium* were significantly abundant in SANGWE (**Figure 3.5 E**).

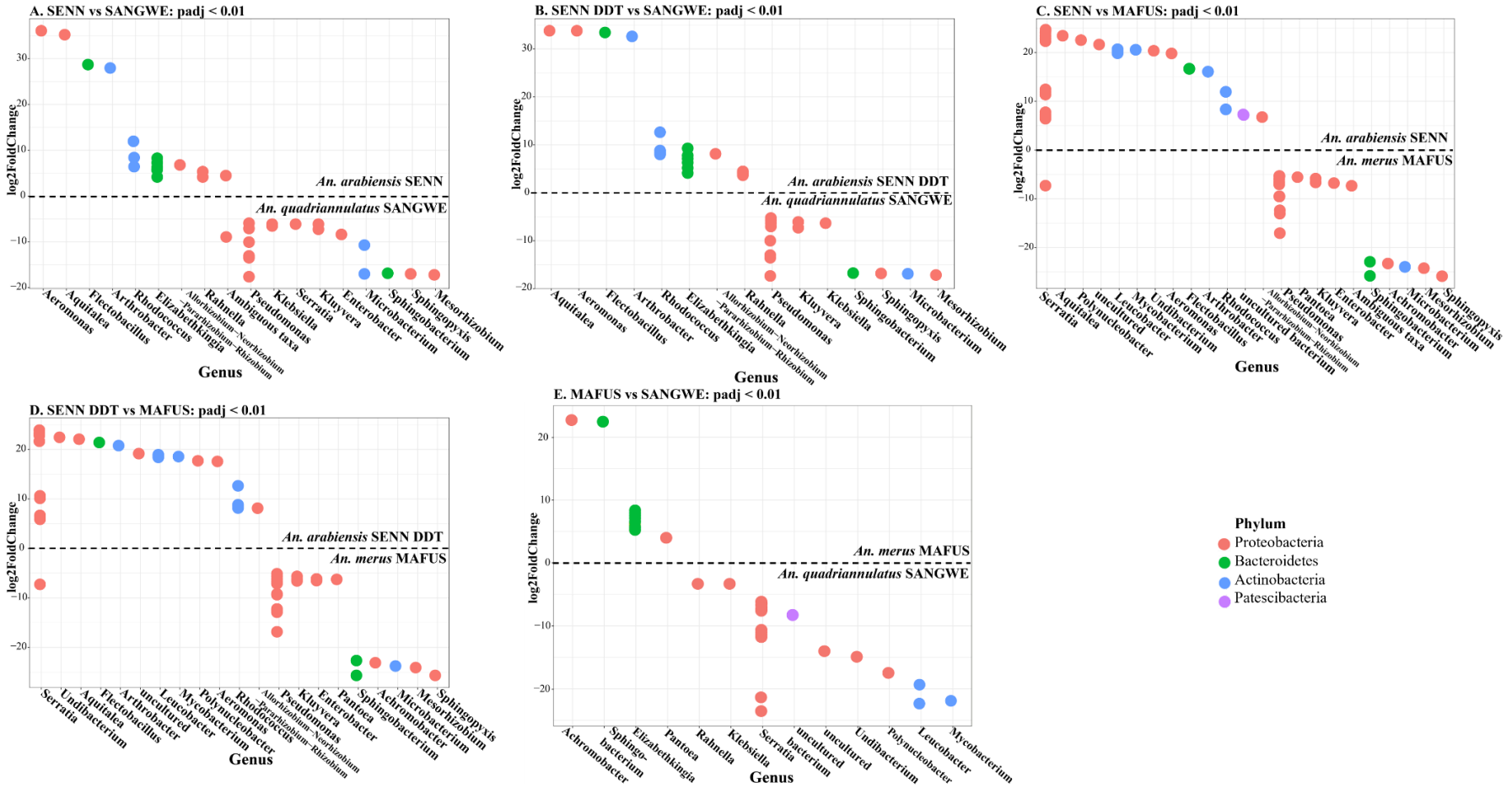

Figure 3.5 Pairwise comparisons of differentially abundant gut microbiota between selected members of the *Anopheles gambiae* complex for all life stages.

(A) Differential abundance of bacterial genera between SENN vs SANGWE. **(B)** Differential abundance of bacterial genera between SENN DDT vs SANGWE. **(C)** Differential abundance of bacterial genera between SENN vs MAFUS. **(D)** Differential abundance of bacterial genera between SENN DDT vs MAFUS. **(E)** Differential abundance of genera in MAFUS vs SANGWE. The mosquito species name on either side of the dotted line indicates where the bacterial genera are more abundant. Differentially abundant genera had a significance threshold of p-value < 0.01.

Differential abundance of the gut microbiota was assessed between the life stages. Differentially abundant genera were considered significant for each pairwise comparison using the adjusted *p*-values < 0.01 of the log2 Fold Change values.

Larvae had significantly abundant genera such as *Sphingopyxis*, *Sphingobacterium*, *Mesorhizobium*, *Arthrobacter*, *Microbacterium*, *Achromobacter*, *Mycobacterium*, *Aquitalea*, *Bosea*, *Pedobacter*, *Comamonas*, *Kaistia*, *Leucobacter*, *Ancylobacter*, and *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* compared to three-day old adults. *Rahnella*, *Elizabethkingia* and *Gluconobacter* were significantly abundant in the three-day old adults than in larvae (**Figure 3.6 A**).

Flectobacillus, *Variovorax*, *Sphingopyxis*, *Sphingobacterium*, *Mesorhizobium*, *Arthrobacter*, *Microbacterium*, *Achromobacter*, *Mycobacterium*, *Aquitalea*, *Bosea*, *Pedobacter*, *Rhodococcus*, *Comamonas*, *Kaistia*, *Leucobacter*, and *Aeromonas* were significantly abundant in larvae compared to fifteen-day old non-blood fed adults. *Rahnella*, *Cedecea*, *Elizabethkingia*, *Serratia*, *Asaia* and *Hafina-Obesumbacterium* were significantly abundant in fifteen-day old non-blood fed adults compared to larvae (**Figure 3.6 B**).

Variovorax, *Flectobacillus*, *Aeromonas*, *Rhodococcus*, *Serratia*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Microbacterium*, and *Sphingobacterium* were significantly abundant in three-day old adults compared to fifteen-day old non-blood fed adults. *Rahnella*, *Raoultella*, *Serratia*, *Asaia*, and *Hafina-Obesumbacterium* were significantly abundant in fifteen-day old non-blood fed compared to three-day old adults (**Figure 3.6 C**).

Aquitalea was significantly abundant in fifteen-day old non-blood fed adults compared to fifteen-day old blood fed adults. *Flectobacillus*, *Achromobacter*, and *Microbacterium* were significantly abundant in fifteen-day old blood fed adults compared to fifteen-day old non-blood fed adults (**Figure 3.6 D**).

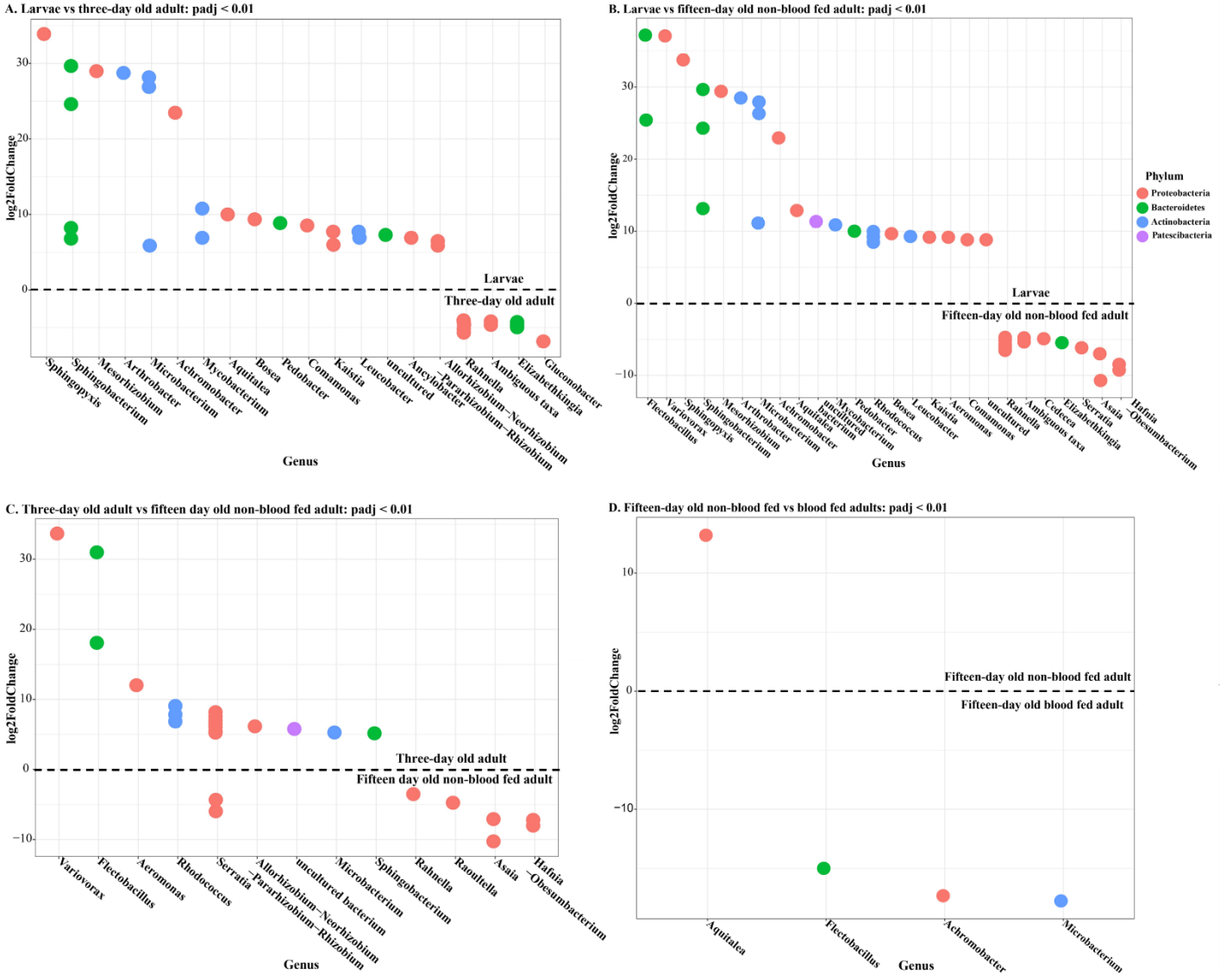

Figure 3.6 Pairwise comparisons of differentially abundant gut microbiota between the life stages of the selected members of the *Anopheles gambiae* complex.

(A) Differential abundance of bacterial genera between larvae vs three-day old adults. **(B)** Differential abundance of bacterial genera between larvae vs fifteen-day old non-blood fed adults. **(C)** Differential abundance of bacterial genera between three-day old adults vs fifteen-day old non-blood fed adults. **(D)** Differential abundance of bacterial genera between fifteen-day old non-blood fed adults vs fifteen-day old blood fed adults. The life stage of the mosquito is on either side of the dotted line, indicating where the relevant bacterial genera are more abundant. Differentially abundant genera had a significance threshold of *p*-value < 0.01.

Differential abundance of the gut microbiota was assessed between the fifteen-day old blood fed adults and fifteen-day old non-blood fed adults for each strain. Differentially abundant genera were considered significant for each pairwise comparison using the adjusted *p*-values < 0.01 of the log2 Fold Change values.

In SENN, genera such as *Morganella* and *Enterobacter* were significantly abundant in non-blood fed adults than in blood fed adults (**Figure 3.7 A**). In SENN DDT non-blood fed adults, *Klebsiella* and *Enterobacter* were significantly abundant in non-blood fed adults compared to blood fed adults. *Aeromonas* and *Serratia* were significantly abundant in SENN DDT blood fed adults compared to non-blood fed adults (**Figure 3.7 B**). *Pseudomonas* was significantly abundant in MAFUS non-blood fed adults compared to blood fed adults (**Figure 3.7 C**). *Morganella* was significantly abundant in SANGWE blood fed adults compared to non-blood fed adults (**Figure 3.7 D**).

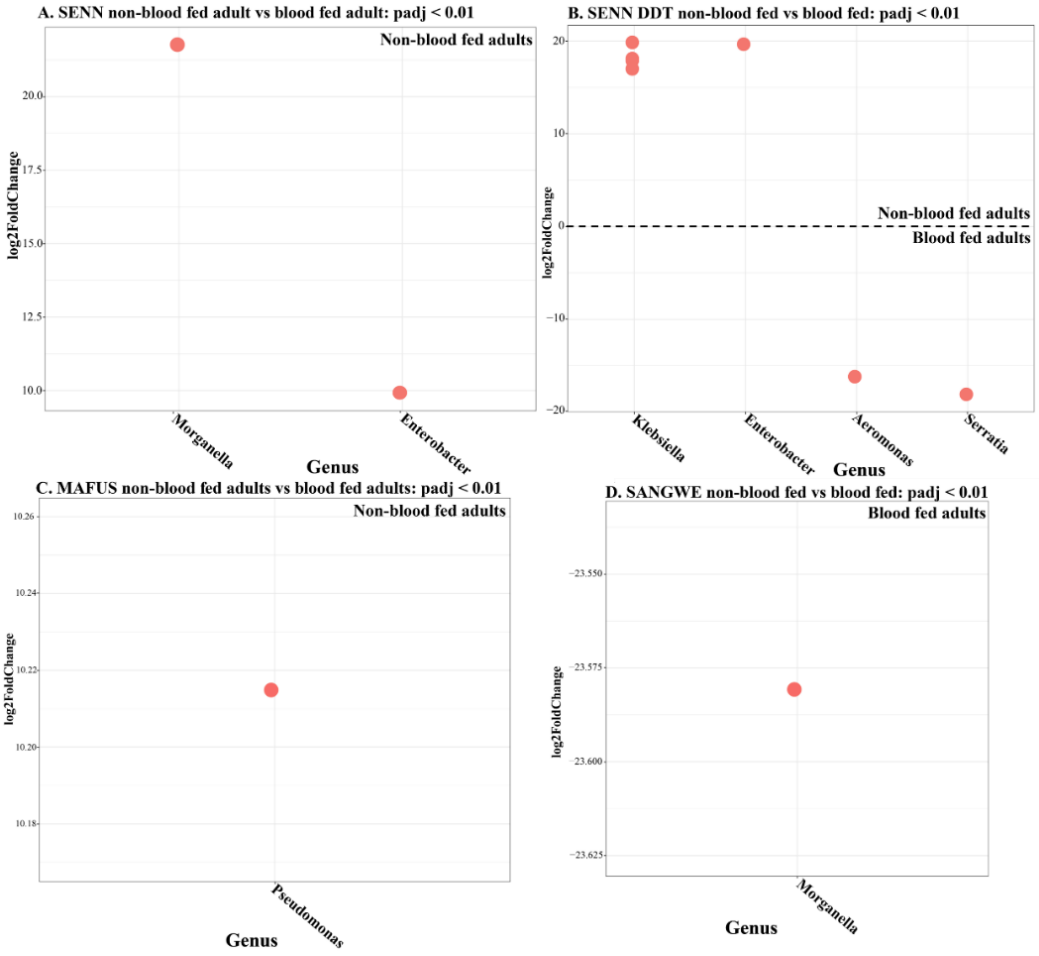

Figure 3.7 Pairwise comparisons of differentially abundant gut microbiota between fifteen-day old blood fed and non-blood fed adults for each strain.

(A) Differential abundance of genera in SENN non-blood fed versus blood fed adults. **(B)** Differential abundance of genera in SENN DDT non-blood fed versus blood fed adults. **(C)** Differential abundance of genera in MAFUS non-blood fed versus blood fed adults. **(D)** Differential abundance of genera in SANGWE non-blood fed versus blood fed adults. The blood feeding status of the fifteen-day-old adult mosquito is on either side of the dotted line, which shows where the relevant bacterial genera are more abundant. Differentially abundant genera had a significance threshold of p-value < 0.01.

3.4 Discussion

Primary vector control methods are insecticide-based and rely on IRS and LLINs [24]. However, the burgeoning problem of insecticide resistance and opportunistic feeding and resting behaviour of *An. arabiensis* populations are hindering the success of these conventional insecticide-based control methods [29,186–189]. Therefore, alternative forms of vector control are necessary. This study is a baseline study to form a scaffold for microbial-mediated control of vector populations. The gut microbiota of members of the *An. gambiae* complex was examined to determine whether the abundance and diversity differ among the major, minor, and non-vectors with similar feeding and biting behaviour. Additionally, we examined the gut microbiota abundance and diversity between the insecticide susceptible (SENN) and the insecticide resistant (SENN DDT) strains of *An. arabiensis*.

Many studies have focused on the gut microbiota of the nominal member of the complex, *An. gambiae* [41–43,46]. There are not many studies on *An. arabiensis* to compare the data to and there are even fewer for *An. quadriannulatus* and *An. merus* [203]. As such, the microbiome data available to compare the data generated in this study is limited. A previous study examined the microbiota of several tissues in nine mosquito species, including *An. gambiae*, *An. arabiensis*, *An. merus* and *An. quadriannulatus* [203]. The current study is similar to another study which examined the dynamic gut microbiota of different life stages of *An. arabiensis* from the field [108]. However, to our knowledge, this study is the first to do a comparative analysis of the gut microbiota of major, minor, and non-malaria vector species from the *An. gambiae* complex for different life stages.

The larvae had the greatest species richness and diversity among all the life stages. This is consistent with previous findings on the dynamic gut microbiota of *An. gambiae* [108]. This phenomenon is likely attributed to the transition of microbiota from the aquatic to aerial life stages [87,204]. As the larvae develop into pupae and, subsequently, adults, there is a notable shift in the abundance of bacteria from aerobic genera to facultatively anaerobic genera. This shift is necessary as the gut environment must become anaerobic to facilitate pupation [87,204]. Analysis comparing different life stages revealed more distinctly abundant bacterial genera in larvae than in older adults. Bacterial genera such as *Asaia*, *Elizabethkingia*, *Serratia*, and *Rahnella* were more prevalent in the adults, whereas *Aquitalea*, *Sphingobacterium*, and *Sphingopyxis* were notably abundant in larvae. This is likely due to

the shift from aerobic to facultatively anaerobic genera in the gut, which leads to reduced diversity in adults [205].

There were differences in differentially abundant genera between the non-blood fed and blood fed adults of the members of the *An. gambiae* complex studied. *Aquitalea* was significantly abundant in non-blood fed adults, while *Flectobacillus*, *Microbacterium* and *Achromobacter* were significantly abundant in blood fed adults. Interestingly, the genera found in abundance in blood fed adults do not possess haemolytic functions. However, haemolytic genera such as *Morganella* [206], *Klebsiella* [207,208], *Enterobacter* [208] and *Serratia* [209] were significantly abundant in both blood fed and non-blood fed adults. This study examined the longer-term effects of multiple blood meals on the gut microbiota. Therefore, it is unsurprising that haemolytic genera would not be abundant as the blood meal had already been digested.

The α-diversity of gut microbiota among members of the *An. gambiae* complex in this study was not significantly influenced by their blood feeding status. This contrasts with previous findings where bacterial richness *An. gambiae* decreased with age and blood feeding status [108]. Interestingly, this trend was not observed in other mosquito species, such as *Ae. aegypti*, as blood fed *Ae. aegypti* had a higher bacterial diversity compared to non-blood fed mosquitoes [58]. The absence of differences between blood fed and non-blood fed adults of the *An. gambiae* complex could be explained by the fact that this study examined the longer-term effects of multiple blood meals on the gut microbiota, where the gut microbiota was examined three days after the last blood meal. Other studies have primarily focused on a blood meal's immediate or direct impact on the gut microbiota [108]. This difference in approach was because multiple blood meals are a frequently used feeding strategy [210]. This is known to increase *Plasmodium* infectivity in *Anopheles* [211].

The richness of the gut microbiota of the insecticide susceptible *An. arabiensis* strain (SENN) was higher than the insecticide resistant strain (SENN DDT). The increased number of gut bacteria in SENN compared to SENN DDT suggests a potential role for gut bacteria in the resistant phenotype or an additional physiological response. The microbiota of fenitrothion resistant *An. albimanus* was lower than that of the susceptible strain [102]. Therefore, the significant decrease in the bacterial richness of SENN DDT could be a result of exposure to DDT, which acts as a stressor on the gut microbiota [199]. Furthermore, the SENN DDT

strain is continuously selected for DDT resistance, however, it is important to note that the specimens were not exposed to DDT prior to these experiments.

When compared, there were no significant increases in the abundance of specific bacterial genera between SENN and SENN DDT. Although insecticide-degrading bacteria are found in both laboratory-reared insecticide susceptible and resistant strains (SENN and SENN DDT, respectively), their resistance profiles differ. SENN DDT is highly resistant to DDT and permethrin, whereas SENN remains susceptible [104,106,212,213]. It is worth noting that dietary supplementation with a single strain of bacteria can significantly alter the expression of resistance in these strains [199]. As such, although it is not a primary driver of the resistance phenotype in SENN DDT, gut bacteria may have the potential to modulate the expression of the phenotype. The presence of pesticide-degrading bacteria found in wild fenitrothion *An. albimanus*, such as *Enterobacter* sp., *Klebsiella* sp., *Serratia* sp., *Kluyvera* sp., and *Salmonella enterica*, were present in SENN and SENN DDT [102]. Although other studies have implicated the gut microbiota in *Anopheles* as a potential contributor to the insecticide resistant phenotype [102,103,198], this was not observed for the *Anopheles* species used in this study. However, this is only by association with previous studies.

An obvious question that arises when assessing these results is that the differences in the results observed may not be due to inherent species differences but rather due to differences related to geographic origins. While this may be possible, it could be expected that such large differences would be reflected in changes in α-diversity. However, the α-diversity is conserved between these *Anopheles* species originating from different geographical areas (Sudan, South Africa, Zimbabwe). Although large differences in the α-diversity were not observed, this may be a function of reduced bacterial diversity typically seen after years of colonisation [87]. Despite the lack of differences in α-diversity, there are apparent differences in β-diversity. The conservation of α-diversity highlights that the changes in β-diversity are, therefore, likely to be an accurate representation of interspecies diversity. Furthermore, the effect of geographical differences in origin would be reduced with time in colony as these differences would arise due to host location affecting factors such as diet and larval environment [53]. Even with the reduced diversity associated with colonisation, the results found in this study are likely to reflect true species differences rather than artefacts of the species' origins.

A study conducted on wild populations of *An. gambiae* and *An. coluzzii* observed no differences in the β-diversity between these major malaria vectors with similar behaviours [42]. This is unsurprising as these closely related species have recently diverged into new species names [167]. By contrast, *An. arabiensis*, *An. merus*, and *An. quadriannulatus* differ in their vector competence despite similar behaviour [18]. From this study, marked differences are observed in the β-diversity between the major malaria vector *An. arabiensis* strains, the minor vector *An. merus*, and non-vector species *An. quadriannulatus*. This may be due to the evolutionary distance between the species [23,27,162]. However, the lack of variation in the β-diversity between *An. quadriannulatus* and *An. merus* should be considered as motivation for the validity of these findings [162]. Therefore, the differences in vector competence may have further molecular underpinnings, such as variable immune responses [32]. The differences in gut microbiota between the major, minor, and non-vectors may, therefore, contribute to vector competence, although this needs to be confirmed by *Plasmodium*-infection studies.

Bacterial genera such as *Asaia*, *Pantoea*, *Pseudomonas*, *Enterobacter*, and *Serratia* have anti-*Plasmodium* activities [203]. Specifically, the bacterial species *P. putida*, *Pantoea* sp. and *S. marcescens* can be introduced via sugar meals and block *Plasmodium* development *in vivo* [203]. Bacteria such as *Comamonas* sp., *Acinetobacter* sp., *P. putida* and *P. rhodesiae*, *Pantoea* sp., *S. marcescens* and *E. anophelis* can block *Plasmodium* activity *in vitro* [72]. The *Enterobacter* isolate Esp_Z inhibits *Plasmodium* development from gametocyte to ookinete through *in vivo* and *in vitro* production of ROS [80]. This is of consequence as these bacterial genera were observed as native symbionts in members of the *An. gambiae* complex examined in this study.

Pseudomonas was significantly abundant in the non-vector *An. quadriannulatus* compared to both *An. arabiensis* strains. Additionally, *Serratia* and *Enterobacter* were significantly abundant in *An. quadriannulatus* compared to the insecticide susceptible *An. arabiensis* strain. *Pseudomonas*, *Pantoea*, and *Enterobacter* had a greater abundance in *An. merus* compared to the *An. arabiensis* strains. The separation in the β-diversity of the gut microbiota between the major vector, *An. arabiensis*, and the minor and non-vectors *An. merus* and *An. quadriannulatus* is possibly driven by the increased abundance of *Plasmodium*-protective genera in the minor and non-vector species. This further suggests that the gut microbiota may contribute to the differences seen in the vector competence between these species.

Serratia had a higher differential abundance in *An. quadriannulatus* and *An. arabiensis* strains compared to *An. merus*. However, the association of *Serratia* with anti-*Plasmodium* defence is strain-dependent. For example, *S. marcescens* Y1 can inhibit *Plasmodium* development by modulating immune-related genes [93]. In addition to anti-*Plasmodium* defence, *S. marcescens* can degrade several insecticides [102]. Therefore, the higher abundance of *S. marcescens* observed in the *An. arabiensis* strains compared to the other species used in this study may be related more to insecticide resistance rather than anti-*Plasmodium* defence.

The gut microbiota of *An. merus* and *An. quadriannulatus* is poorly examined. This study has contributed to the existing body of information on these species and provides a good baseline for microbiota studies. However, there are limitations to this study, this experiment needs to be replicated in field specimens. As reviewed by Romoli and Gendrin [77], field specimens provide a more relevant gut microbiota that can be used as paratransgenesis candidates to target mosquitoes in the wild effectively [77]. However, field specimens in South Africa are difficult to obtain as South Africa is among the frontrunners to eliminate malaria [214,215]. Although our studies provide potential bacterial genera implicated in protection against *Plasmodium*, this must be confirmed. Both from the aspect of directly implicating the relevant species in transmission-blocking and establishing its field relevance. Additionally, the experiments should examine the changes in gut microbiota before and after *Plasmodium* infection.

3.5 Conclusion

In conclusion, this study characterised the dynamic gut microbiota of the members of the *An. gambiae* complex found in South Africa. Although α-diversity did not differ greatly, there was a significant difference in β-diversity between the major vector *An. arabiensis* and the minor vector *An. merus* and non-vector *An. quadriannulatus* laboratory reared strains. This diversity, in conjunction with the decreased relative abundance of genera associated with anti-*Plasmodium* effects in *An. arabiensis* suggests that this difference may be implicated in vector competence. Therefore, this study suggests a role for differential microbial diversity in the life history of zoophilic members of the *An. gambiae* complex.

CHAPTER 4

Characterisation of the gut microbiota of *Anopheles arabiensis* and the effect of heavy metal pollutants

4.1 Introduction

As discussed previously, the gut microbiota of mosquitoes plays a critical role in the life history of mosquitoes, as reviewed by Romoli and Gendrin [77]. Like other factors, the gut microbiota differs between laboratory reared strains and their wild counterparts [43,216,217]. For example, field-caught *Anopheles gambiae* were found to have a greater diversity in their gut microbiota than laboratory reared *An. gambiae* [43,216]. In *Aedes aegypti*, samples collected from different locations had similar gut microbiota when reared in the same insectary [217]. Such contrast studies between the gut microbiota of laboratory reared strains and their wild counterparts are valuable as assessing each population has limitations when considered in isolation.

In low vector density countries like South Africa, laboratory reared mosquitoes are more easily sampled than their wild counterparts, as they are readily available. These mosquitoes have a lower bacterial diversity than their wild counterparts [218]. This has been suggested to be due to the larval environment. The adaptations to insectary environments drive specific selection for bacteria, which results in reduced microbiota [87,218]. As such, analysis of their gut microbiota may not represent the full spectrum of potential bacteria that may be encountered in wild populations. Additionally, the gut microbiota composition of laboratory reared mosquitoes varies between insectaries due to local variations and differences in husbandry, as reviewed by Romoli and Gendrin [77]. For example, different larval diets may influence the gut microbiota [219]. Despite these variations, the characterisation of bacterial diversity and its effect on mosquito physiology in laboratory reared strains is a useful starting point. This is particularly true for understanding these dynamics in the context of the wild.

Wild mosquitoes, which are of more interest operationally, may be challenging to attain. This may be due to seasonal population fluctuations and operational circumstances [220]. However, they remain optimal study specimens due to their natural gut microbiota. Adults caught in the field have unknown life history traits. For example, the blood-feeding status and age of the adult mosquito would be unknown [221]. Larval collection and first filial (F_1)

progeny of adults collected from the field would not be as useful for understanding field larval dynamics as they would be reared in laboratory water with an artificial diet. However, these adults are more practical targets for analysis. Both the age and blood-feeding status of these mosquitoes are known and can be controlled.

The larval environment of mosquitoes has been suggested to play a role in the acquisition of gut microbiota in the adult life stages [41]. As reviewed by Strand [40], larvae could acquire their gut microbiota through drinking and feeding on what is available in the surrounding water [40]. A portion of the gut microbiota from larvae may be transstadially transmitted to the adult during metamorphosis [41,50,51]. Additionally, adults drinking water from the larval water during emergence can alter their gut microbiota [41,50,51]. The larval environments of wild mosquitoes are suggested to impact the composition of their gut microbiota although data to support this has been contradictory [42,43,108]. Investigations have indicated that the larval environment has no significant association with the gut microbiota as laboratory reared *An. gambiae* adults have a gut microbiota composition similar to that of field-collected *An. gambiae* from Kenya [108]. However, other studies have determined that the larval environment influences the composition and diversity of the gut microbiota in field-collected *An. gambiae* and *An. coluzzii* [42,43]. Therefore, variations in the gut microbiota between laboratory reared and wild populations could potentially be attributed to the differences in the larval environment. As such, factors that influence the larval environment are of interest.

Pollution is prevalent in mosquito breeding sites and, to our knowledge, has not yet been considered in microbiota studies. Heavy metal pollutants are primarily introduced into the environment in both rural and urban areas by human activities [222]. In rural areas, heavy metals can contaminate water sources through inorganic fertilisers, which increase the amount of heavy metal trace elements [223,224]. In South Africa, mining also contributes to heavy metal pollution, the run-off tends to pollute water sources [225,226]. This has implications for the life history of mosquitoes that have adapted to breeding in these polluted waters.

Members of the *An. gambiae* complex typically breed in clean, sunlit temporary bodies of water [18]. However, there have been reports of members of the *An. gambiae* complex expanding their breeding sites to polluted water throughout Africa [114,227]. This adaptation has caused significant physiological shifts in these species [111–114,228]. More specifically, *An. arabiensis* has demonstrated adaptability to metal pollutants [111,227]. Therefore, the presence of metal pollutants in mosquito breeding sites could impact the acquisition and

diversity of the gut microbiota in *An. arabiensis*, given the interplay between the larval environment and gut microbiota.

The Mamfene district in Northern Kwa-Zulu Natal currently experiences ongoing residual malaria transmission [31]. This is a rural area that is engaged in agricultural activities [229], including the use of fertilisers, which may contribute to the contamination of water with heavy metals. *Anopheles arabiensis*, the primary malaria vector in this region, has shown low-level insecticide resistance [31]. This population of *An. arabiensis* is associated with such agricultural areas, with their larvae developing in bodies of water that occur from agricultural run-off [113,230]. Therefore, this population is more likely to be exposed to metal pollutants associated with agriculture. As such, it would be valuable to investigate the impact of metal pollutants in the larval environment of *An. arabiensis*. In this study, cadmium and copper were selected as representative heavy metal pollutants as they are commonly present in organic and inorganic fertilisers [231].

Metal pollutant exposure in laboratory reared and wild populations of *An. arabiensis* larvae have resulted in increased tolerance of insecticide resistance in adults [111,212]. In addition, the insecticide resistant phenotype in adults has been associated with increased larval pollutant tolerance [212]. Detoxification enzymes such as β-esterases and the cytochrome P450 proteins may be associated with increased insecticide tolerance [212]. Furthermore, a single generation of laboratory reared *An. arabiensis* larvae exposed to metal pollutants resulted in transgenerational effects in adults [232]. These adults had reduced longevity and increased insecticide tolerance, which persisted even after the second generation emerged from unpolluted water [232]. Considering the interplay between the insecticide resistant phenotype and microbiota and the interaction with metal pollutants, larval exposure to heavy metals would likely differ between insecticide susceptible and resistant *An. arabiensis* adults. Such differences could have significant implications for understanding the response of insecticide susceptible and resistant populations to environmental stressors.

This study compared the gut microbiota of laboratory reared insecticide susceptible and resistant strains of *An. arabiensis* adults (SENN and SENN DDT), *An. arabiensis* offspring of females collected from the field (hereafter referred to as the F_1 population), and *An. arabiensis* adults collected as larvae from the field (hereafter referred to as the wild population) of *An. arabiensis* adults from Mamfene. In addition, the effect of larval heavy

metal exposure on the adult gut microbiota of laboratory reared (SENN and SENN DDT) and the F_1 population of *An. arabiensis* adults were determined.

4.2 Materials and Methods

The characterisation of the gut microbiota between three-day old *An. arabiensis* was conducted as described in **CHAPTER 2 General Materials and Methods**.

4.2.1 Sample collection and preparation of the laboratory reared strains, F_1 population, and wild population of *An. arabiensis* adults for gut microbiota analysis

Collection and preparations of the laboratory reared strains (SENN and SENN DDT), F_1 population, and a wild population of three-day old *An. arabiensis* adults were conducted as follows:

Laboratory reared insecticide susceptible *An. arabiensis* (SENN) and insecticide resistant *An. arabiensis* (SENN DDT) strains, as described in **2.1.1 Laboratory reared *Anopheles* species/strains and their phenotypes**, were collected as three-day old adults. Wild *An. arabiensis* larvae and adults were collected from Section 9 (S 27°27′34.3″; E 032° 10′43.7″) in the Mamfene district in Kwa-Zulu Natal, South Africa. The direct larval collections from this region were used as the wild population specimens. The offspring of identified isofemale *An. arabiensis* were used as the F_1 population specimens. All adults were allowed *ad libitum* access to a 10% (w/v) sucrose solution. All *An. arabiensis* specimens were maintained as described in **2.1.2 Insectary conditions for laboratory reared *Anopheles* mosquitoes**. Species identification of wild population adults was conducted using conventional Polymerase Chain Reaction (PCR) to amplify species-specific regions within the intergenic spacer (IGS) of the small subunit of the ribosomal RNA (rRNA) gene as described by Scott *et al.* [233].

Identification and analysis of the gut microbiota for the laboratory reared strains, F_1 population, and wild population of *An. arabiensis* were conducted as follows: The gut microbiota for all specimens under investigation were characterised once the adults were three days old. Three replicates consisting of five midguts were prepared for SENN and SENN DDT. Twelve replicates of the wild population adults were prepared, and seventeen replicates were made for the F_1 population. Each replicate contained one midgut due to the limited availability of samples. Total genomic DNA from the mosquito's midgut was extracted using

the DNeasy PowerSoil® Kit (QIAGEN: 12888-50) as described in **2.1.4 Midgut dissection and DNA extraction**. The gut microbiota from the specimens were then identified as described in **2.2 Next-Generation Sequencing of the gut microbiota**.

4.2.2 Sample collection and preparation of the laboratory reared strains and F_1 population of *An. arabiensis* adults for gut microbiota analysis post-larval exposure to heavy metals

Collection and preparation of laboratory reared strains (SENN and SENN DDT) and F_1 population of *An. arabiensis* adults to examine the effect of larval metal exposure on the gut microbiota of three-day old adults was conducted as follows:

Laboratory reared *An. arabiensis* insecticide susceptible strain (SENN) and insecticide resistant strains (SENN DDT) as described in **2.1.1 Laboratory reared *Anopheles* species/strains and their phenotypes** were collected as first instar larvae. *Anopheles arabiensis* females were collected from Section 9 (S 27°27′34.3″; E 032° 10′43.7″) in the Mamfene district in Kwa-Zulu Natal, South Africa. From the identified isofemale *An. arabiensis* first instar larvae were collected (F_1 population). All *An. arabiensis* larvae were maintained as described in **2.1.2 Insectary conditions for laboratory reared *Anopheles* mosquitoes**.

The metal-polluted treated specimens under investigation were collected as first instar larvae. The larvae of each population were reared in one litre of reverse osmosis water supplemented with the maximum acceptable toxic concentration (MATC) [234] of either cadmium chloride (0.36 µg/L) or copper nitrate (1.86 µg/L) [234]. First instar larvae reared in untreated reverse osmosis water served as a control. All adults that emerged from these treatments were allowed *ad libitum* access to a 10% (w/v) sucrose solution.

The effect of larval exposure to heavy metals on the gut microbiota for laboratory reared and F_1 population of *An. arabiensis* adults were conducted as follows: The gut microbiota for all specimens under investigation was characterised once the adults were three days old. Three replicates of SENN and three replicates of SENN DDT samples were prepared for each treatment. For the F_1 population, five replicates of untreated samples, five replicates of cadmium-treated samples and ten replicates of copper-treated samples were prepared. Each replicate contained three midguts. Total genomic DNA from the mosquito's midgut was

extracted using the Qiagen DNeasy Blood & Tissue kit (Cat no. 69506) as described in **2.1.4 Midgut dissection and DNA extraction**. The gut microbiota from the specimens were then identified as described in **2.2 Next-Generation Sequencing of the gut microbiota**.

4.3 Results

Comparison of the gut microbiota between the laboratory reared (SENN and SENN DDT), the F_1 population and the wild population of *An. arabiensis* adults

4.3.1 α-diversity of the gut microbiota

The α-diversity (within-sample diversity) [147] was assessed for each laboratory reared, F_1 and wild populations of three-day old *An. arabiensis* adults. Boxplots were used to visualise and compare differences in the α-diversity of the laboratory reared insecticide susceptible strain (SENN), the laboratory reared insecticide resistant strain (SENN DDT), the F_1 population, and the wild population of *An. arabiensis* three-day old adults. Significant differences between the indices were assessed using a Kruskal-Wallis rank-sum test [157]. The Chao1 and Abundance-based Coverage Estimator (ACE) diversity indices were used as estimators of species richness by considering the number of bacterial species in the gut [148–152]. Shannon-Weiner and Simpson's dominance indices were used as estimators of species richness and evenness by considering the number of different bacterial species and the relative abundance of each species [153–156].

There were no significant differences in the Chao1 ($p = 0.82$), ACE ($p = 1.00$), Shannon-Weiner ($p = 0.07$) and Simpson's dominance ($p = 0.07$) diversity indices between the insecticide susceptible and insecticide resistant laboratory reared *An. arabiensis* adults (**Figure 4.1**). No significant differences were observed in the Chao1 diversity indices ($p = 0.11$) and ACE diversity indices ($p = 0.15$) between the F_1 population and the wild population of *An. arabiensis* (**Figure 4.1 A** and **B**). In contrast, the Shannon-Weiner diversity indices ($p = 0.01$) and the Simpson's dominance diversity indices ($p = 0.03$) were significantly higher for the F_1 population than the wild population (**Figure 4.1 C** and **D**).

Significant differences in the α-diversity indices Chao1 and ACE, indicating species richness, were observed between the laboratory reared, the F_1 population and the wild population of *An. arabiensis* adults. The Chao1 index was significantly higher for the F_1 population of *An. arabiensis* compared to laboratory reared *An. arabiensis* strains SENN ($p < 0.01$) and SENN

DDT ($p < 0.01$) (**Figure 4.1 A**). Similarly, the Chao1 index was significantly higher for the wild population of *An. arabiensis* compared to laboratory reared *An. arabiensis* strains SENN ($p = 0.01$) and SENN DDT ($p = 0.02$) (**Figure 4.1 A**). The ACE index was significantly higher for the F_1 population of *An. arabiensis* compared to the laboratory reared SENN DDT strain ($p = 0.03$) (**Figure 4.1 B**). Conversely, no significant differences were observed in the ACE diversity indices between the F_1 population and the laboratory reared strain SENN ($p = 0.13$) nor between the wild population and the laboratory reared strains SENN ($p = 0.29$) and SENN DDT ($p = 0.07$) (**Figure 4.1 B**).

Significant differences in the α-diversity indices indicating species richness and evenness were observed between the laboratory reared insecticide susceptible strain (SENN) and the F_1 population and wild population of *An. arabiensis* adults. The Shannon-Weiner diversity index for the wild population was significantly higher compared to SENN ($p < 0.01$). By contrast, no significant differences in the Shannon-Weiner diversity indices between the wild population and SENN DDT ($p = 0.09$) were observed (**Figure 4.1 C**). The Shannon-Weiner diversity indices were significantly higher in the F_1 population compared to SENN ($p < 0.01$) and SENN DDT ($p < 0.01$) (**Figure 4.1 C**). The Simpson's dominance diversity indices were not significantly different between the wild population and SENN DDT ($p = 0.20$) nor between the F_1 population and SENN DDT ($p = 0.09$) (**Figure 4.1 D**). In contrast, the Simpson's dominance diversity indices were significantly higher for the wild population compared to SENN ($p < 0.01$) and the F_1 population and SENN ($p < 0.01$) (**Figure 4.1 D**).

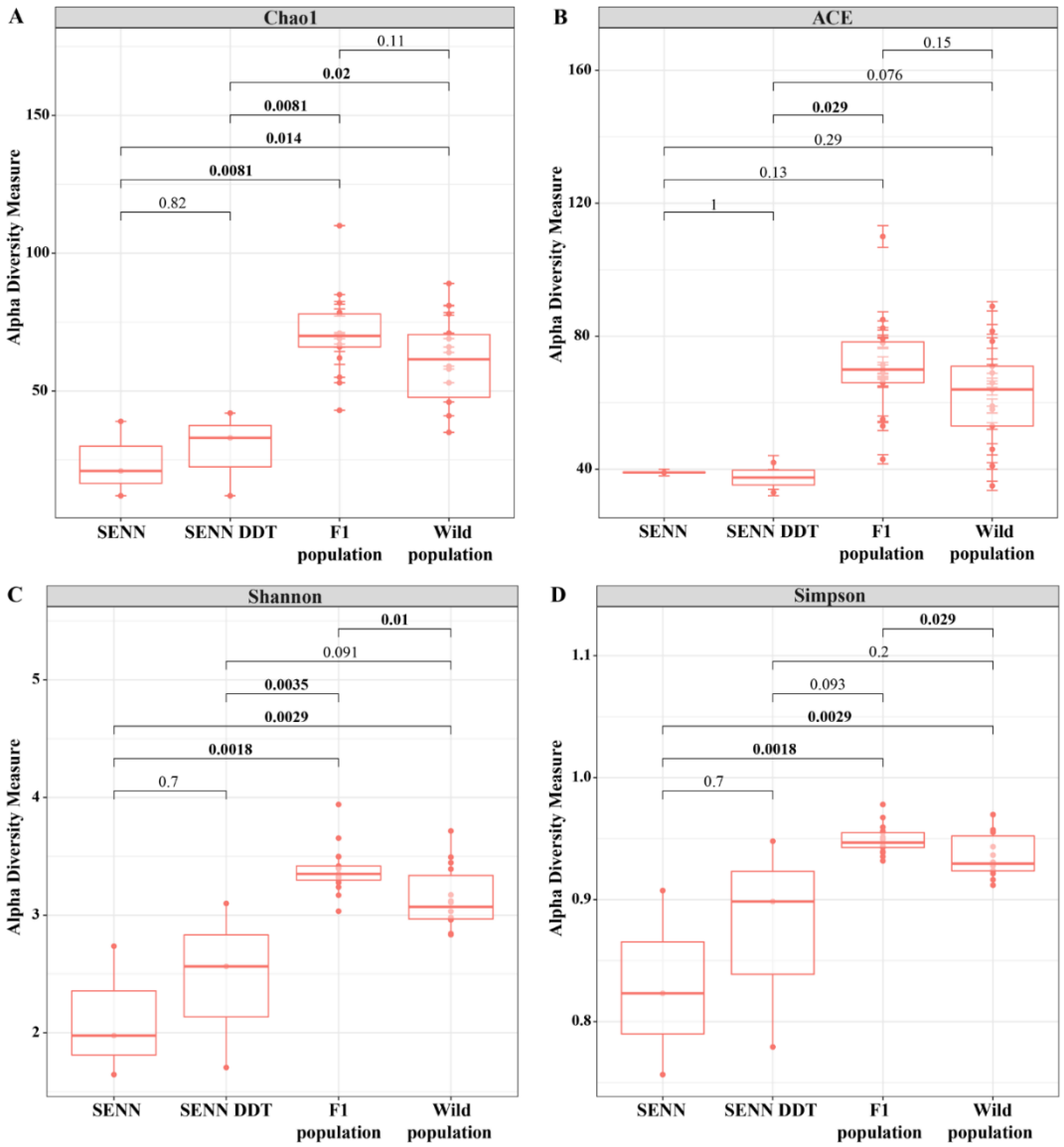

Figure 4.1 Comparison of the α-diversity of gut microbiota between *Anopheles arabiensis* adults.

(A) Chao1 diversity indices of the gut microbiota of laboratory reared, F_1, and wild populations. **(B)** ACE diversity indices of the gut microbiota of laboratory reared, F_1, and wild populations. **(C)** Shannon-Weiner diversity indices of the gut microbiota of laboratory reared, F_1, and wild populations. **(D)** Simpson's dominance diversity indices of the gut microbiota of laboratory reared, F_1, and wild populations. Comparisons of the diversity indices between the laboratory reared (SENN and SENN DDT) laboratory reared, the F_1 population, and the wild population of three-day old *An. arabiensis* adults were assessed using a Kruskal-Wallis rank-sum test. The dots represent the number of biological replicates used for each group. Significant differences in the diversity indices between samples are indicated by bold print.

4.3.2 β-diversity of the gut microbiota

The β-diversity analysis was used to determine interspecies differences [147] in the gut bacteria diversity between the laboratory reared strains (SENN and SENN DDT), the F_1 population, and the wild population of three-day old *An. arabiensis* adults. This was visualised using two-dimensional Non-Metric Dimensional Scaling (NMDS) ordination. The differences in the bacterial communities of the gut were assessed using the Bray-Curtis dissimilarity distances. Permutational multivariate analysis (PERMANOVA) was used to determine if there were any significant interspecies differences in the gut microbiota diversity.

There were similar clustering patterns in the diversity (number and abundance of bacterial communities) between the wild population and F_1 population adults. Notably, the bacterial diversity of laboratory reared adults was dissimilar from the F_1 population and wild population adults as they clustered separately. The gut microbiota of the wild population and the F_1 population of *An. arabiensis* adults had a greater diversity than their laboratory reared counterparts. The diversity of the gut microbiota between the insecticide susceptible and insecticide resistant laboratory reared adults (SENN and SENN DDT) was significantly different to that of the F_1 population and wild population (PERMANOVA; $p = 0.01$, $F = 4.28$, stress value = 0.15) (**Figure 4.2**).

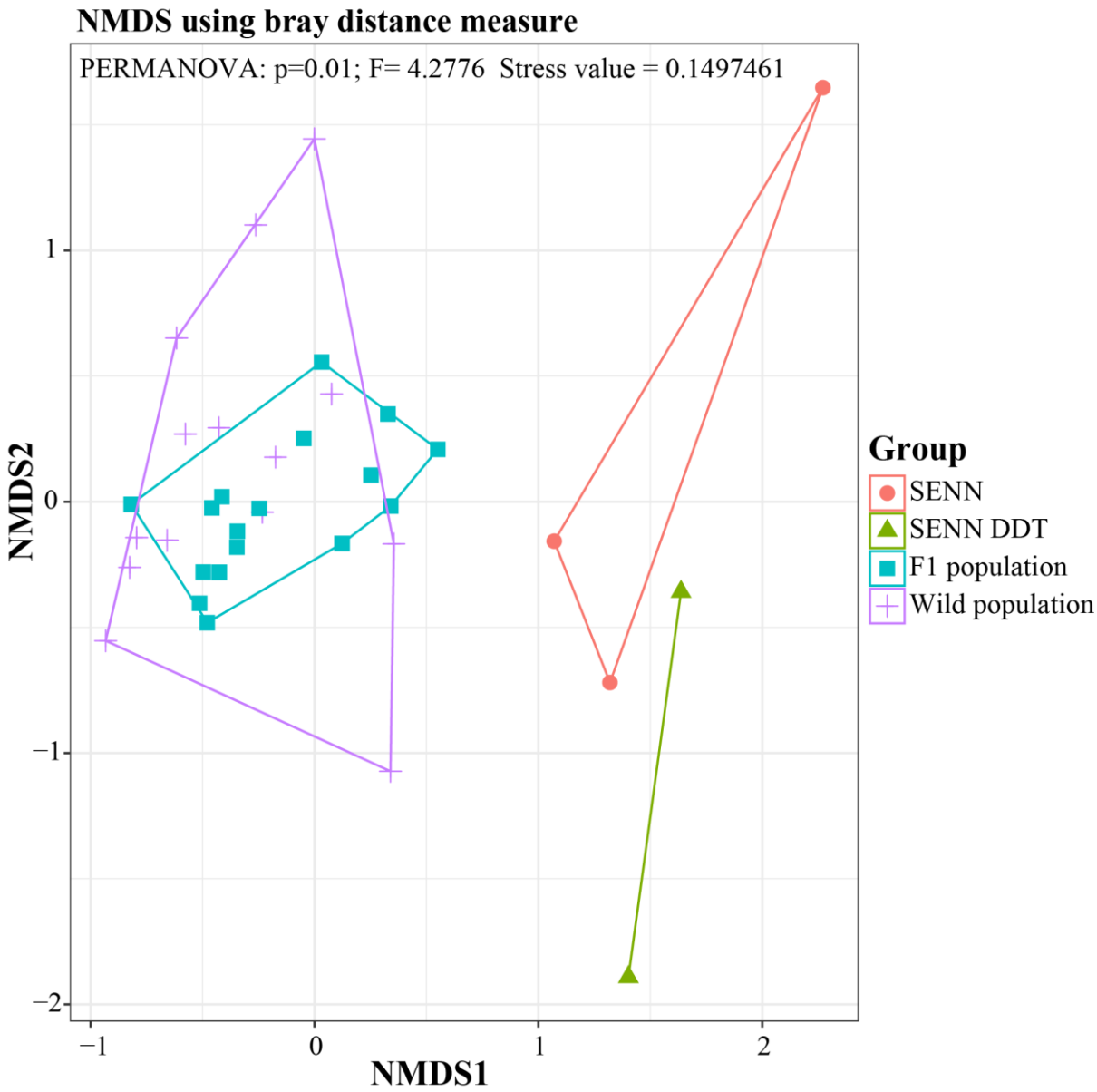

Figure 4.2 Differences in the gut microbiota diversity between *Anopheles arabiensis* adults.

A visual representation of the β-diversity analysis of the gut bacteria between the laboratory reared strains (SENN and SENN DDT), the F_1 population, and the wild population of three-day old *An. arabiensis* adults. Different colours represent the different *An. arabiensis* adults, the insecticide susceptible laboratory reared *An. arabiensis* (SENN) as red, the insecticide resistant laboratory reared *An. arabiensis* (SENN DDT) as green, the F_1 population of *An. arabiensis* as blue and the wild population of *An. arabiensis* as purple. Significant differences in the bacterial diversity between the laboratory reared, the F_1 population, and the wild population of *An. arabiensis* were assessed using a PERMANOVA (p = 0.01).

4.3.3 Overlapping bacterial communities

Shared and unique bacteria were represented numerically on UpSet plots for the laboratory reared strains (SENN and SENN DDT), the F_1 population, and the wild population of three-day old *An. arabiensis* adults. The number of shared and unique bacterial families is shown in **Figure 4.3 A**, genera in **Figure 4.3 B**, and species in **Figure 4.3 C**. Bacterial communities that were identified until family, genus, and species level are listed below. Note that not all bacterial communities were identified to the respective taxonomic levels, as this is a limitation of 16S rRNA gene sequencing [200–202]. Therefore, the identified bacterial communities may not correspond to the numbers below.

Two bacterial families, including *Yersiniaceae* and *Enterobacteriaceae*, were shared between the laboratory reared and the wild population of *An. arabiensis*. Three bacterial families, including *Weeksellaceae*, *Rhizobiaceae*, and *Nocardiaceae*, were shared between the laboratory reared *An. arabiensis* strains. Two bacterial families, including *Acetobacteraceae* and *Pseudomonadaceae*, were shared between the F_1 population and the wild population of *An. arabiensis*. *Enterobacteriaceae* overlapped between SENN DDT and F_1 and wild populations. Two bacterial families, *Hafniaceae* and *Aeromonadaceae*, were unique to SENN. *Sphingobacteriaceae* was unique to SENN DDT, and *Burkholderiaceae* was unique to the wild population (**Figure 4.3 A**).

Three bacterial genera and species, including *Rahnella* and *Yersinia*, were shared between all specimens. Three bacterial genera and five bacterial species, including *Yersinia aldovae*, *Elizabethkingia anophelis*, *Elizabethkingia bruuniana*, and *Rhodococcus qingshengii* and representatives of the group *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* were found in the laboratory reared strains of *An. arabiensis*. In the F_1 and wild population, three bacterial genera and seven bacterial genera, including *Asaia krungthepensis*, *Clavibacter michiganensis*, *Cedecea neteri*, *Pseudomonas proteolytica*, *Aeromonas veronii*, *Klebsiella* and *Pseudomonas* were shared. Three bacterial genera and species, including *Klebsiella oxytoca* and *Enterobacter*, were shared between SENN DDT, the F_1 population, and the wild population. Two bacterial genera and species, including *Hafnia paralvei* and *Aeromonas*, were unique to SENN. One bacterial genus and six bacterial species, including *Sphingobacterium multivorum*, *Cupriavidus metallidurans*, *Enterobacter hormaechei*, *Rhodococcus erythropolis*, *Yersinia pekkanenii*, and *Elizabethkingia*, were unique to SENN DDT. One bacterial genus and three bacterial species, including *Robbsia andropogonis*, *Enterobacter bugandensis*, and

Pseudomonas gessardii, were unique to the wild population. One unique genus and four bacterial genera, including *Pseudomonas palleroniana*, *Enterobacter roggenkampii*, *Asaia*, and *Kluyvera*, were unique to the F_1 population (**Figure 4.3 B** and **C**).

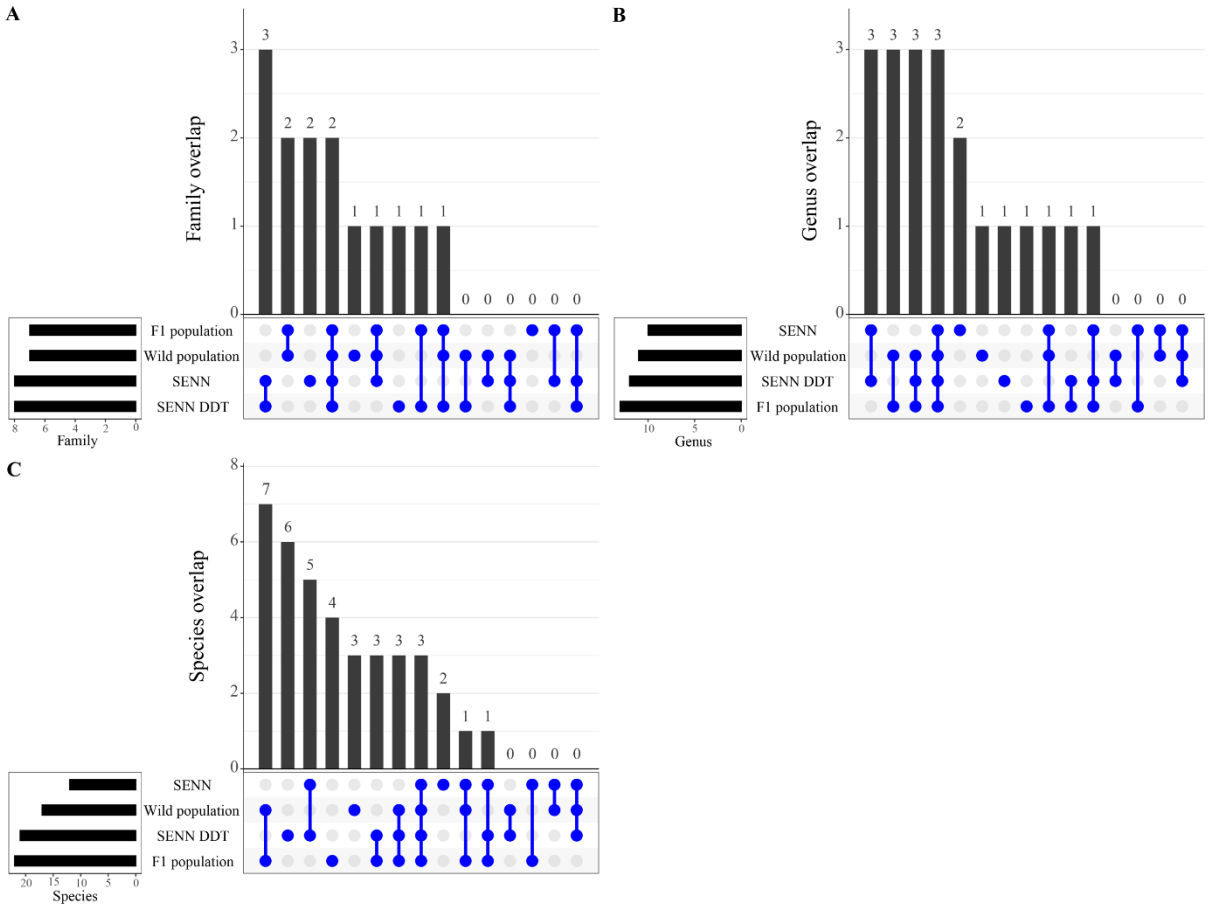

Figure 4.3 Comparison of overlapping gut bacteria between *Anopheles arabiensis* adults.

Comparison of common **(A)** bacterial families, **(B)** bacterial genera, and **(C)** bacterial species between the laboratory reared strains (SENN and SENN DDT), the F_1 population, and the wild population of three-day old *An. arabiensis* adults. The blue dots with a joined line represent the shared bacteria between laboratory reared strains (SENN and SENN DDT), the F_1 population, and the wild population of *An. arabiensis* three-day old adults, with single dots indicating the unique bacteria per strain. Each numbered column represents the number of bacterial families **(A)**, genera **(B),** or species **(C)** that overlap.

4.3.4 Differential abundance of bacterial genera

Differential abundance of the gut microbiota with pairwise comparisons was assessed between the laboratory reared strains (SENN and SENN DDT), the F_1 population, and the wild population of three-day old *An. arabiensis* adults. Differentially abundant genera were considered significantly different when the assigned log2 Fold Change values were compared and had a *p*-value < 0.01.

The most represented bacterial genera present belonged to the phyla Proteobacteria, Bacteroidota, and Acinetobacteriota. Six bacterial genera, including *Aeromonas*, *Rhodococcus*, *Yersinia*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Rahnella*, and *Elizabethkingia*, were significantly abundant in SENN compared to the wild population (**Figure 4.4 A**) and F_1 population (**Figure 4.4 B**). Four bacterial genera, including *Cedecea*, *Klebsiella*, *Rahnella* and *Pseudomonas*, were significantly abundant in the wild population (**Figure 4.4 A**) and F_1 population (**Figure 4.4 B**) compared to SENN.

Five bacterial genera, including *Yersinia*, *Aeromonas*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, and *Elizabethkingia*, were significantly abundant in SENN DDT compared to the wild population (**Figure 4.4 C**) and F_1 population (**Figure 4.4 D**). Four bacterial genera, including *Rahnella*, *Pseudomonas*, *Klebsiella*, and *Cedecea*, were significantly abundant in the wild population (**Figure 4.4 C**) and F_1 population (**Figure 4.4 D**) compared to SENN DDT. Additionally, three bacterial genera, including *Rhodococcus*, *Rahnella*, and *Klebsiella*, were significantly abundant in SENN DDT compared to the wild population (**Figure 4.4 C**). Two bacterial genera, including *Enterobacter* and *Klebsiella*, were also significantly abundant in the F_1 population compared to SENN DDT (**Figure 4.4 D**).

Three bacterial genera, including *Cedecea*, *Klebsiella*, and *Rahnella*, were significantly abundant in the F_1 population compared to the wild population (**Figure 4.4 E**). *Pseudomonas* was significantly abundant in both the wild population and F_1 population (**Figure 4.4 E**).

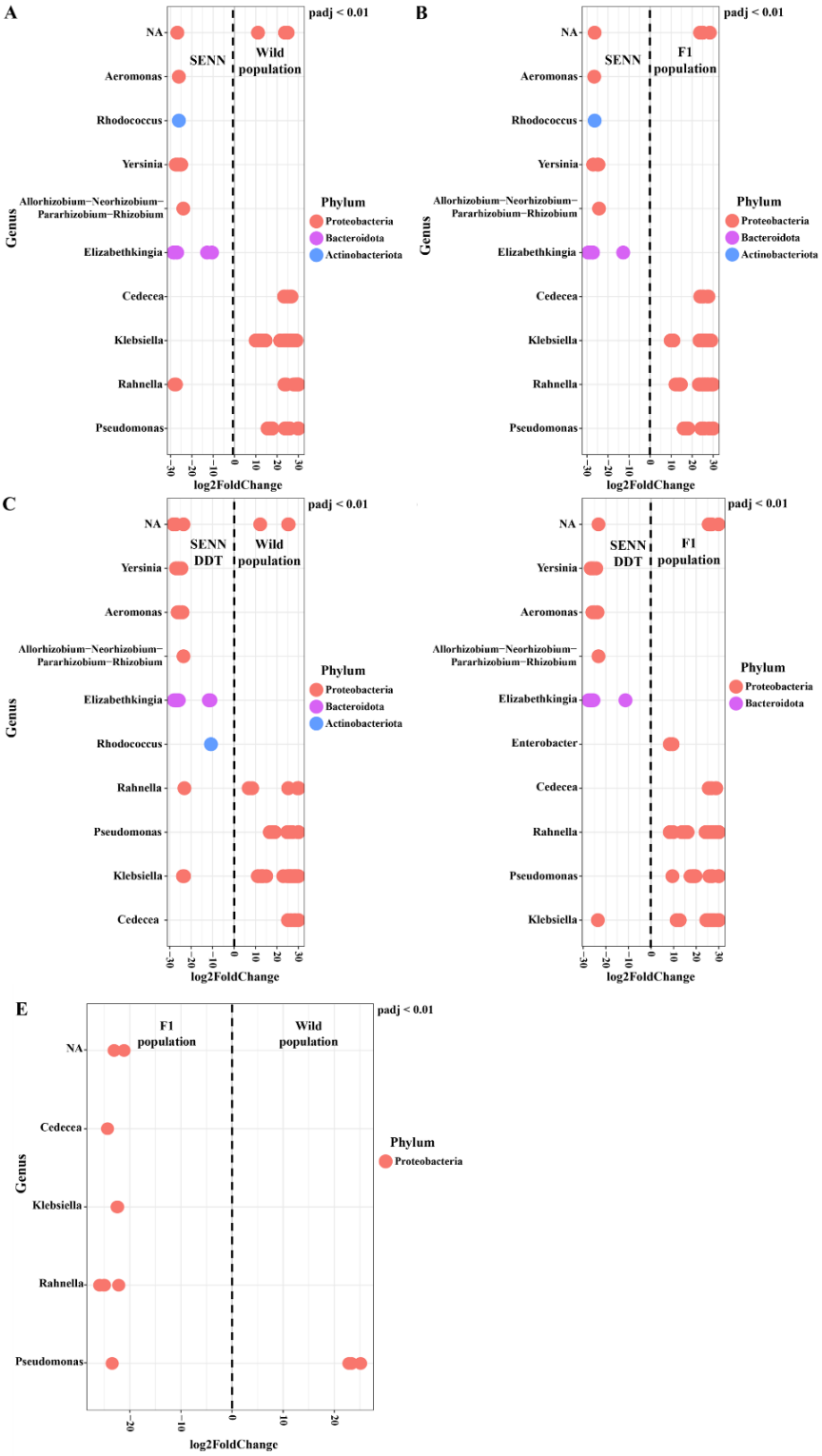

Figure 4.4 Pairwise comparisons of differentially abundant bacterial genera between *Anopheles arabiensis* adults.

Differential abundance of the gut bacteria of three-day old *An. arabiensis* adults between **(A)** SENN vs the wild population. **(B)** SENN vs the F_1 population. **(C)** SENN DDT vs the wild population. **(D)** SENN DDT vs F_1 population. **(E)** The F_1 population vs the wild population. Differentially abundant genera had a significance threshold of p-value < 0.01. The strain name on either side of the dotted line shows where the relevant bacterial genera are abundant.

The effects of larval metal exposure on the gut microbiota between laboratory reared (SENN and SENN DDT) and the F_1 population *An. arabiensis* adults

4.3.5 α-diversity of the gut microbiota

Comparative analysis of α-diversity was used to assess the effect of larval exposure to heavy metals on gut microbiota diversity within each of the laboratory reared and the F_1 population of three-day old *An. arabiensis* adults. Boxplots were used to visualise and compare differences in the α-diversity between the laboratory reared insecticide susceptible strain (SENN), the laboratory reared insecticide resistant strain (SENN DDT), and the F_1 population of *An. arabiensis* three-day old adults exposed to copper or cadmium as larvae and their untreated counterparts. A Kruskal-Wallis rank-sum test was used to determine if these differences were significant. The Chao1 diversity index was used as an estimator of species richness by considering the number of bacterial species in the gut [148].

No significant differences were observed between the Chao1 indices between untreated adults and those exposed to cadmium or copper (**Figure 4.5 A-C**). The Chao1 diversity indices between untreated SENN adults and those exposed to cadmium ($p = 0.40$) and copper ($p = 0.51$) as larvae were not significantly different (**Figure 4.5 A**). There was no significant difference in the Chao1 indices between untreated SENN DDT adults and those exposed to cadmium ($p = 0.40$) or copper ($p = 0.20$) as larvae (**Figure 4.5 B**). The Chao1 indices were not significantly different between untreated F1 population adults and those exposed to cadmium ($p = 0.40$) or copper ($p = 0.51$) as larvae (**Figure 4.5 C**).

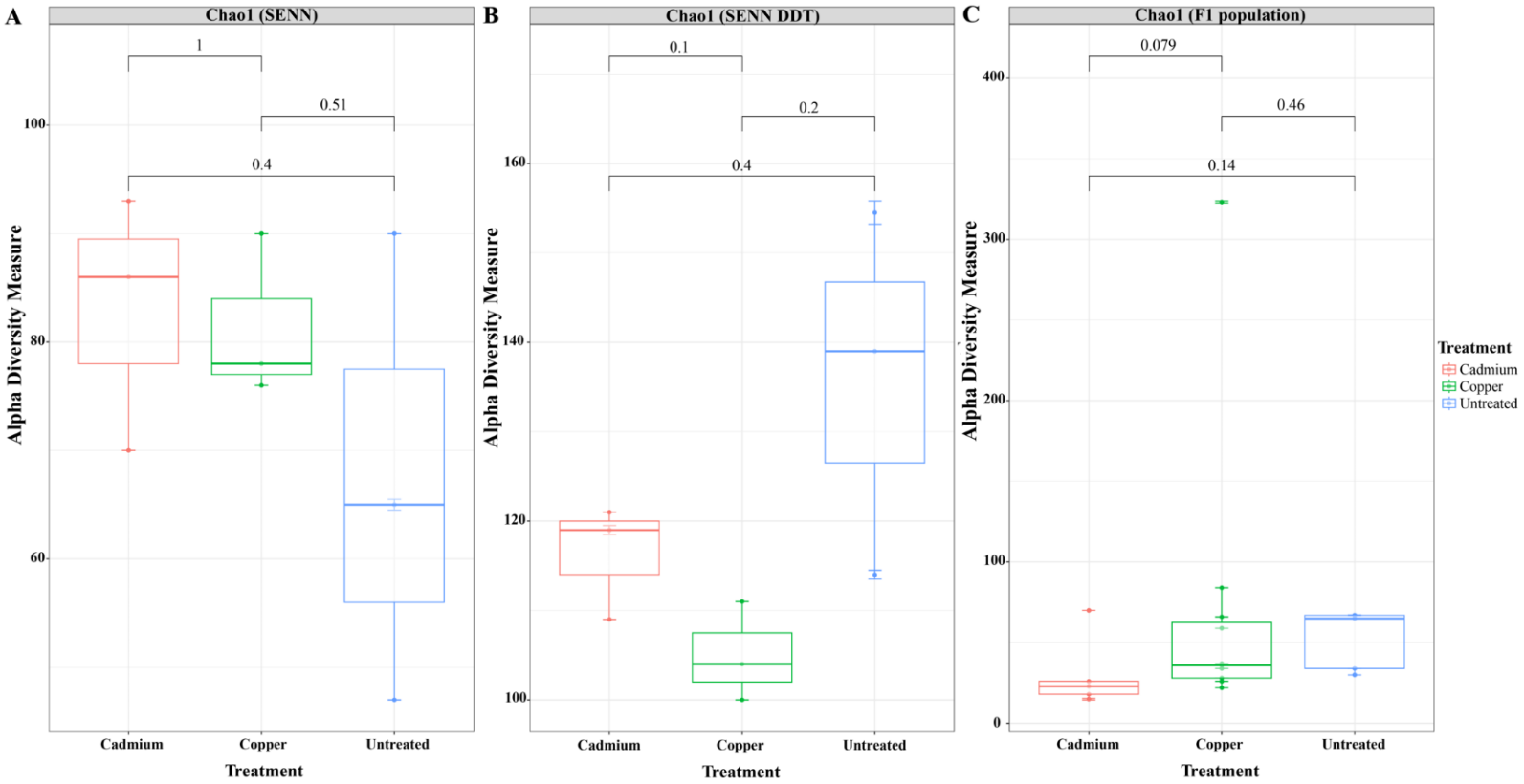

Figure 4.5 Comparison of the α- diversity (species richness) of the gut microbiota between untreated *Anopheles arabiensis* adults and those exposed to heavy metals as larvae.

Chao1 diversity indices of the gut microbiota for untreated three-day old *An. arabiensis* adults and those exposed to cadmium or copper as larvae. **(A)** For laboratory reared insecticide susceptible *An. arabiensis* (SENN). **(B)** For laboratory reared insecticide resistant *An. arabiensis* (SENN DDT). **(C)** For the F_1 population of wild *An. arabiensis*. Comparisons of the diversity indices between untreated *An. arabiensis* adults and those exposed to cadmium or copper as larvae were assessed using a Kruskal-Wallis rank-sum test. The dots represent the number of biological replicates used for each group. Significant differences in the diversity indices between samples are indicated by bold print.

The Shannon-Weiner diversity index was used as an estimator of species richness and evenness which considers both the number of different bacterial species and the relative abundance of each species [153,155,156].

No significant differences were observed in the Shannon-Weiner indices between untreated adults and those exposed to cadmium or copper as larvae (**Figure 4.6**). There were no significant differences in the Shannon-Weiner indices between untreated SENN adults and those exposed to cadmium ($p = 0.70$) or copper ($p = 0.07$) as larvae (**Figure 4.6 A**). Similarly, there were no significant differences in the Shannon-Weiner indices between untreated SENN DDT adults and those exposed to cadmium ($p = 1.00$) or copper ($p = 0.20$) as larvae (**Figure 4.6** B). The Shannon-Weiner indices were not significantly different between untreated F1 population adults and those exposed to cadmium ($p = 0.31$) or copper ($p = 1.00$) as larvae (**Figure 4.6 C**).

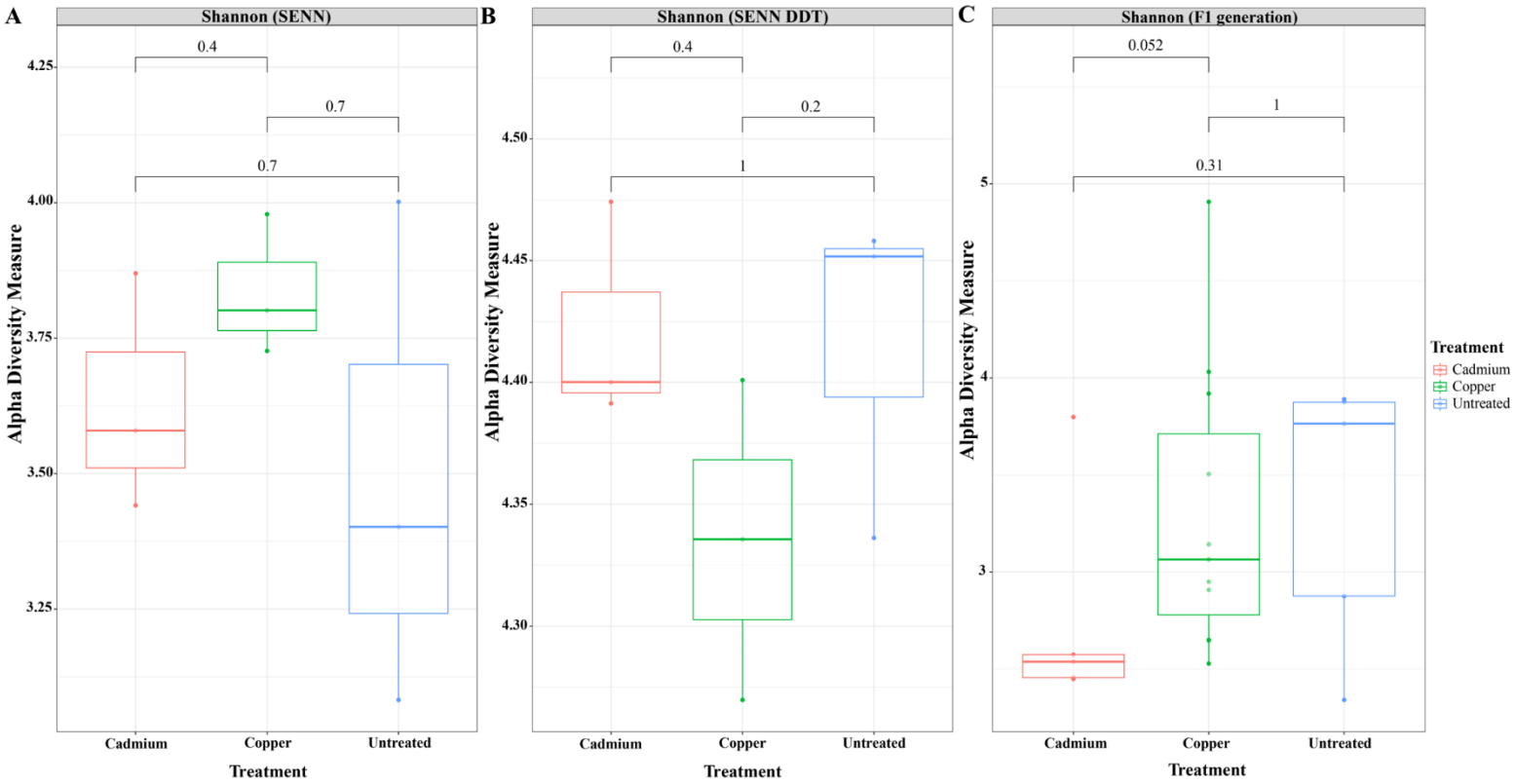

Figure 4.6 Comparison of the α- diversity (species richness and evenness) of the gut microbiota between untreated *Anopheles arabiensis* adults and those exposed to heavy metals as larvae.

Shannon-Weiner diversity indices of the gut microbiota for untreated three-day old *An. arabiensis* adults and those exposed to cadmium or copper as larvae. **(A)** For laboratory reared insecticide susceptible *An. arabiensis* (SENN). **(B)** For laboratory reared insecticide resistant *An. arabiensis* (SENN DDT). **(C)** For the F_1 population of *An. arabiensis*. Comparisons of the diversity indices between untreated three-day old *An. arabiensis* adults and those exposed to cadmium or copper as larvae were assessed using a Kruskal-Wallis rank-sum test. The dots represent the number of biological replicates used for each group. Significant differences in the diversity indices between samples are indicated by bold print.

4.3.6 β-diversity of the gut microbiota

β-diversity analysis was used to determine interspecies differences [147] in the gut microbiota diversity between the laboratory reared (SENN and SENN DDT) and the F_1 population of untreated three-day old *An. arabiensis* adults and those exposed to heavy metals, cadmium, or copper as larvae. This was visualised using two-dimensional Non-Metric Dimensional Scaling (NMDS) ordination. The differences in the diversity of gut bacteria were assessed using the Bray-Curtis dissimilarity distances. Permutational multivariate analysis (PERMANOVA) was used to determine if there were any significant interspecies differences in the gut microbiota diversity.

The bacterial communities between the insecticide susceptible and resistant laboratory reared adults (SENN and SENN DDT) across all treatments did not overlap but clustered together. By contrast, the bacterial communities identified in the F_1 population had a greater diversity and overlapped across all treatments. Notably, there were significant differences in the gut microbiota diversity between the different *An. arabiensis* groups and their treatments (PERMANOVA; $p = 0.01$, $F = 6.77$, stress value = 0.09) (**Figure 4.7**).

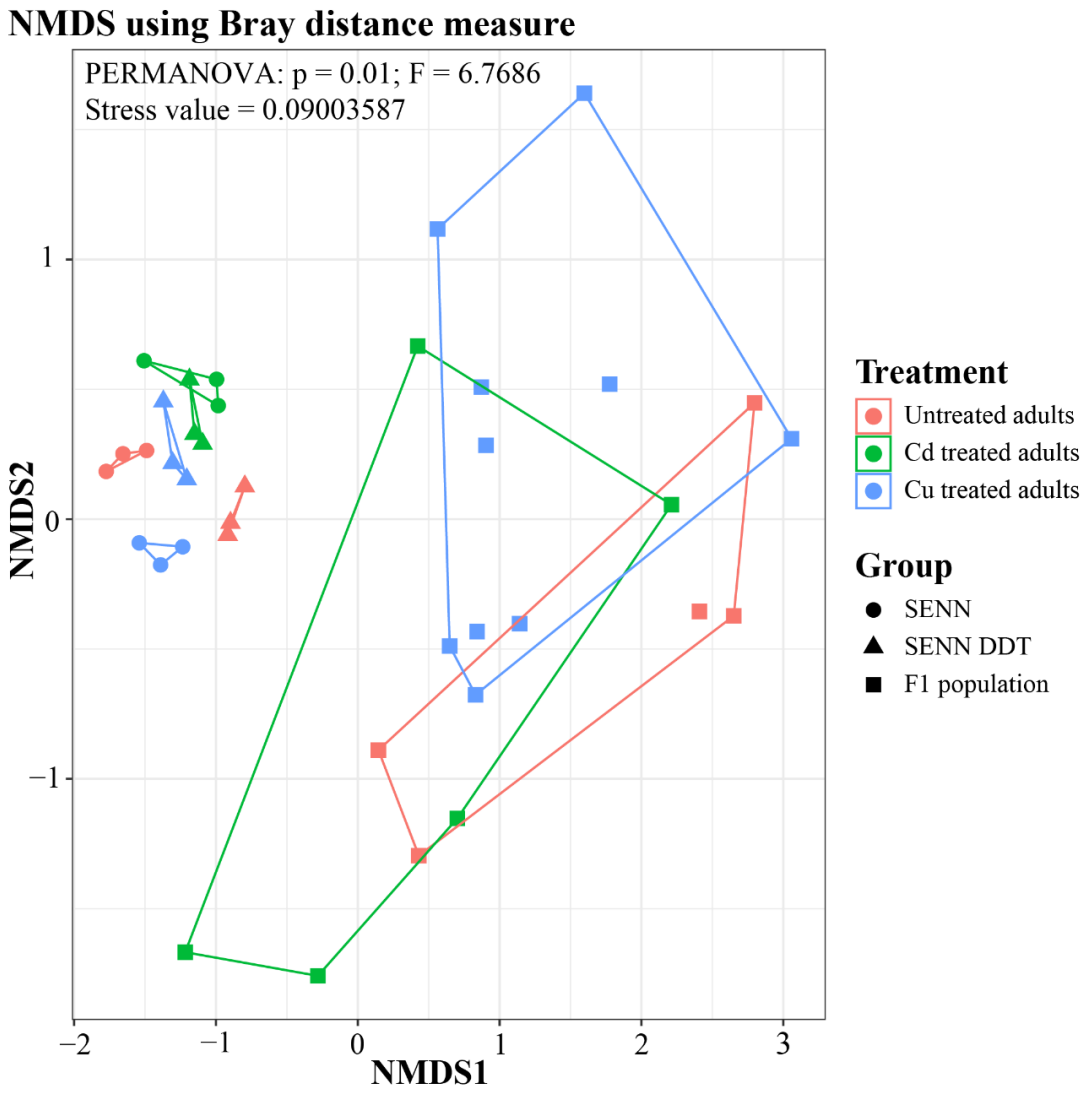

Figure 4.7 Differences in the gut microbiota diversity between untreated *Anopheles arabiensis* adults and those exposed to heavy metal treatments as larvae.

A visual representation of the β-diversity analysis of the gut bacteria between untreated the laboratory reared strains (SENN and SENN DDT) and the F_1 population of *An. arabiensis* three-day old adults and those exposed to cadmium or copper treatments as larvae. The different colours represent the various treatments used to rear *An. arabiensis*, red represents untreated adults, green represents adults exposed to cadmium as larvae and blue represents adults exposed to copper as larvae. The shapes represent the different strains of *An. arabiensis* adults, circles represent insecticide susceptible laboratory reared *An. arabiensis* (SENN), triangles represent insecticide resistant laboratory reared *An. arabiensis* (SENN DDT) and squares represent the F_1 population of *An. arabiensis*. Significant differences in the bacterial diversity between the laboratory reared and the F_1 population of *An. arabiensis* across metal treatments were assessed using a PERMANOVA (p = 0.01).

4.3.7 Overlapping bacterial communities

Shared and unique bacterial genera were represented numerically on UpSet plots between the untreated laboratory reared strains (SENN and SENN DDT) and the F_1 population of three-day old *An. arabiensis* adults and those exposed to cadmium and copper as larvae. The number of shared and unique bacterial genera between untreated adults and those exposed to heavy metals as larvae is shown in **Figure 4.8**. Bacterial communities that were identified to the genus level are listed below. Note that not all bacterial communities were identified to the respective taxonomic levels, as this is a limitation of 16S rRNA gene sequencing [200–202]. Therefore, the identified bacterial communities may not correspond to the numbers below.

Seventeen bacterial genera, including *Acidovorax, Acinetobacter, Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium, Armatimonas, Bosea, Kaistia, Microbacterium, Novosphingobium, Pedobacter, Phenylobacterium, Phreatobacter, Rurimicrobium, Variovorax,* and *Yersinia*, were shared between untreated and heavy metal treated SENN adults. Seven bacterial genera, including *Azospirillum, Chryseobacterium, Rhodopseudomonas,* and *Sphingobacterium,* were shared between SENN adults exposed to copper or cadmium as larvae. Eight bacterial genera, including *Aeromonas, Alsobacter, Asticcacaulis, Iamia, Pseudarcicella, Reyranella,* and *Xylophilus*, were shared between untreated SENN adults and those exposed to copper as larvae. Fourteen bacterial genera, including *Bdellovibrio, Fibrella, Methylophilus, Pseudomonas, Siphonobacter,* and *Sphingomonas,* were unique to SENN adults exposed to cadmium as larvae. Fifteen bacterial genera, including *Abditibacterium, Ancylobacter, Caulobacter, Delftia, Elizabethkingia, Heliimonas, Paucibacter, Pelomonas, Pseudochrobactrum, Rahnella, Shinella,* and *Spirosoma,* were unique to SENN adults exposed to cadmium as larvae (**Figure 4.8 A**).

Twenty-nine bacterial genera, including *Aeromonas, Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium, Alsobacter, Armatimonas, Asinibacterium, Asticcacaulis, Bosea, Bradyrhizobium, Chryseobacterium, Curvibacter, Elizabethkingia, Kaistia, Luteolibacter, Microbacterium, Mucilaginibacter, Novosphingobium, Nubsella, Pedobacter, Reyranella, Rhodoferax, Rurimicrobium, Siphonobacter, Sphingobacterium, Undibacterium, Variovorax,* and *Yersinia,* were shared between untreated and heavy metal treated SENN DDT adults. Three bacterial genera, including *Paracoccus* and *Terrimicrobium,* were shared between SENN DDT adults exposed to copper and cadmium as larvae. Six bacterial genera, including *Camelimonas, Hephaestia, Lacunisphaera, Methylophilus,* and *Pelomonas,* were shared

between untreated SENN DDT adults and those exposed to cadmium as larvae. Nine bacterial genera, including *Acidovorax*, *Chitinimonas*, *Delftia*, *Heliimonas*, *Mycobacterium*, *Phreatobacter*, *Pseudomonas*, and *Variovorax*, were shared between untreated SENN DDT adults and those exposed to copper as larvae. Ten bacterial genera, including *Abditibacterium*, *Caulobacter*, *Ferruginibacter*, *Fibrella*, *Paucibacter*, *Phenylobacterium*, *Rubritepida*, and *Shinella*, were unique to SENN DDT adults exposed to cadmium as larvae. Seven bacterial genera, including *Brevundimonas*, *Clostridium sensu stricto* 13, *Ochrobactrum*, *Roseomonas*, and *Stenotrophomonas*, were unique to SENN DDT adults exposed to copper as larvae (**Figure 4.8 B**).

Twenty bacterial genera, including *Bacillus*, *Cloacibacterium*, *Corynebacterium*, *Enterobacter*, *Enterococcus*, *Escherichia-Shigella*, *Gluconobacter*, *Klebsiella*, *Kluyvera*, *Limosilactobacillus*, *Listeria*, *Pseudomonas*, *Rahnella*, *Staphylococcus*, *Streptococcus*, and *Veillonella*, were shared between untreated and heavy metal treated wild (F_1 population) adults. One unidentified genus was shared between the F_1 population adults exposed to copper and cadmium as larvae. Six bacterial genera, including *Cutibacterium*, *Elizabethkingia*, *Porphyromonas*, *Peptoniphilus*, *Salmonella*, and *Serratia*, were shared between the untreated F_1 population adults and those exposed to cadmium as larvae. Three bacterial genera, including *Anaerococcus*, *Anoxybacillus*, and *Fenollaria*, were shared between the untreated F_1 population adults and those exposed to copper as larvae. Six bacterial genera, including *Lactococcus*, *Morganella*, *Rhodopseudomonas*, and *Truepera*, were unique to the F_1 population of adults exposed to cadmium as larvae. Fifteen bacterial genera, including *Acetobacter*, *Acinetobacter*, *Asaia*, *Blattabacterium*, *Dyella*, *Ezakiella*, *Flavobacterium*, *Frateuria*, *Gluconacetobacter*, *Herbaspirillum*, *Sphingobacterium*, and *Tepidimonas*, were unique to the F_1 population adults exposed to copper as larvae (**Figure 4.8 C**).

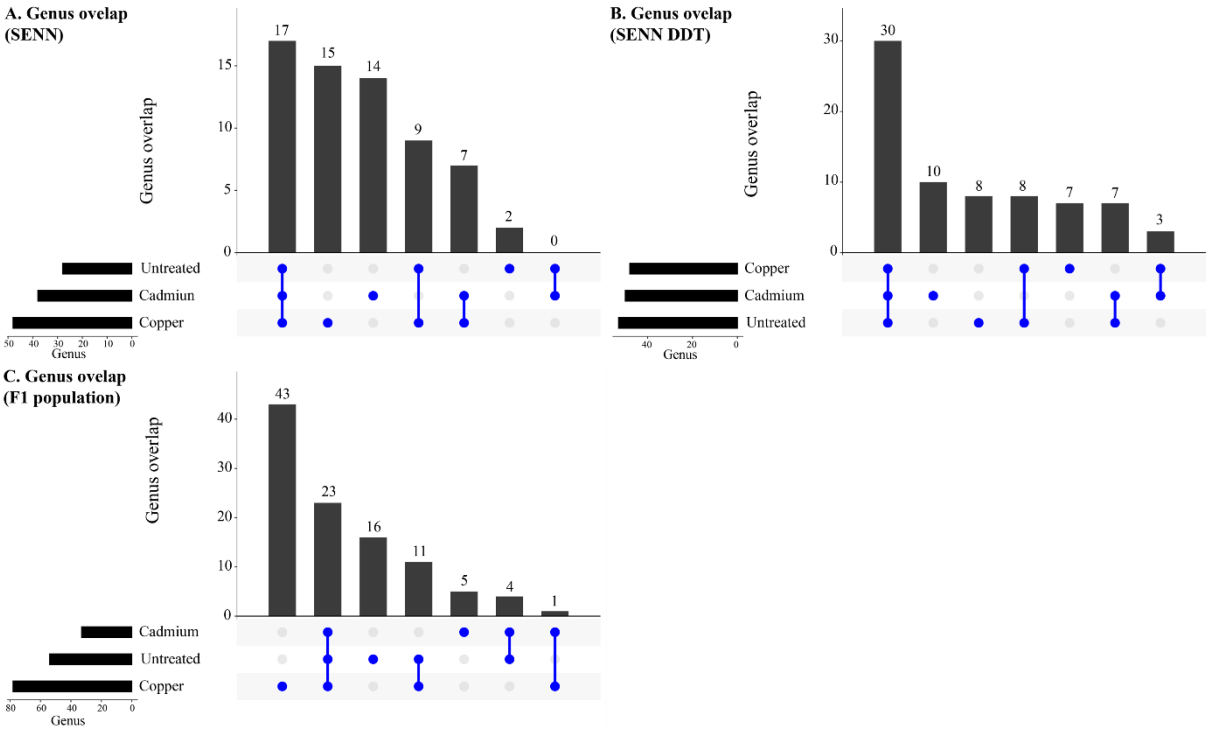

Figure 4.8 Comparison of overlapping gut bacteria between untreated *Anopheles arabiensis* adults and those exposed to heavy metal treatments as larvae.

(A) Overlapping bacterial genera between the untreated, cadmium-treated, and copper-treated laboratory reared *An. arabiensis* (SENN) adults. **(B)** Overlapping bacterial genera between the untreated, cadmium-treated, and copper-treated laboratory reared *An. arabiensis* (SENN DDT) adults. **(C)** Overlapping bacterial genera between the untreated, cadmium-treated, and copper-treated F_1 population of *An. arabiensis* adults. The blue dots with a joined line represent the overlaps of bacteria between untreated, cadmium-treated, and copper-treated three-day old *An. arabiensis* adults, with single dots indicating the unique bacteria per strain. Each numbered column represents the number of bacterial genera that overlap.

4.3.8 Differential abundance of bacterial genera

Differential abundance of the gut microbiota with pairwise comparisons was assessed between the untreated insecticide susceptible laboratory reared (SENN) adults and those exposed to heavy metals (cadmium or copper) as larvae. Differentially abundant genera were considered significantly different when the assigned log2 Fold Change values were compared and had a *p*-value < 0.01.

Fifteen bacterial genera, including *Alsobacter*, *Terrimicrobium*, *Bradyrhizobium*, *Reyranella*, *Iamia*, *Aeromonas*, *Acinetobacter*, *Phreatobacter*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Variovorax*, *Armatimonas*, *Bosea*, *Kaistia*, *Pedobacter*, and *Microbacterium*, were significantly abundant in untreated SENN adults compared to SENN adults exposed to cadmium as larvae (**Figure 4.9 A**).

Nineteen bacterial genera, including *Chryseobacterium*, *Acinetobacter*, *Phreatobacter*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Siphonobacter*, *Novosphingobium*, *Rurimicrobium*, *Azospirillum*, *Enterobacter*, *Blastocatella*, *Phenylobacterium*, *Variovorax*, *Fibrella*, *Armatimonas*, *Bosea*, *Kaistia*, *Pedobacter*, *Sphingobacterium*, and *Microbacterium*, were significantly abundant in SENN adults exposed to cadmium as larvae compared to untreated SENN adults (**Figure 4.9 A**).

Seventeen bacterial genera, including *Yersinia*, *Terrimicrobium*, *Bradyrhizobium*, *Pedobacter*, *Acidovorax*, *Armatimonas*, *Alsobacter*, *Microbacterium*, *Variovorax*, *Iamia*, *Acinetobacter*, *Novosphingobium*, *Bosea*, *Phreatobacter*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Kaistia*, and *Aeromonas*, were significantly abundant in untreated SENN adults compared to SENN adults exposed to copper as larvae (**Figure 4.9 B**).

Thirty bacterial genera, including *Pedobacter*, *Caulobacter*, *Paucibacter*, *Acidovorax*, *Armatimonas*, *Alsobacter*, *Sphingobacterium*, *Rhodopseudomonas*, *Phenylobacterium*, *Microbacterium*, *Pelomonas*, *Elizabethkingia*, *Pseudarcicella*, *Heliimonas*, *Chryseobacterium*, *Ancylobacter*, *Spirosoma*, *Variovorax*, *Iamia*, *Azospirillum*, *Asticcacaulis*, *Acinetobacter*, *Novosphingobium*, *Bosea*, *Shinella*, *Phreatobacter*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Kaistia*, *Aeromonas*, and *Reyranella*, were significantly abundant in SENN adults exposed to copper as larvae compared to untreated SENN adults (**Figure 4.9 B**).

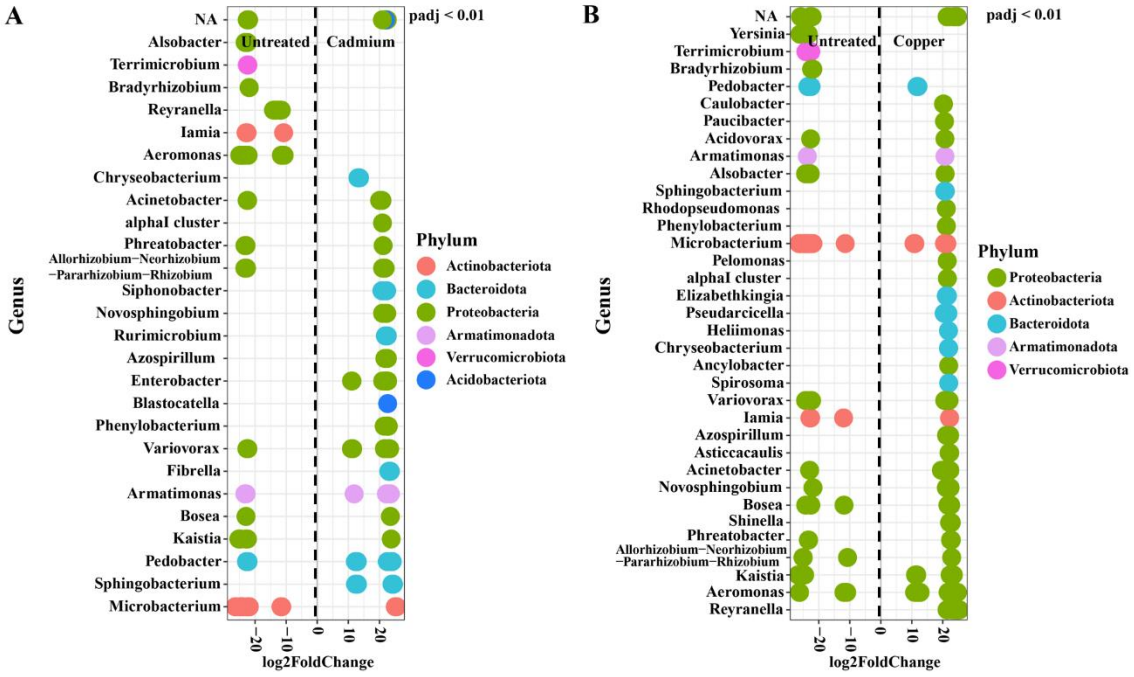

Figure 4.9 Pairwise comparisons of differentially abundant bacterial genera between untreated insecticide susceptible laboratory reared *Anopheles arabiensis* (SENN) adults and those exposed to heavy metal treatments as larvae.

Differential abundance of the gut microbiota of insecticide susceptible laboratory reared *An. arabiensis* (SENN) three-day old adults between **(A)** untreated SENN adults vs SENN adults exposed to cadmium as larvae, and **(B)** untreated SENN adults vs SENN adults exposed to copper as larvae. Differentially abundant genera had a significance threshold of *p*-value < 0.01. The treatment is on either side of the dotted line, showing where the relevant bacterial genera are more abundant.

Differential abundance of the gut microbiota with pairwise comparisons was assessed between the untreated insecticide resistant laboratory reared (SENN DDT) adults and those exposed to heavy metals (cadmium or copper) as larvae. Differentially abundant genera were considered significantly different when the assigned log2 Fold Change values were compared and had a *p*-value < 0.01.

Twenty-nine bacterial genera, including *Pseudacidovorax*, *Reyranella*, *Delftia*, *Pelomonas*, *Sphingobacterium*, *Variovorax*, *Undibacterium*, *Phreatobacter*, *Heliimonas*, *Pseudomonas*, *Comamonas*, *Elizabethkingia*, *Pedobacter*, *Aeromonas*, *Rhodoferax*, *Asticcacaulis*, *Allorhizobium−Neorhizobium−Pararhizobium−Rhizobium*, *Bradyrhizobium*, *Rurimicrobium*, *Siphonobacter*, *Novosphingobium*, *Bosea*, *Kaistia*, *Asinibacterium*, *Luteolibacter*, *Mucilaginibacter*, *Microbacterium*, *Armatimonas*, and *Chryseobacterium*, were significantly abundant in untreated SENN DDT adults compared to SENN DDT adults exposed to cadmium as larvae (**Figure 4.10 A**).

Twenty-seven bacterial genera, including *Shinella*, *Rhodoferax*, *Asticcacaulis*, *Abditibacterium*, *Allorhizobium−Neorhizobium−Pararhizobium−Rhizobium*, *Methylophilus*, *Paracoccus*, *Alsobacter*, *Nubsella*, *Bradyrhizobium*, *Camelimonas*, *Lacunisphaera*, *Rurimicrobium*, *Caulobacter*, *Hephaestia*, *Phenylobacterium*, *Siphonobacter*, *Novosphingobium*, *Bosea*, *Kaistia*, *Asinibacterium*, *Luteolibacter*, *Mucilaginibacter*, *Microbacterium*, *Armatimonas*, *Terrimicrobium*, and *Chryseobacterium*, were significantly abundant in SENN DDT adults exposed to cadmium as larvae compared to untreated SENN DDT adults (**Figure 4.10 A**).

Thirty bacterial genera, including *Pseudacidovorax*, *Pedobacter*, *Pelomonas*, *Pseudomonas*, *Hephaestia*, *Elizabethkingia*, *Comamonas*, *Aeromonas*, *Reyranella*, *Asticcacaulis*, *Luteolibacter*, *Nubsella*, *Novosphingobium*, *Variovorax*, *Delftia*, *Chryseobacterium*, *Siphonobacter*, *Heliimonas*, *Allorhizobium−Neorhizobium−Pararhizobium−Rhizobium*, *Undibacterium*, *Armatimonas*, *Rhodoferax*, *Bradyrhizobium*, *Phreatobacter*, *Rurimicrobium*, *Asinibacterium*, *Bosea*, *Kaistia*, *Microbacterium*, and *Sphingobacterium*, were significantly abundant in untreated SENN DDT adults compared to SENN DDT adults exposed to copper as larvae (**Figure 4.10 B**).

Thirty-two bacterial, genera including *Reyranella*, *Paracoccus*, *Brevundimonas*, *Ochrobactrum*, *Acidovorax*, *Asticcacaulis*, *Luteolibacter*, *Nubsella*, *Novosphingobium*, *Variovorax*, *Delftia*, *Chryseobacterium*, *Alsobacter*, *Stenotrophomonas*, *Mycobacterium*, *Siphonobacter*, *Yersinia*, *Heliimonas*, *Undibacterium*, *Armatimonas*, *Rhodoferax*, *Allorhizobium−Neorhizobium−Pararhizobium−Rhizobium*, *Bradyrhizobium*, *Phreatobacter*, *Rurimicrobium*, *Curvibacter*, *Terrimicrobium*, *Asinibacterium*, *Bosea*, *Kaistia*, *Microbacterium*, *Sphingobacterium*, were significantly abundant in SENN DDT adults exposed to copper as larvae compared to untreated SENN DDT adults (**Figure 4.10 B**).

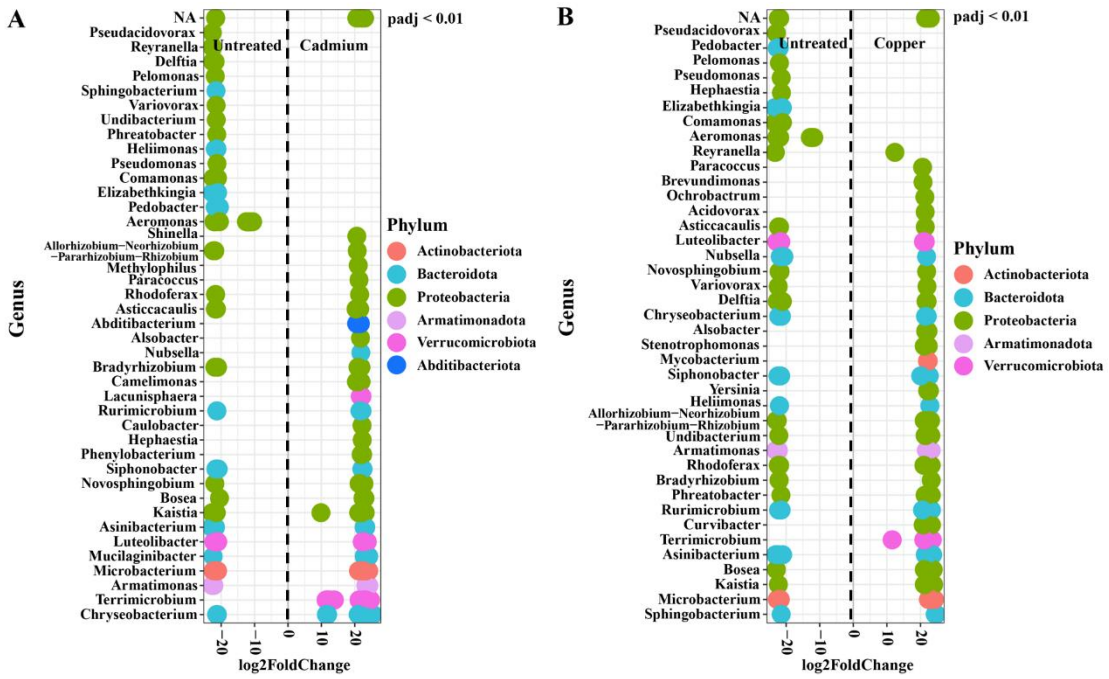

Figure 4.10 Pairwise comparisons of differentially abundant bacterial genera between untreated insecticide resistant laboratory reared *Anopheles arabiensis* (SENN DDT) adults and those exposed to heavy metal treatments as larvae.

Differential abundance of the gut microbiota of insecticide resistant laboratory reared *An. arabiensis* (SENN DDT) three-day old adults between **(A)** untreated SENN DDT adults vs SENN DDT adults exposed to cadmium as larvae, and **(B)** untreated SENN DDT adults vs SENN DDT adults exposed to copper as larvae. Differentially abundant genera had a significance threshold of p-value < 0.01. The treatment is on either side of the dotted line, showing where the relevant bacterial genera are more abundant.

Differential abundance of the gut microbiota with pairwise comparisons was assessed between the untreated F_1 population adults and those exposed to heavy metals (cadmium or copper) as larvae. Differentially abundant genera were considered significantly different when the assigned log2 Fold Change values were compared and had a *p*-value < 0.01.

Thirty-two bacterial genera, including *Thermicanus*, *Delftia*, *Salmonella*, *Pseudomonas*, *Anoxybacillus*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Peptoniphilus*, *Petrimonas*, *Elizabethkingia*, *Fenollaria*, *Proteiniphilum*, *Acidibacter*, *Massilia*, *Mycobacterium*, *Glutamicibacter*, *Geobacillus*, *Novosphingobium*, *Moheibacter*, *Bacillus*, *Escherichia-Shigella*, *Staphylococcus*, *Cloacibacterium*, *Anaerococcus*, *Porphyromonas*, *Veillonella*, *Streptococcus*, *Cutibacterium*, *Corynebacterium*, *Enterobacter*, *Enterococcus*, *Listeria*, and *Klebsiella*, were significantly abundant in the untreated F_1 population adults compared to the F_1 population adults exposed to cadmium as larvae (**Figure 4.11 A**).

Fourteen bacterial genera, including *Porphyromonas*, *Veillonella*, *Streptococcus*, *Lactococcus*, *Cutibacterium*, *Corynebacterium*, *Gluconobacter*, *Rahnella*, *Enterobacter*, *Enterococcus*, *Limosilactobacillus*, *Listeria*, *Morganella*, and *Klebsiella*, were significantly abundant in the F_1 population adults exposed to cadmium as larvae compared to the untreated F_1 population adults (**Figure 4.11 A**).

Thirty-four bacterial genera, including *Listeria*, *Thermicanus*, *Delftia*, *Salmonella*, *Pseudomonas*, *Streptococcus*, *Cutibacterium*, *Anoxybacillus*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Limosilactobacillus*, *Fenollaria*, *Petrimonas*, *Elizabethkingia*, *Peptoniphilus*, *Massilia*, *Proteiniphilum*, *Acidibacter*, *Mycobacterium*, *Bacillus*, *Glutamicibacter*, *Geobacillus*, *Cloacibacterium*, *Enterococcus*, *Moheibacter*, *Porphyromonas*, *Veillonella*, *Novosphingobium*, *Corynebacterium*, *Escherichia-Shigella*, *Anaerococcus*, *Staphylococcus*, *Enterobacter*, and *Klebsiella*, were significantly abundant in the untreated F_1 population adults compared to the F_1 population adults exposed to copper as larvae (**Figure 4.11 B**).

Eight bacterial genera, including *Frateuria*, *Enterobacter*, *Asaia*, *Kluyvera*, *Klebsiella*, *Acetobacter*, *Gluconobacter*, and *Rahnella*, were significantly abundant in the F_1 population adults exposed to copper as larvae compared to the untreated F_1 population adults (**Figure 4.11 B**).

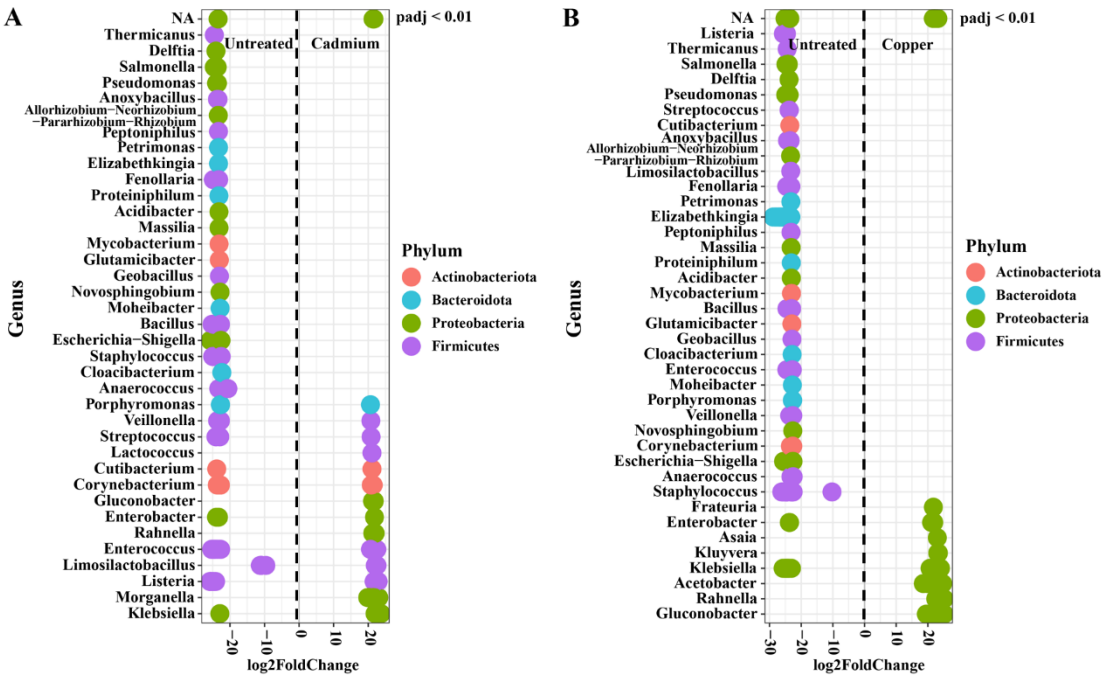

Figure 4.11 Pairwise comparisons of differentially abundant bacterial genera between the untreated F_1 population of wild *Anopheles arabiensis* adults and those exposed to heavy metal treatments as larvae.

Differential abundance of the gut microbiota of three-day old F_1 population *An. arabiensis* adults between **(A)** untreated F_1 population adults vs F_1 population adults exposed to cadmium as larvae, and **(B)** untreated F_1 population adults vs F_1 population adults exposed to copper as larvae. Differentially abundant genera had a significance threshold of *p*-value < 0.01. The treatment is on either side of the dotted line, showing where the relevant bacterial genera are more abundant.

4.4 Discussion

Although this chapter covers a substantial body of data, the results can broadly be divided into two categories. The first is a comparison of the two laboratory strains from Sudan and a wild and F_1 population. The second is a characterisation of the effect of larval exposure to copper and cadmium on the two laboratory strains and an F_1 population. For both these sections, the results look at the microbial diversity and abundance of specific genera. To the best of our knowledge, this is the first investigation conducted to determine the changes in the gut microbiota of *An. arabiensis* adults caused by metal pollutants in the larval breeding water.

This study compared the bacterial dynamics of *An. arabiensis* from South Africa to that of two laboratory strains from Sudan. Ideally, it would have been best to do the comparison with a laboratory strain from South Africa. This would have given information on the effect of keeping mosquitoes in colony. However, a specific aim was to understand the effect of the pre-existence of a resistant phenotype, and for this reason, the SENN strain and its' resistant sister strain, SENN DDT, were used. The role of microbiota in the construction of a resistant phenotype is of interest. For example, fenitrothion resistant wild *An. albimanus* had a lower bacterial diversity compared to insecticide susceptible individuals [102]. This was not seen in the current study, which may seem like a contradiction, but there are a few considerations. In a study on *An. albimanus*, the mosquitoes were classified as moderately resistant (surviving 5X the diagnostic dose), but there is no description of the resistance mechanism. The resistant phenotype in SENN DDT is complex. It is highly resistant to DDT, but the resistance to pyrethroids is at low intensity. The resistance phenotype also consists of both target site resistance and metabolic detoxification [104,235]. Therefore, it is unlikely that insecticide-degrading bacteria play a role in the construction of the insecticide resistant phenotype in SENN DDT. This could be confirmed by comparison to a fully susceptible strain of the same species, as SENN is not a standard susceptible strain, and it is relatively easy to select for resistance in this strain [107]. Crucially, to confirm this, the specific bacterial strains must be demonstrated to degrade pesticides. However, it is interesting to note that of the six unique species in SENN DDT, four species can degrade herbicides. As *Sphingobacterium multivorum* [236], *Cupriavidus metallidurans* [237], *Enterobacter hormaechei* [238], and *Rhodococcus erythropolis* [239] can all degrade herbicides and, in some cases, pesticides, the role of specific gut microbiota in the construction of the resistance phenotype in SENN DDT could still be investigated further.

Consistent with previous studies [43,216], the bacterial diversity in laboratory reared strains was significantly lower compared to F_1 and wild populations of *An. arabiensis*. This reduction in diversity could be due to the loss of native bacteria over multiple generations of laboratory-rearing [43,240]. A study done on the conservation of diversity and composition of different populations of *An. gambiae* collected from the field demonstrated that the α-diversity of the mosquitoes reduced significantly after ten generations under laboratory reared conditions [87]. This is likely due to the reduced diversity of larval rearing water. In the Botha de Meillon insectary, reverse osmosis water is used to rear larvae. Other laboratories may use tap water, but regardless of this, insectary water will always be less bacterially diverse than water in the field [87]. The homogenous larval diet would also contribute less to the microbial diversity than the material that would constitute food in the field. This would contribute to a reduced diversity of gut bacteria [241].

The results of the β-diversity analysis echo that of the α-diversity analysis. There is a lack of overlap between the laboratory strains and the F_1 and wild populations. The laboratory strains did not overlap, but the diversity of the insecticide susceptible strain was more diverse. Notably, although the wild and F_1 populations are not directly related, as they were collected at different periods, the F_1 population falls within the wild population. This is expected for individuals from the same geographical location. This would also be expected from the significantly increased diversity of the F_1 population compared to that of the wild population. This suggests that in the colonisation procedure, the microbial diversity will become more even before a reduction in the richness that comes with the selection of strains that will become prevalent in laboratory strains.

The gut microbiota of laboratory reared and wild *Anopheles* mosquitoes are dominated by Gram-negative Proteobacteria [43,117,242–244]. Several studies have characterised a suggested core microbiota in field-collected *Anopheles* adults [43,243,245,246]. In wild *An. stephensi* adults, collected as larvae, the core gut microbiota comprised of *Serratia*, *Cedecea*, *Elizabethkingia*, *Klebsiella*, and *Asaia* [245]. In *An. gambiae* adults obtained from larval collections, the genera *Asaia, Burkholderia*, *Serratia, Ralstonia, Acinetobacter, Pseudomonas*, *Sphingomonas*, *Staphylococcus*, *Streptococcus*, and *Escherichia-Shigella* were suggested to be part of the core microbiota [43]. Other *Anopheles* species outside of the *An. gambiae* complex, such as larval-collected and wild adult *Anopheles atroparvus*, have a core microbiota that is suggested to include *Pantoea*, *Thorsellia*, *Serratia*, *Asaia*, and *Pseudomonas* [246]. In this study, the core gut microbiota from field-collected adults from

Mamfene in Kwa-Zulu Natal, South Africa, is suggested to include the bacterial genera *Rahnella, Yersinia, Asaia, Clavibacter, Cedecea, Pseudomonas, Aeromonas, Klebsiella,* and *Enterobacter*. This is further emphasised as *Cedecea, Klebsiella*, *Rahnella,* and *Pseudomonas* were abundant in the F_1 population and wild population compared to the laboratory strains SENN and SENN DDT. The suggested core microbiota is consistent with previous studies as these bacterial genera were found to dominate the gut of field-collected *An. stephensi* and *An. gambiae* [43,245].

Several *Plasmodium*-protective bacterial genera were identified in this study, including *Enterobacter, Pseudomonas,* and *Asaia*. For example, *Enterobacter* Esp_Z fed to *An. gambiae* demonstrated *P. falciparum* inhibition [71,80]. Other *Enterobacter* species, such as *E. cloacae,* can activate immune responses to *P. berghei* in laboratory-reared *An. stephensi* [247,248]. *Pseudomonas putida,* isolated from the gut of wild *An. arabiensis* mosquitoes from Zambia, was introduced into laboratory reared *An. gambiae* via a sugar meal and inhibited the development of *P. falciparum* [72]. In another study, exposure to *P. stutzeri* reduced the prevalence and intensity of *P. falciparum* infection in *An. gambiae* [249]. *Pseudomonas* can be transstadially transmitted as it was able to colonise the Malpighian tubules *in An. stephensi* [51]. This highlights the potential role of this bacterium as a paratransgenesis candidate. *Pseudomonas* is therefore an important candidate for paratransgenesis interventions. In this study, *P. proteolytica* and other *Pseudomonas* species, which could not be identified to species level, were observed in the F_1 population and wild population of *An. arabiensis*. *Pseudomonas gessardii* was unique to the F_1 population, and *Pseudomonas palleroniana* was unique to the wild population. Additionally, *Pseudomonas* was abundant in the F_1 and F_0 populations compared to the laboratory reared SENN and SENN DDT strains. *Enterobacter* and *Pseudomonas* are important as they are naturally occurring symbionts in *An. arabiensis* found in Mamfene, KZN. Whole genome sequencing would be required to identify the *Enterobacter* and *Pseudomonas* to the species and strain levels.

In this study, *Asaia krungthepensis* and *Asaia* sp., which were not identified to species level, were only found in the gut of the F_1 and wild populations of *An. arabiensis*. *Asaia* isolates were introduced into *An. stephensi* and had induced basal-level immunity that resulted in a significant reduction in *P. berghei* oocyst development [250]. Another study yielded similar results in *An. stephensi,* where modified *Asaia* strains could suppress the number of oocysts formed after consuming a blood meal infected with *P. berghei* more effectively than wild-type *Asaia bogorensis* [251]. *Asaia* colonises the female gut and salivary glands and the male

reproductive glands [47]. This bacterium can be transmitted maternally or paternally to their offspring [45,47]. Additionally, *Asaia* spp. are suggested to play a beneficial role in the development of laboratory reared *An. Stephensi* larvae and there are no fitness costs associated with the introduction of this bacterium [49]. This highlights the role of *Asaia* as a naturally occurring symbiont for paratransgenesis. In addition to the current study, *Asaia* has also been identified in field-collected *Anopheles* adults from Cameroon and their F_1 progeny [252]. Since *Asaia* has been identified in several *Anopheles* species, including *An. coluzzii*, *An. arabiensis*, *An. funestus* and *An. gambiae*, it has been suggested as a potential symbiont for controlling malaria in sub-Saharan Africa [252]. The presence of *Asaia* in the gut of the F_1 population and wild population of *An. arabiensis* further emphasises the possibility of this symbiont for controlling malaria in sub-Saharan Africa. Additionally, *Wolbachia* may not be a suitable microbial-based vector control intervention for wild *An. arabiensis* from KZN as bacterial competition between *Asaia* and *Wolbachia* would inhibit the vertical transmission and colonisation of *Wolbachia* in *Anopheles* mosquitoes [125].

The information is useful for considering a paratransgenesis for the KZN province. This is particularly appealing, as this province is the closest in the country to eliminating malaria [31]. The final hurdle is the problem with residual malaria, largely driven by the outdoor biting activity of *An. arabiensis* [253]. Therefore, paratransgenesis is an attractive option to augment the elimination agenda in this province. If native symbionts are to be used, it would be critical to understand the effects of the larval environment on the bacterial dynamics. With increasing examples of *An. arabiensis* breeding in polluted areas [111,254]. Heavy metals, which are common pollutants, were chosen as representative pollutants.

It is expected that heavy metal exposure on larvae will affect the diversity of the gut microbiota. In other insects, such as pygmy grasshoppers (*Eucriotettix oculatus*), populations living in mining areas polluted with metal pollutants displayed significantly decreased bacterial species richness and evenness of the gut microbiota [255]. A study on the gut microbiota of black soldier fly larvae (*Hermetia illucens*) revealed a notable reduction in the microbial diversity in their guts when exposed to metal pollutants (copper and cadmium) [256]. Surprisingly, this study showed no significant differences in the bacterial diversity of the gut microbiota between untreated adults and adults exposed to heavy metals copper and cadmium as larvae. This was true for laboratory reared (SENN and SENN DDT) strains and the F_1 population of *An. arabiensis*. Therefore, exposure to heavy metals, such as copper and cadmium, does not affect the bacterial diversity.

Despite the lack of differences in α-diversity, β-diversity analysis indicated significant differences in the gut microbiota diversity between untreated and those exposed to heavy metals as larvae. Like with the untreated samples, the diversity of the F_1 population was much greater than that of the laboratory strains. There was also more overlap in the diversity of the two treatments and the untreated individuals for the F_1 than for the laboratory strains. There was no overlap between the different treatments in any of the two laboratory strains. This indicates that larval exposure to heavy metals had a more profound effect on the F_1 population.

The laboratory reared SENN strain had a combined total of 64 bacterial genera present among untreated adults and those exposed to heavy metals, and the laboratory reared SENN DDT strain had a combined total of 74 bacterial genera present among all treatments. The F_1 population had a combined total of 104 bacterial genera present among all treatments. The number of unique genera differed in the three strains for each treatment. For the SENN strain, the untreated adults had only two unique genera. For SENN DDT and the F_1 population, the greatest number of unique genera were found in metal treatments (cadmium and copper treatment, respectively). When examining which genera were differentially abundant, there were large-scale variations. In general, SENN had less unique differentially abundant genera in the untreated samples (6:11 for cadmium, 3:17 for copper). The difference in SENN DDT was not marked (14:11 for cadmium, 8:10 for copper). However, many more uniquely differentially abundant genera were in the untreated adults of the F_1 population compared to those exposed to the metal treatments (19:3 for cadmium, 31:6 for copper). This has potential implications for identifying native symbionts. Metal treatment reduces the number of differentially abundant genera in the F_1 population. This means that culturing bacteria from the F_1 population reared in polluted water may be more difficult, which is likely true for larvae collected in the wild. As such, even though wild larvae would have the greatest diversity in gut microbiota, the optimal samples for obtaining culturable specimens may be in F_1 populations reared in laboratory water.

Larval exposure to heavy metals caused significant changes in the gut microbiota composition in SENN, SENN DDT, and F_1 population adults. Several bacterial genera were present in adults exposed to the heavy metals as larvae. Larval exposure to cadmium and copper increased the bacterial genera present in the gut microbiota of all adults under investigation compared to their untreated counterparts. However, adults exposed to copper as larvae had more bacterial genera than those exposed to cadmium. As reviewed by Samanovic *et al*. [257]

and Argüello *et al*. [258], copper is an essential micronutrient required by microorganisms, such as bacteria, for growth and development [257,258]. Therefore, the increase in bacterial genera in adults exposed to copper is likely attributed to copper acting as a micronutrient for bacteria. Copper is an essential micronutrient that acts as a cofactor to various proteins involved in cellular processes [259]. As reviewed by Samanovic *et al*. [257], copper, however, can act as an antimicrobial agent, and high concentrations of copper are toxic to organisms [257]. Thus, the concentration of copper used in this study likely aids in the growth of bacterial communities in the gut of adults exposed to copper as larvae. Additionally, copper can act as a micronutrient for bacteria in the surrounding larval environment of mosquito larvae, which has been suggested to play a role in the diversity of the gut microbiota [42,43]. Therefore, additional studies on the concentrations and the abundance of specific heavy metals in the larval breeding sites are required to gain insight into the role of heavy metals on the microbiota of mosquitoes obtained in the wild.

Studies on the gut microbiota of other insects, such as pygmy grasshoppers and chironomid larvae, show an increased abundance of specific bacterial genera when exposed to metal pollutants [255,260]. In pygmy grasshoppers, *Aeromonas*, *Wolbachia*, *Chryseobacterium*, *Cupriavidus*, and *Pseudomonas* were abundant [255]. The bacterial genera *Yersinia*, *Acinetobacter*, *Shinella*, *Dysgonomonas*, *Delftia*, *Enterococcus*, and *Chryseobacterium* had increased abundances in chironomid larvae [260]. Bacterial genera such as *Aeromonas*, *Chryseobacterium*, *Pseudomonas*, *Yersinia*, *Acinetobacter*, *Shinella*, *Delftia*, and *Enterococcus* observed in these insects were also identified in the gut of SENN, SENN DDT, and F_1 populations used in this study. This suggests a potential relationship between these bacterial genera and insect gut microbiota and the presence of heavy metals.

Bacterial genera such as *Bacillus*, *Klebsiella*, *Enterobacter*, *Microbacterium*, *Pseudomonas*, *Sphingomonas*, *Acinetobacter*, *Corynebacterium*, *Streptococcus*, *Rahnella*, *Staphylococcus*, and *Rhodopseudomonas* are tolerant to heavy metals and are involved in heavy metal degradation [261–264]. Other bacterial genera shared between insects exposed to heavy metals, including *Aeromonas*, *Chryseobacterium*, *Enterococcus*, *Yersinia*, *and Delftia*, are tolerant to heavy metals [65,265–268]. These genera were identified and were differentially abundant in the gut of SENN, SENN DDT, and F_1 populations used in this study (**Appendix 4: Bacteria Involved in Heavy Metal Metabolism**).

Both laboratory reared strains (SENN and SENN DDT) had predominantly heavy metal-tolerant bacterial genera. Notably, these genera were differentially abundant in untreated adults and those exposed to copper as larvae. SENN adults also had more genera that were able to degrade heavy metals. This is unsurprising as previous studies have demonstrated insecticide resistance affects how mosquitoes respond to starvation, oxidative stress, blood meals, and thermal stress [104–107]. As such, in the absence of a pre-existing resistant phenotype, the gut microbiota of SENN possibly aids in the degradation of heavy metals such as cadmium. This, however, would need to be empirically confirmed. The gut microbiota of SENN DDT adults is not as affected as SENN adults when exposed to cadmium in the larval environment. This further suggests that gut microbiota plays less of a role in protection against metal stress in this strain.

Notably, bacterial genera tolerant to heavy metals and involved in heavy metal degradation are predominantly present in the F_1 populations in both untreated adults and those exposed to either copper or cadmium as larvae. Furthermore, these bacterial genera are significantly abundant in untreated adults and those exposed to either copper or cadmium as larvae. This could be expected from the offspring of adults from Mamfene. This is a rural area where agricultural activities [229] require fertilisers, which may contribute to the contamination of the larval breeding site with heavy metals. Exposure to insecticides resulted in *An. albimanus* acquiring insecticide-degrading bacteria, which was suggested to be a result of selective pressure driven by insecticide exposure [103]. Furthermore, the effects of metal pollutant exposure on life history traits are transgenerational [232]. Exposure of the previous generations may have resulted in a selective pressure for heavy metal degrading bacteria which has been transmitted to the following generation.

Limitations in mosquito microbiome studies with wild mosquitoes are due to sample availability, which varies with region and seasonal changes [101]. This study obtained wild larvae and adults from a malaria-endemic area in KZN. In South Africa, acquiring wild specimens is particularly challenging as South Africa is a frontline candidate for eliminating malaria in the Southern African Development Community (SADC) region [214,215]. Due to this, there was a limited availability of wild specimens that could be used.

This relates to another limitation: the number of midguts used would vary based on sample availability. To our knowledge, there is no standardised procedure for sample processing for mosquito microbiota studies. As reviewed by Dada *et al*. [101], a standardised method for

mosquito microbiota studies is required [101]. From the current study, different processes in sample processing resulted in differences between experiments. This was likely due to differences in the type of DNA extraction kit used, the number of midguts used per biological replicate, and the number of biological replicates used. This was all due to the availability of samples and reagents throughout the study. Therefore, these parts of sample processing should be considered in future mosquito microbiota studies.

4.5 Conclusion

In conclusion, the gut microbiota diversity between the laboratory reared strains differed from the F_1 population and the wild population of *An. arabiensis*. The loss of bacterial diversity in the laboratory reared strains is suggested to be due to the differences in the larval environment caused by insectary conditions. This is suggested to start with an increase in the microbiota diversity before a significant decrease in the bacterial richness. Insecticide-degrading bacteria are present in the gut of the laboratory-reared insecticide resistant strain (SENN DDT) and the F_1 and wild populations, which are known to have low-level insecticide resistance. Therefore, it is likely, that specific gut bacteria may be associated with the insecticide resistant phenotype in these populations. Furthermore, there is evidence of a core gut microbiota in adults from the Mamfene district in Kwa-Zulu Natal, South Africa, which includes the bacterial genera *Rahnella, Yersinia, Asaia, Clavibacter, Cedecea, Pseudomonas, Aeromonas, Klebsiella,* and *Enterobacter*. The bacterial genera *Enterobacter, Pseudomonas,* and *Asaia* are present in the F_1 and wild populations and are associated with inhibiting *Plasmodium* activities. However, this does not necessarily equate to increased protection against *Plasmodium*. It implies that microbial-based interventions with bacterial strains from these genera can potentially be used for vector control as these are native symbionts. Additionally, *Asaia* was present in the F_1 population and wild population. Therefore, microbial-based interventions with *Wolbachia* could potentially be difficult.

Insects exposed to heavy metals in their larval environment have bacterial genera in common. As such, there is a possibility of an interplay between specific bacterial genera in the insect gut microbiota and exposure to heavy metals in the larval environment. Furthermore, heavy metal exposure increased the number of gut bacteria present in all strains of *An. arabiensis* adults. The untreated and heavy metal-treated F_1 population specimens had an increased presence of heavy metal-degrading bacteria. This was possibly a result of heavy metal exposure on the previous generation which resulted in a selective pressure for heavy metal

degrading bacteria that was transmitted to the offspring. Larval exposure to copper increased the number of bacterial genera present in SENN, SENN DDT, and F_1 generation adults. This is likely because heavy metals, such as copper, are micronutrients for bacteria. Therefore, the presence of specific heavy metals in the larval environment needs to be considered. Furthermore, insecticide susceptible SENN adults had more heavy metal degrading bacteria than insecticide resistant SENN DDT adults when exposed to cadmium. This means that the gut microbiota of SENN may be involved in the response to the stress induced by heavy metals, and other mechanisms that confer insecticide resistance in SENN DDT may aid in responding to the stress caused by heavy metals. These findings have implications in microbiota studies as the larval environment caused significant shifts in the gut microbiota that may be of physiological importance. Therefore, the larval environment needs to be considered in microbial-based interventions, as changes in the larval environments cause a shift in the native symbionts.

CHAPTER 5

Characterisation of the dynamic gut mycobiota of *Anopheles arabiensis*

5.1 Introduction

The gut microbiota consists of many microorganisms, including bacteria, fungi, viruses, archaea, and protozoa, as reviewed by Berg *et al*. [38] and Gurung *et al*. [269]. The mosquito gut mycobiota is the fungal component of the microbiome. Fungi are a group of eukaryotic microorganisms [270]. They can occur as solitary cells that reproduce by budding, known as yeasts, or they can also occur as long filaments (hyphae) that grow by apical extension, known as moulds [270]. As reviewed by Malassigné *et al*. [271] and Djihinto *et al*. [272], fungi can have a commensal or symbiotic relationship with the mosquito host, which plays a role in nutrition, development, physiology, fitness, and immunity [271,272]. As such, the mycobiota is of interest in mosquito microbiota studies.

Fungi are ubiquitous, and larvae are suggested to acquire fungi in their gut from consuming the surrounding water in larval breeding sites [273]. Adults acquire fungi from the larval environment during emergence, sugar sources, and blood meals [274,275]. Like with the bacterial component of the microbiota, the mycobiota diversity in *Culex* mosquitoes decreased from the larval stage to adulthood. This was suggested due to the partial sterilisation of the midgut from the pupal to the adult life stage [276,277]. Additionally, a decrease in fungal diversity was observed in *Aedes* mosquitoes after consuming a blood meal [278]. This suggests that the mosquito life stage and food source are important considerations in gut mycobiota studies.

Fungi have been implicated in the physiological responses of the mosquito [61,276,279–281]. For example, in female *Ae*. *albopictus*, the yeast genera *Malassezia* and *Cyberlindnera* and filamentous fungi genera *Pezoloma* and *Ganoderma* were active during fructose digestion [61]. Therefore, fungi play an active role in mosquito digestion. Furthermore, fungi have been suggested to impact the mosquito host's life history traits such as development, longevity, and reproduction. Yeasts serve as a food source for larvae and aid in the development of larvae into adults [276,279,280]. A study observed that yeasts *Candida albicans*, *C. glabrata*, *C. pseudolambica*, *Cryptococcus gattii*, *Metschnikowia bicuspidata*, *Saccharomyces cerevisiae* and *Wickerhamomyces anomalus* were essential for larval development, survival, and

pupation of *Culex pipiens* [276]. Additionally, Brewer's yeast, *S. cerevisiae*, induced hypoxic conditions in the gut of *Ae. aegypti* and *An. gambiae*, which is vital for the growth and moulting of larvae [281]. As such, specific fungi play a role in mosquito fitness, a crucial aspect of vector biology.

The current knowledge of the interactions between fungi and the mosquito host has mainly focused on fungal entomopathogens. Entomopathogens are pathogenic microbes such as fungi, viruses, protozoa, and bacteria that infect and colonise an insect host. As reviewed by Sharma and Sharma [282], entomopathogens have been used as microbial control agents against insect pests [282]. In mosquitoes, entomopathogenic fungi result in an overall decrease in mosquito fitness, as reviewed by Tawidian [283]. For example, *Beauveria bassiana* is a well-studied entomopathogen capable of infecting *Anopheles* vectors. It has been implicated in decreased longevity of both laboratory reared and field-caught *An. arabiensis* as well as field-caught *An. gambiae* and *An. funestus* [284,285]. Hence, most studies have focused on entomopathogens due to their potential as microbial-based vector control strategies for mosquitoes.

Some studies have shown non-entomopathogenic fungal interactions in the gut mycobiota of mosquito vectors. The gut mycobiota of both laboratory reared and field-caught mosquitoes have been characterised in several studies [273,274,278,286–289]. However, most of the fungal communities characterised are from the guts of *Culex* and *Aedes* mosquitoes [273,274,278,286–289]. Studies that characterised the gut mycobiota of *Anopheles* vectors are limited. Gut mycobiota studies on *Anopheles* vectors include *An. stephensi*, *An. gambiae* [274], and *An. coluzzii* [290]. It has been suggested that the fungal community in larval guts may be generalised across mosquito species [273,287]. However, the studies conducted on *Anopheles* species have highlighted different fungal communities and a core mycobiota has not yet been established for Anophelines. Yeasts, including *Meyerozyma guilliermondii* and *Rhodotorula glutinis*, were identified in the guts of laboratory reared *An. gambiae* and *An. stephensi* adults [274]. Common fungal genera found in field-caught *An. coluzzii* adults included *Aspergillus*, *Malassezia*, *Cladosporium*, and *Phoma* [290]. Consequently, additional baseline studies characterising the gut mycobiota of other *Anopheles* vectors are required.

As studies on mosquito-associated fungi are broadening, the gut mycobiome has become a potential new candidate for novel vector control strategies. One such approach involves using non-entomopathogenic fungi as potential microbes for paratransgenesis, as reviewed by

Romoli and Gendrin, [77]. For example, the mould *Leptosphaerulina* sp., isolated from the midgut of *An. gambiae* was re-introduced into *An. gambiae* through inoculating the larvae with crushed mycelium and spores [291]. This mould was transstadially transmitted across developmental stages and to their progeny, resulting in lower fitness levels for this host. Furthermore, fungi are advantageous as they can infect the mosquito host through the cuticle, unlike bacteria that need to be ingested [292]. As such, fungi are beneficial paratransgenesis candidates, especially since they are easily transmissible to the host and can be transmitted to the offspring.

In addition, fungi in *Anopheles* vectors can induce anti-*Plasmodium* responses [293–295]. For example, the yeast *Wickerhamomyces anomalus* isolated from the gut of *An. stephensi* was found to inhibit *P. berghei* ookinetes *in vitro* [295]. Furthermore, the microsporidian *Vavraia culicis* reduced *P. berghei* effectiveness in *An. gambiae* through eliciting the host's antimicrobial peptide system [293]. However, the anti-*Plasmodium* responses of these fungi were only effective against the *Plasmodium* parasite that infects rodents. Therefore, studies demonstrating the effectiveness of this microsporidian on *P. falciparum* or *P. vivax* are needed. However, not all fungi are promising candidates for paratransgenesis as they are not associated with *Plasmodium* suppression in *Anopheles* vectors. For example, *Penicillium chrysogenum* in the *An. gambiae* midgut increases the mosquito's susceptibility to *Plasmodium* infection by suppressing the mosquito's immune system [294]. Therefore, it is important to catalogue the fungi found in malaria vectors to gain insight into the dynamics of fungal communities in the gut.

A more diverse catalogue of the gut mycobiota in other *Anopheles* vectors is required to use fungi as candidates for paratransgenesis. Therefore, expanding on the current knowledge will contribute to an essential understanding of the dynamic fungal component of the microbiota needed for microbial-based vector control interventions. As such, this study, to our knowledge, is the first to contribute to the understanding of the gut mycobiota composition and diversity in *An. arabiensis*, a dominant vector species in southern Africa.

5.2 Materials and Methods

The gut mycobiota of insecticide susceptible *An. arabiensis* (SENN), insecticide resistant *An. arabiensis* (SENN DDT), and F_1 population *An. arabiensis* was conducted as described in **General Materials and Methods.**

Laboratory reared insecticide susceptible *An. arabiensis* (SENN) and insecticide resistant *An. arabiensis* (SENN DDT) strains, as described in **2.1.1 Laboratory reared *Anopheles* species/strains and their phenotypes**, were collected at different life stages. Wild *An. arabiensis* adults were collected from Section 9 (S 27°27′34.3″; E 032° 10′43.7″) in the Mamfene district in Kwa-Zulu Natal, South Africa. The offspring of identified isofemale *An. arabiensis* were used as the F_1 population specimens and were collected at different life stages. All emerged adults were allowed *ad libitum* access to a 10% sucrose solution. All *An. arabiensis* specimens were maintained as described in **2.1.2 Insectary conditions for laboratory reared *Anopheles* mosquitoes**. The gut mycobiota were identified for different life stages, including fourth instar larvae, three-day old adults, fifteen-day old non-blood fed adults, and fifteen-day old blood fed adults. Blood feeding was conducted as described in **2.1.3 Sample details of *Anopheles* specimens and blood feeding protocol**.

Five replicates for each life stage were prepared for laboratory reared specimens (SENN and SENN DDT). Each replicate contained five midguts. For the F_1 population specimens, five replicates of larvae, thirteen replicates of three-day old adults, four fifteen-day old non-blood fed adults, and seven replicates of fifteen-day old blood fed adults were prepared. Each replicate contained one midgut per replicate. Total genomic DNA from the mosquito's midgut was extracted using the DNeasy PowerSoil® Kit (QIAGEN: 12888-50) as described in **2.1.4 Midgut dissection and DNA extraction**.

The gut mycobiota of the samples were then identified as described in **2.2 Next-Generation Sequencing of the gut microbiota**. However, different primers were used to amplify the fungal internal transcribed spacer (ITS) region of the ribosomal RNA (rRNA) cistron (**Appendix 5: Fungal Primers**). These primers targeted the noncoding region between the 18S and 5.8S rRNA genes, known as the fungal ITS1 region. This region can identify fungi using the MiSeq® Illumina (2 ×300 bp reads) [296,297].

5.3 Results

Comparison of the gut mycobiota between laboratory reared (SENN and SENN DDT) and F_1 population of *An. arabiensis* at different life stages

5.3.1 α-diversity of the gut mycobiota

The α-diversity was assessed for each laboratory reared and the F_1 population of *An. arabiensis* at different life stages. Boxplots were used to visualise and compare differences in the α-diversity of the laboratory reared insecticide susceptible strain (SENN), the laboratory reared insecticide resistant strain (SENN DDT), and the F_1 population of *An. arabiensis*. Significant differences between the indices were assessed using a Kruskal-Wallis rank-sum test [157]. The Chao1 diversity index was used as an estimator of species richness, which considers the number of different fungal species present in the gut [148].

The Chao1 index was significantly higher in the F_1 population fourth instar larvae compared to SENN DDT larvae ($p = 0.01$) (**Figure 5.1 A**). Three-day old SENN adults had a significantly lower Chao1 index compared to the F_1 population adults ($p < 0.01$) (**Figure 4.5 B**). By contrast, no significant differences were observed in the Chao1 indices between the laboratory reared strains of fourth instar larvae ($p = 0.17$) (**Figure 4.5 A**), three-day old adults ($p = 0.17$) (**Figure 4.5 B**), fifteen-day old non-blood fed adults ($p = 0.25$) (**Figure 4.5 C**), and fifteen-day old blood fed adults ($p = 0.67$) (**Figure 4.5 D**). No significant differences were observed in the Chao1 indices between SENN and F_1 population fifteen-day old non-blood fed adults ($p = 1.00$) nor between SENN DDT and the F_1 population fifteen-day old non-blood fed adults ($p = 0.62$) (**Figure 4.5 C**). Similarly, no differences were observed in the Chao1 indices between SENN and the F_1 population fifteen-day old blood fed adults ($p = 0.06$) as well as between SENN DDT and the F_1 population fifteen-day old blood fed adults ($p = 0.10$) (**Figure 4.5 D**).

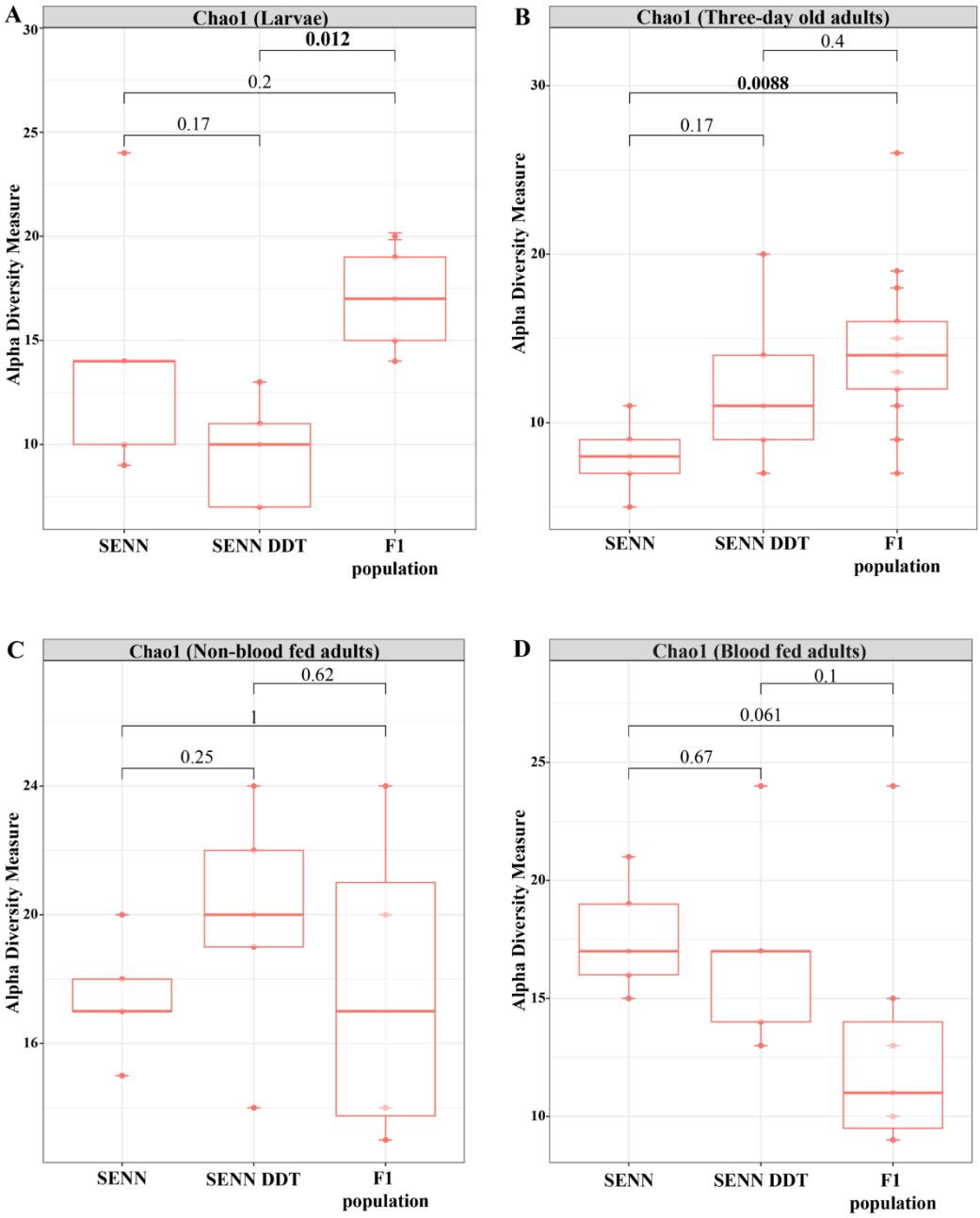

Figure 5.1 Comparison of the α-diversity (species richness) of the gut mycobiota between laboratory reared and F_1 populations of *Anopheles arabiensis* at different life stages.

Chao1 diversity indices of the gut mycobiota for laboratory reared (SENN and SENN DDT) and the F_1 populations of *An. arabiensis* for different life stages. **(A)** For fourth instar larvae. **(B)** For three-day-old adults. **(C)** For fifteen-day old non-blood-fed adults. **(D)** For fifteen-day old blood-fed adults. Comparisons of the diversity indices between laboratory reared (SENN and SENN DDT) and F_1 populations of *An. arabiensis* across life stages were assessed using a Kruskal-Wallis rank-sum test. The dots represent the number of biological replicates used for each group. Significant differences in the diversity indices between samples are indicated by bold print.

The Shannon-Weiner index was used as an estimator of species richness and evenness, which considers the number of fungal species and the relative abundance of each species [153,155,156].

In fourth instar larvae, the Shannon-Weiner diversity indices were significantly higher in SENN and the F_1 population when compared to SENN DDT ($p < 0.01$). However, there were no significant differences in the diversity between SENN and the F_1 population ($p = 0.55$) (**Figure 5.2 A**).

In three-day old adults, the F_1 population had a significantly higher diversity than both SENN ($p < 0.01$) and SENN DDT ($p < 0.01$) (**Figure 5.2 B**). No significant differences were observed between three-day old laboratory reared strains ($p = 1.00$) (**Figure 5.2 B**).

The Shannon-Weiner diversity index in SENN was significantly higher than the F_1 population of fifteen-day-old non-blood fed adults ($p = 0.02$) (**Figure 5.2 C**). No significant differences were observed in the Shannon-Weiner diversity indices between the SENN DDT and the F_1 population of fifteen-day old non-blood fed adults ($p = 0.06$) (**Figure 5.2 C**). No significant differences were observed in the Shannon-Weiner diversity indices between the fifteen-day old non-blood fed laboratory reared strains ($p = 0.42$) (**Figure 5.2 C**).

The Shannon-Weiner diversity indices of fifteen-day-old blood fed adults were significantly higher in SENN compared to SENN DDT ($p < 0.01$) and the F_1 population ($p < 0.01$) (**Figure 5.2 D**). The Shannon-Weiner diversity index of fifteen-day-old blood fed adults was significantly higher in SENN DDT compared to the F_1 population ($p < 0.01$) (**Figure 5.2 D**).

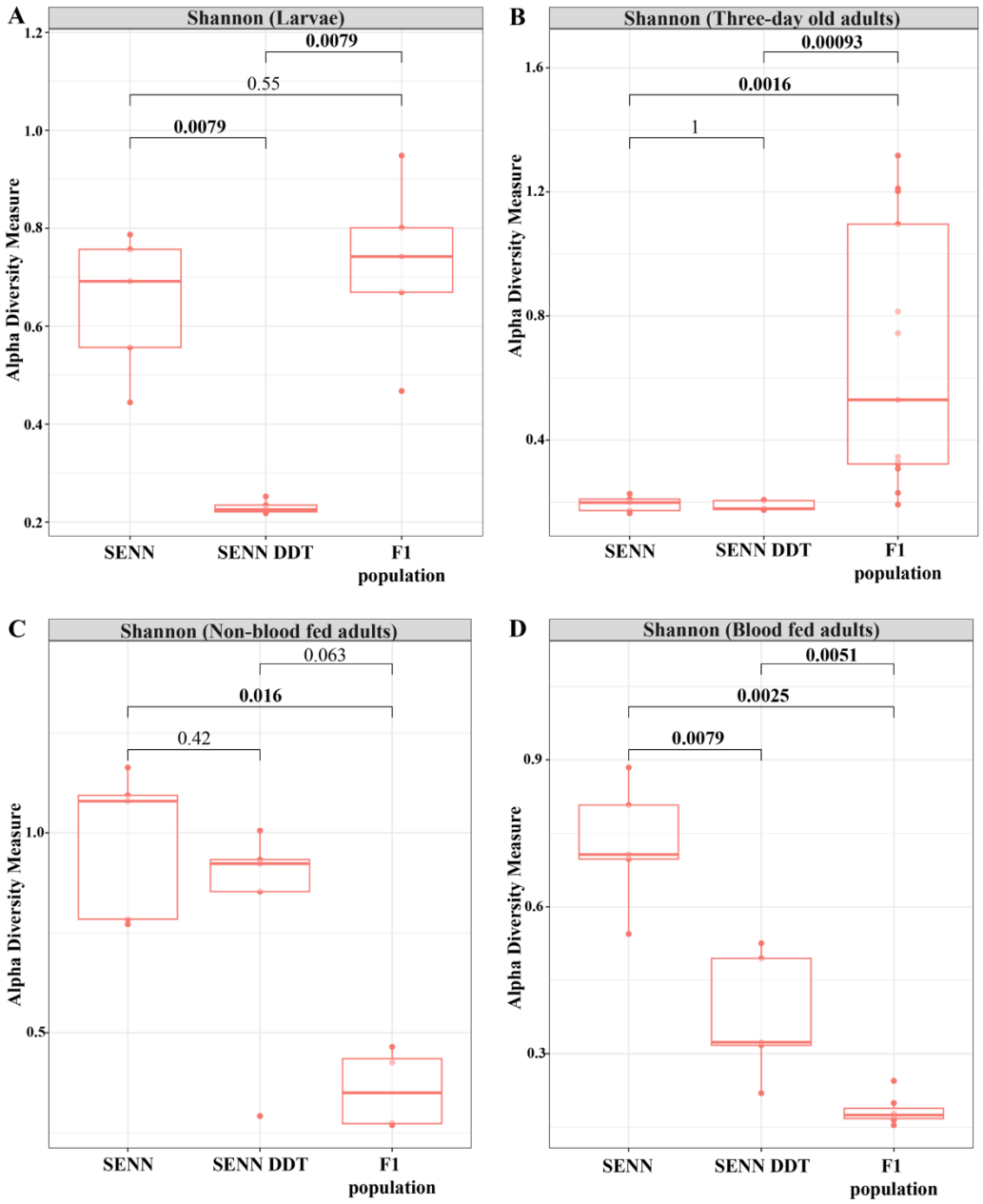

Figure 5.2 Comparison of the species richness and evenness of the gut mycobiota between laboratory reared and F_1 populations of *Anopheles arabiensis* at different life stages.

Shannon-Weiner diversity indices of the gut mycobiota for laboratory reared (SENN and SENN DDT) and the F_1 population of *An. arabiensis* at different life stages **(A)** For fourth instar larvae. **(B)** For three-day-old adults. **(C)** For fifteen-day old non-blood-fed adults. **(D)** For fifteen-day old blood-fed adults. Comparisons of the diversity indices between laboratory reared (SENN and SENN DDT) and the F_1 population of *An. arabiensis* across life stages were assessed using a Kruskal-Wallis rank-sum test. The dots represent the number of biological replicates used for each group. Significant differences in the diversity indices between samples are indicated by bold print.

5.3.2 β-diversity of the gut mycobiota

The β-diversity between the gut mycobiota of laboratory reared (SENN and SENN DDT) and the F_1 population of *An*. *arabiensis* for each life stage was visualised using Non-Metric Dimensional Scaling (NMDS) ordination. The differences in the fungal communities were assessed using the Bray-Curtis dissimilarity distances. Permutational multivariate analysis (PERMANOVA) was used to determine if there were any significant differences in the diversity between SENN, SENN DDT, and the F_1 population. Bray-Curtis dissimilarity distances showed significant differences in the diversity between the F_1 population and the laboratory reared strains for each life stage. Larvae (PERMANOVA; $p = 0.01$, $F = 3.10$, stress value = 0.11), three-day-old adults (PERMANOVA; $p = 0.01$, $F = 3.10$, stress value = 0.11), fifteen-day-old non-blood-fed adults (PERMANOVA; $p = 0.01$, $F = 1.11$, stress value = 0.10), and fifteen-day-old blood-fed adults (PERMANOVA; $p = 0.01$, $F = 3.67$, stress value = 0.11).

The fungal diversity in fourth instar larvae differed between all the strains, as no overlapping was observed (**Figure 5.3 A**). There were similar clustering patterns and overlapping in the diversity of fungal communities across adult life stages. The fungal communities in adult SENN and SENN DDT strains (laboratory reared) clustered closely and overlapped. The fungal communities in the F_1 population adults clustered separately from the laboratory reared adults (**Figure 5.3 B-D**).

Notably, the fungal diversity of three-day old F_1 population adults was greater than that of three-day old laboratory reared adults (**Figure 5.3 B**). Additionally, the fungal diversity in fifteen-day old non-blood fed SENN adults was greater than SENN DDT and the F_1 population (**Figure 5.3 C**). The fungal diversity in fifteen-day old blood fed SENN DDT adults was greater than SENN and the F_1 population (**Figure 5.3 D**).

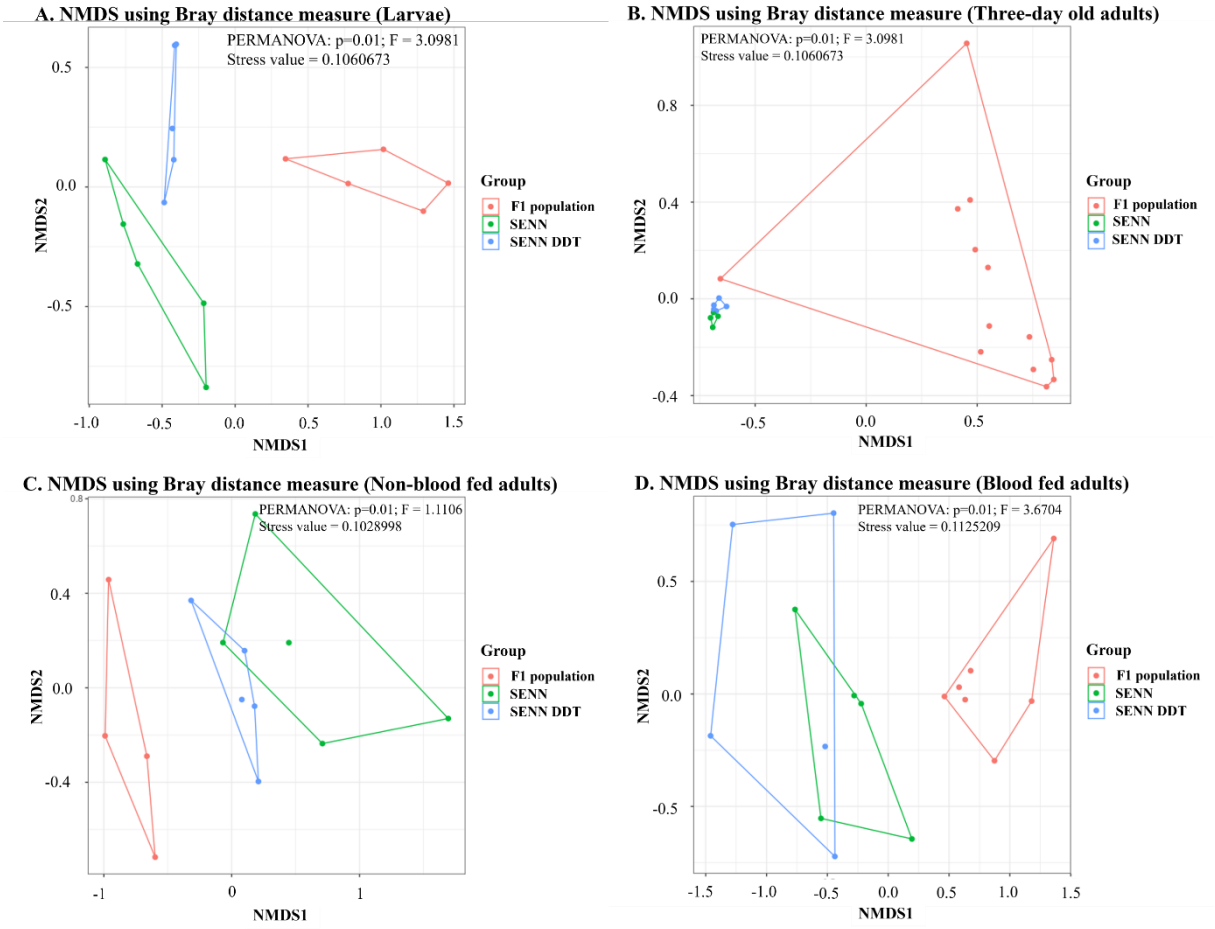

Figure 5.3 Differences in the fungal diversity of the gut mycobiota between laboratory reared strains and F_1 populations of *Anopheles arabiensis* across different life stages.

A visual representation of the β-diversity analysis of the gut mycobiota between laboratory reared strains (SENN and SENN DDT) and the F_1 population of *An. arabiensis* for different life stages, including **(A)** Fourth instar larvae. **(B)** Three-day-old adults. **(C)** Fifteen-day old non-blood fed adults. **(D)** Fifteen-day old blood-fed adults. Different colours represent the different *An. arabiensis* strains, insecticide susceptible laboratory reared *An. arabiensis* (SENN) as green, insecticide resistant laboratory reared *An. arabiensis* (SENN DDT) as blue, and F_1 population of *An. arabiensis* as red. Significant differences in the fungal diversity between the strains were assessed using a PERMANOVA (p = 0.01).

5.3.3 Overlapping fungal communities

The overlapping fungal genera between laboratory reared (SENN and SENN DDT) and the F_1 population of *An. arabiensis* for the different life stages were analysed and numerically represented on UpSet plots. Fungi identified up to the genus level are listed below. Not all fungal communities were identified to the respective taxonomic levels, which is a limitation of ITS sequencing [298]. Therefore, the identified fungal communities may not correspond to the numbers below.

Eight fungal genera were shared between SENN, SENN DDT, and the F_1 population fourth instar larvae, including *Saccharomyces*, *Penicillium*, *Epicoccum*, *Cladosporium*, and *Candida*. *Moesziomycesone* was shared between SENN and SENN DDT larvae. Four genera were shared between the F_1 population and SENN larvae, including *Papiliotrema*, *Chaetomium*, and *Auricularia*. Seven unique genera were identified in the F_1 population, including *Colacogloea*, *Mucor*, *Rhodotorula*, *Sporobolomyces*, and *Wallemia*. The insecticide susceptible strain SENN had seven unique genera, including *Alternaria*, *Aspergillus*, *Curvibasidium*, *Exophiala*, *Malassezia*, *Neoascochyta*, and *Paraconiothyrium*. The insecticide resistant strain SENN DDT had two unique genera, including *Cystobasidium* and *Meira* (**Figure 5.4 A**).

Five genera were shared between SENN, SENN DDT, and the F_1 population in three-day old adults, including *Candida* and *Cladosporium*. There were no shared genera between three-day old laboratory reared adults. *Auricularia* was common between three-day old SENN and the F_1 population adults. Three genera were shared between three-day old SENN DDT and the F_1 population adults, including *Wallemia* and *Chaetomium*. Twelve fungal genera were unique to three-day old F_1 population adults, including *Candida*, *Chytridiomycota*, *Cryptococcus*, *Dimennazyma*, *Fonsecazyma*, *Kurtzmaniella*, *Meira*, *Neoascochyta*, *Papiliotrema*, *Phaeococcomyces*, *Saccharomyces*, *Vishniacozyma* and *Zygoascus*. Five genera were unique to three-day old SENN adults, including *Alternaria*, *Aureobasidium*, *Penicillium*, *Rhodotorula*, and *Didymella*. *Peniophora* was unique to three-day old SENN DDT adults (**Figure 5.4 B**).

Candida was common in fifteen-day old non-blood fed SENN, SENN DDT, and F_1 population adults. Five fungal genera were shared between fifteen-day old non-blood fed SENN and SENN DDT adults, including *Kurtzmaniella, Epicoccum, Aureobasidium, Papulaspora*, and *Rhodotorula*. Fifteen-day old non-blood fed SENN DDT and the F_1 population adults shared three genera, including *Penicillium, Malassezia,* and *Alternaria*. Three genera were unique to fifteen-day old non-blood fed F_1 population adults: *Auricularia* and *Chaetomium*. Eight genera were unique to fifteen-day old non-blood fed SENN adults, including *Cordyceps, Marasmius, Neoascochyta, Spissiomyces, Wallrothiella, Wickerhamiella, Beauveria*, and *Zygoascus*. Ten genera were unique to fifteen-day old non-blood fed SENN DDT adults, including *Cladosporium, Cystofilobasidium, Fusarium, Meyerozyma, Naganishia, Papiliotrema, Plectosphaerella, Saccharomyces,* and *Toxicocladosporium* (**Figure 5.4 C**).

Fifteen-day-old blood-fed SENN, SENN DDT, and F_1 population adults shared seven genera, including *Candida, Kurtzmaniella, Cladosporium*, and *Malassezia*. Fifteen-day-old blood-fed laboratory reared strains shared eight genera, including *Alternaria, Cordyceps, Epicoccum, Papulaspora, Penicillium, Rhodotorula, Beauveria*, and *Saccharomyces. Papiliotrema* was shared between fifteen-day old non-blood fed SENN DDT and the F_1 population adults. Fourteen fungal genera were unique to fifteen-day old non-blood fed F_1 population adults, including *Chaetomium, Cladophialophora, Didymella, Fuscoporia, Genolevuria, Yurkovia, Mycoarthris, Oberwinklerozyma, Oliveonia,* and *Petrophila*. Four unique genera were unique to fifteen-day old non-blood fed SENN adults, including *Spissiomyces, Sarcinomyces, Pseudopithomyces,* and *Aureobasidium*. Twelve genera were unique to fifteen-day old non-blood fed SENN DDT adults, including *Aspergillus, Blumeria, Cryptococcus, Exserohilum, Filobasidium, Fusarium, Naganishia, Neodendryphiella,* and *Toxicocladosporium* (**Figure 5.4 D**).

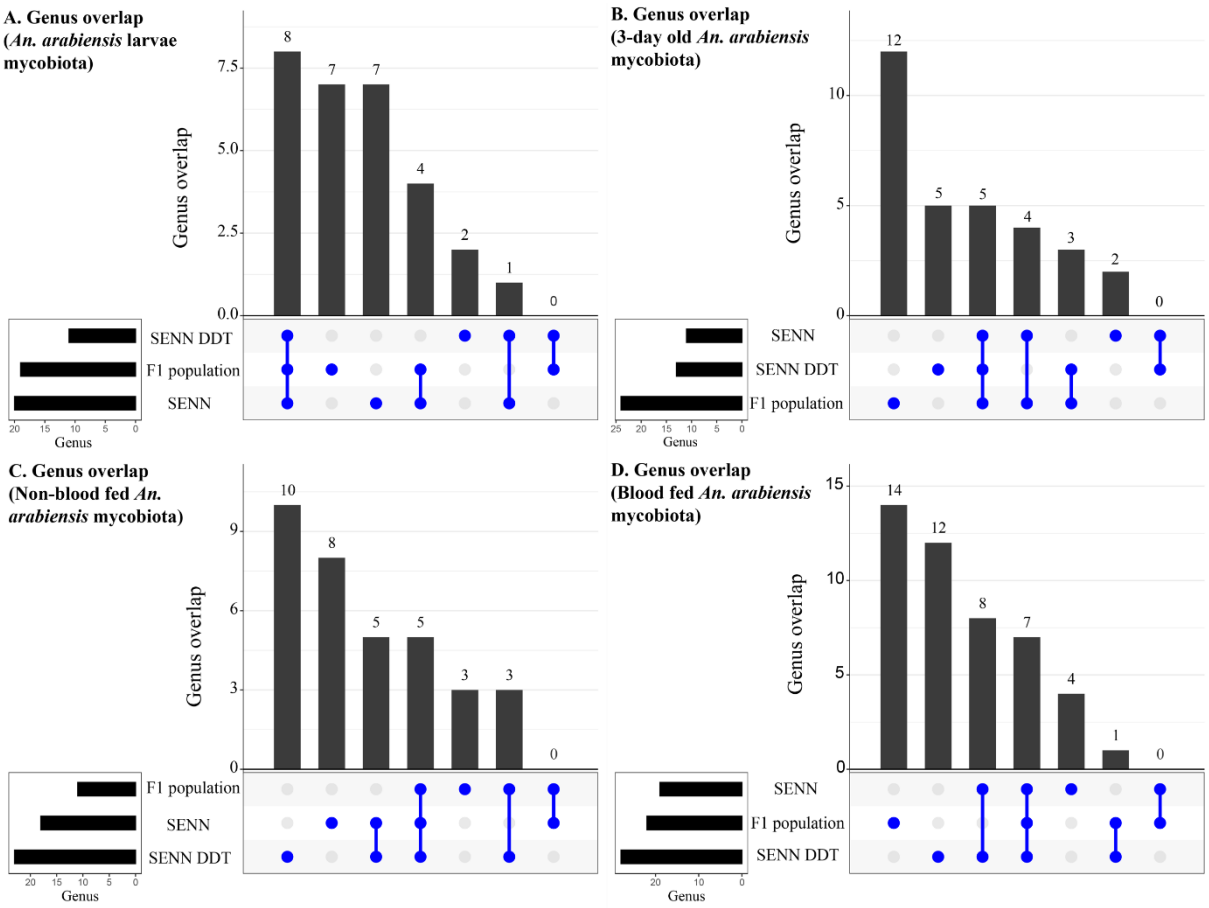

Figure 5.4 Comparison of overlapping fungal genera between laboratory reared and F_1 populations of *Anopheles arabiensis* across different life stages.

Comparisons between shared fungal genera for the laboratory reared insecticide susceptible strain (SENN), the laboratory reared insecticide resistant strain (SENN DDT), and the F_1 population of *An. arabiensis* were assessed for the following life stages: **(A)** fourth instar larvae, **(B)** three-day old adults, **(C)** fifteen-day old non-blood-fed adults, and **(D)** fifteen-day old blood-fed adults.

5.3.4 Differential abundance of fungal genera

Differential abundance of the gut mycobiota of fourth instar larvae with pairwise comparisons was assessed between the insecticide susceptible laboratory reared (SENN), insecticide resistant laboratory reared (SENN DDT), and the F_1 population. Differentially abundant genera were considered significantly different when the assigned log2 Fold Change values were compared and had a *p*-value < 0.01.

When comparing the relative abundance of fungal communities between fourth instar larvae in laboratory reared strains, *Meira* was significantly abundant in SENN DDT (**Figure 5.5 A**).

When comparing the relative abundance of fungal communities in the SENN and F_1 population fourth instar larvae, *Curvibasidium*, *Malassezia*, *Cladosporium*, *Moaesziomyces*, and *Candida* were differentially abundant in SENN larvae. *Colacogloea*, *Penicillium*, *Rhodotortula, papiliotrema,* and *Candida* were significantly abundant in the F_1 population larvae (**Figure 5.5 B**).

When comparing the relative abundance of fungal communities between SENN DDT and F_1 population fourth instar larvae, *Meira* and *Candida* were differentially abundant in SENN DDT larvae. *Colacogloea*, *Rhodotortula*, *Papiliotrema,* and *Candida* were significantly abundant F_1 population larvae (**Figure 5.5 C**).

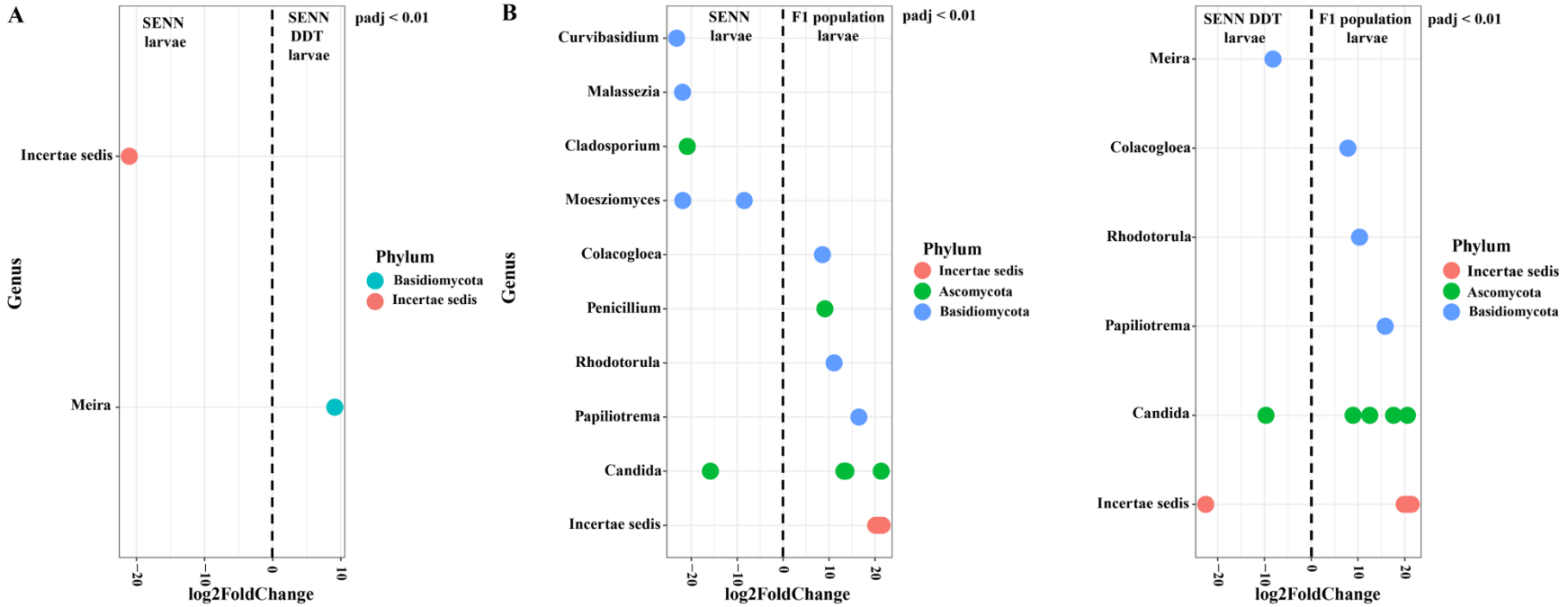

Figure 5.5 Pairwise comparisons of differentially abundant fungal genera between SENN, SENN DDT, and the F_1 population of fourth instar larvae.

(A) Differentially abundant fungal genera in SENN vs SENN DDT. **(B)** Differentially abundant fungal genera in SENN vs the F_1 population. **(C)** Differentially abundant fungal genera in SENN DDT vs the F_1 population. Fungal genera were considered differentially abundant when the Log2 Fold Change value had a significance threshold of $p < 0.01$. The genus name on either side of the dotted line shows where the relevant fungal genera are more abundant.

Differential abundance of the gut mycobiota of three-day old adults with pairwise comparisons was assessed between the insecticide susceptible laboratory reared (SENN), insecticide resistant laboratory reared (SENN DDT), and the F_1 population. Differentially abundant genera were considered significantly different when the assigned log2 Fold Change values were compared and had a *p*-value < 0.01.

There were no differentially abundant fungal genera when comparing the relative abundance of fungal communities between three-day old adults in laboratory reared strains. *Candida* was significantly abundant in the F_1 population of three-day old adults when compared to the laboratory reared three-day old adults (data not shown).

Differential abundance of the gut mycobiota of fifteen-day old non-blood fed adults with pairwise comparisons was assessed between the insecticide susceptible laboratory reared (SENN), insecticide resistant laboratory reared (SENN DDT), and the F_1 population. Differentially abundant genera were considered significantly different when the assigned log2 Fold Change values were compared and had a *p*-value < 0.01.

A comparison of the relative abundance in fungal communities between fifteen-day old non-blood fed SENN and SENN DDT adults showed that *Candida* and *Fusarium* were significantly abundant in SENN DDT (**Figure 5.6 A**).

When comparing the relative abundance of fungal communities between the fifteen-day old non-blood fed SENN and F_1 population adults, *Candida*, *Kurtzmaniella*, and *Chaetomium* were significantly abundant in SENN adults (**Figure 5.6 B**).

When comparing the relative abundance of fungal communities between the fifteen-day old non-blood fed SENN DDT and F_1 population adults. *Fusarium*, *Kurtzmaniella*, *Aureobasidium*, and *Candida* were significantly abundant in SENN DDT adults. *Chaetomium* was significantly abundant in F_1 population adults (**Figure 5.6 C**).

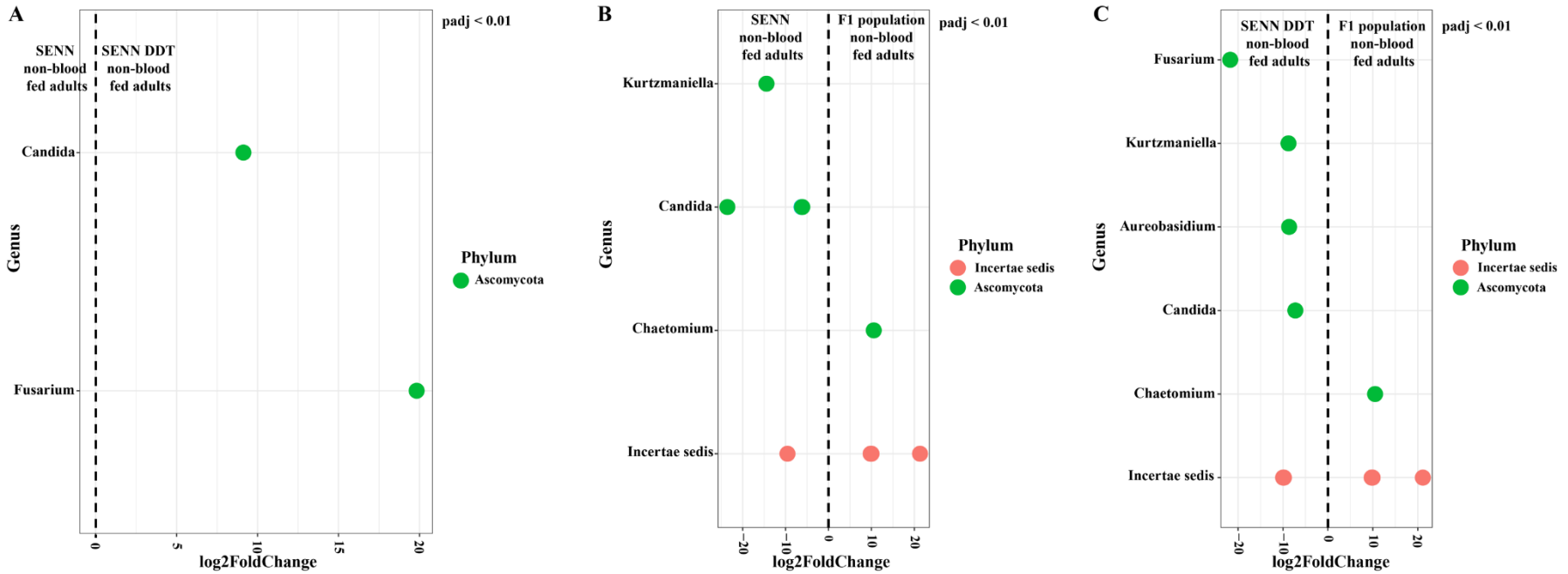

Figure 5.6 Pairwise comparisons of differentially abundant fungal genera between SENN, SENN DDT, and the F_1 population of fifteen-day old non-blood fed adults.

(A) Differentially abundant fungal genera in SENN vs SENN DDT. **(B)** Differentially abundant fungal genera in SENN vs the F_1 population. **(C)** Differentially abundant fungal genera in SENN DDT vs the F_1 population. Fungal genera were considered differentially abundant when the Log2 Fold Change value had a significance threshold of $p < 0.01$. The genus name on either side of the dotted line shows where the relevant fungal genera are more abundant.

Differential abundance of the gut mycobiota of fifteen-day old blood fed adults with pairwise comparisons was assessed between the insecticide susceptible laboratory reared (SENN), insecticide resistant laboratory reared (SENN DDT), and the F_1 population. Differentially abundant genera were considered significantly different when the assigned log2 Fold Change values were compared and had a *p*-value < 0.01.

When comparing, the relative abundance of fungal communities between fifteen-day old blood fed SENN and SENN DDT adults, *Fusarium*, *Malasseizia*, and *Blumeria* were significantly abundant in SENN DDT adults (**Figure 5.7 A**).

When comparing the relative abundance of fungal communities between SENN and F_1 population fifteen-day old blood fed adults, *Candida* and *Chaetomium* were significantly abundant in F_1 population adults (**Figure 5.7 B**).

When comparing the relative abundance of fungal communities between SENN DDT and F_1 population fifteen-day old blood fed adults. *Fusarium*, *Malasseizia*, and *Blumeria* were significantly abundant in SENN DDT adults. *Candida* and *Chaetomium* were significantly abundant in F_1 population adults (**Figure 5.7 C**).

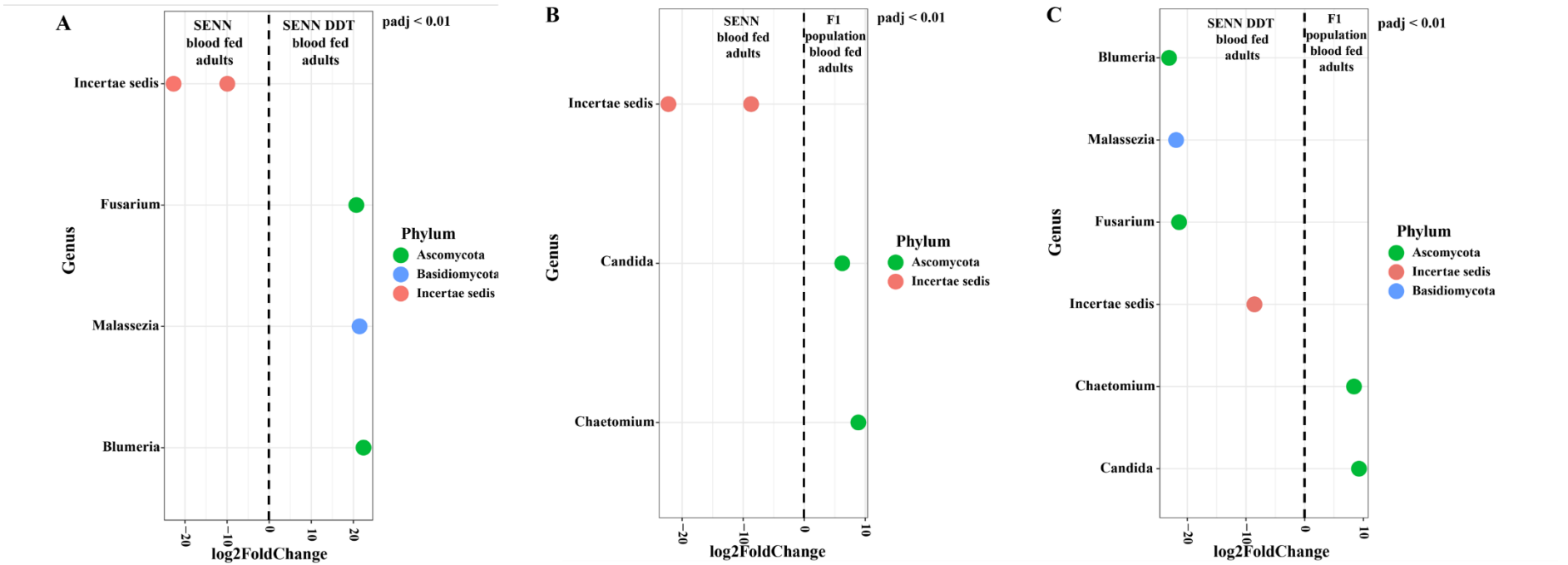

Figure 5.7 Pairwise comparisons of differentially abundant fungal genera between SENN, SENN DDT, and F_1 population of fifteen-day old blood fed adults.

(A) Differentially abundant fungal genera in SENN vs SENN DDT. **(B)** Differentially abundant fungal genera in SENN vs the F_1 population. **(C)** Differentially abundant fungal genera in SENN DDT vs the F_1 population. Fungal genera were considered differentially abundant when the Log2 Fold Change value had a significance threshold of $p < 0.01$. The genus name on either side of the dotted line shows where the relevant fungal genera are more abundant.

Differential abundance of the gut mycobiota of the insecticide susceptible laboratory reared (SENN), insecticide resistant laboratory reared (SENN DDT), and the F_1 population with pairwise comparisons were assessed between the fifteen-day old blood-fed and non-blood-fed adults. Differentially abundant genera were considered significantly different when the assigned log2 Fold Change values were compared and had a *p*-value < 0.01.

When comparing the relative abundance of fungal communities between fifteen-day old blood fed and non-blood fed F_1 population adults, *Candida* and *Kurtzmaniella* were significantly abundant in the F_1 population blood fed adults (**Figure 5.8 A**).

When comparing the relative abundance of fungal communities between fifteen-day old blood fed and non-blood fed SENN adults, *Candida* was significantly abundant in SENN non-blood fed adults (**Figure 5.8 B**).

When comparing the relative abundance of fungal communities between fifteen-day old blood fed and non-blood fed SENN DDT adults. *Fusarium*, *Malasseizia*, and *Blumeria* were significantly abundant in blood fed SENN DDT adults. *Aureobasidium* and *Candida* were significantly abundant in non-blood fed SENN DDT adults (**Figure 5.8 C**).

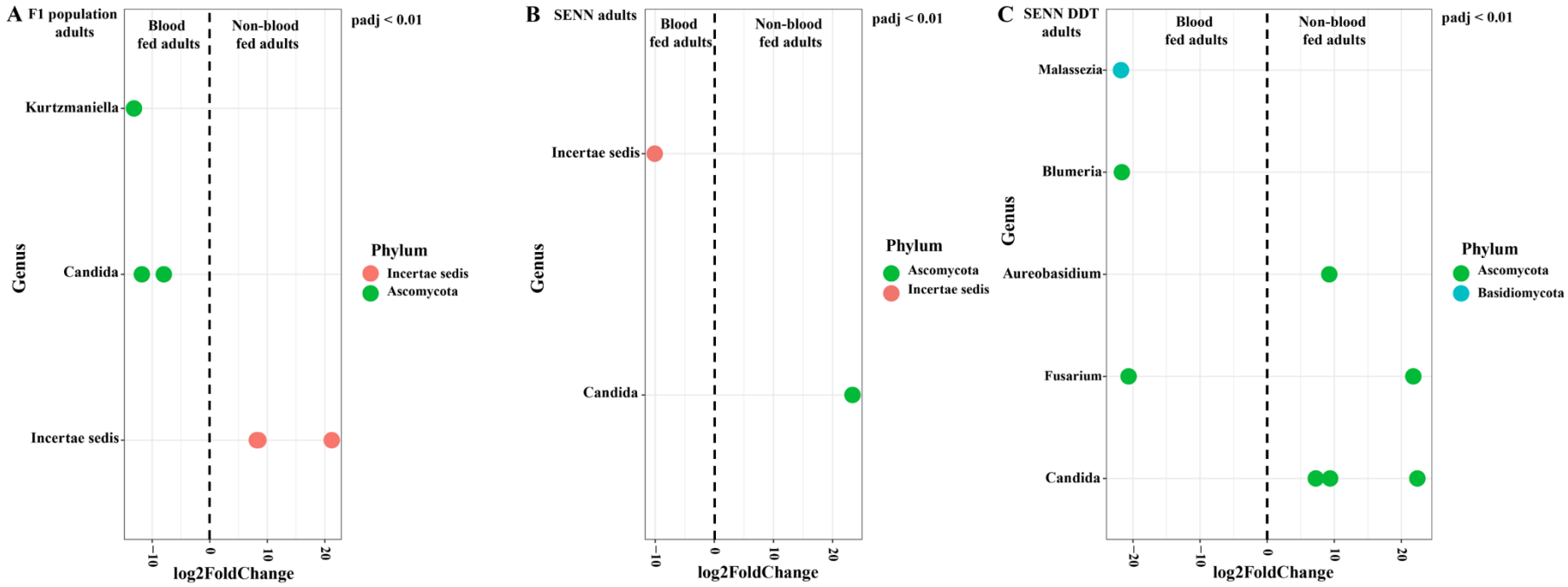

Figure 5.8 Pairwise comparisons of differentially abundant fungal genera between fifteen-day old non-blood fed and blood fed adults for the F_1 population, SENN, and SENN DDT.

(A) Differentially abundant fungal genera in blood fed vs non-blood fed F_1 population adults. **(B)** Differentially abundant fungal genera in blood fed vs non-blood fed SENN adults. **(C)** Differentially abundant fungal genera in blood fed vs non-blood fed SENN DDT adults. Fungal genera were considered differentially abundant when the Log2 Fold Change value had a significance threshold of $p < 0.01$. The genus name on either side of the dotted line shows where the relevant fungal genera are more abundant.

5.4 Discussion

The gut mycobiome of *Anopheles* is less examined than the bacterial component of the microbiome. This is particularly true for the species found in Southern Africa, such as *An. arabiensis*. This experiment aimed to add to the body of knowledge on this subject. This study characterised the gut mycobiome of fourth instar larvae, three-day old adults, fifteen-day old non-blood fed adults, and fifteen-day old blood fed adults in laboratory reared insecticide susceptible (SENN) and insecticide resistant (SENN DDT) strains of *An. arabiensis* as well as F_1 population *An. arabiensis*.

As previously described in **CHAPTER 3**, the bacterial diversity was not significantly different between the laboratory reared strains SENN and SENN DDT. However, this pattern does not apply to the gut mycobiota. Although there is no significant difference in the richness between the laboratory reared strains, there are differences in the diversity of the gut mycobiota. This is particularly notable at the larval stage as well as after the consumption of a blood meal. The mycobiota of SENN was significantly more even than SENN DDT. This suggests that the differences in diversity of the mycobiota could be a result of the larval environment and because of blood feeding.

The larval food used in the insectary consists of powdered dog biscuits and Brewer's yeast *Saccharomyces cerevisiae*. Therefore, the difference in the diversity of the gut mycobiota in larvae may be in part accounted for by different amounts of larval food consumed by the individual mosquitoes assayed [281]. The difference in the diversity of the gut mycobiota in fifteen-day old blood fed adults could be the result of physiological differences between the laboratory reared strains. A previous study noted that SENN and SENN DDT have a differential response to blood feeding. The SENN DDT strain had a lower oxidative stress in response to blood feeding than SENN [106]. This physiological difference could have affected the diversity of the gut mycobiota, while it did not affect the gut bacteria. To verify this, additional investigation into potential strain-specific variations in gut pH, which could impact the effectiveness of fungal growth, would be necessary.

In contrast to the gut bacteria, the α-diversity of the F_1 population gut mycobiota does not exhibit a marked difference, as only the larvae have a significantly higher fungal richness than the laboratory reared strains. However, the diversity of the gut mycobiota is more variable than the richness, as the larvae display a significantly higher diversity in SENN but not SENN

DDT. The diversity was greatest in the three-day old F_1 population, but the diversity was the lowest in fifteen-day old blood fed adults compared to the laboratory reared strains. This indicates that the gut mycobiota is more affected by the effects of multiple blood meals than the gut bacteria.

The β-diversity analysis between SENN, SENN DDT and F_1 population across various life stages differs and appears to be a function of the α-diversity. Interestingly, it could be expected to see the greatest difference in the β-diversity of the F_1 population larvae. However, the three-day old F_1 population adults display the greatest diversity. This is followed by a significant decrease thereafter. Notably, in the fifteen-day old non-blood fed adults, there is an overlap between the laboratory reared strains. The fifteen-day old non-blood fed F_1 population adults does not overlap with the fifteen-day old non-blood fed laboratory reared adult strains. This suggests that regardless of whether the mosquitoes are laboratory reared or F_1 population, the diversity of the gut mycobiome undergoes significant changes with age. Consequently, the mycobiota diversity of epidemiologically relevant females may be the least diverse among the life stages examined.

The gut mycobiota of the different life stages of *An. arabiensis* strains used in this study are predominantly made up of fungi from the phyla Ascomycota and Basidiomycota. This is consistent with previous mycobiome studies conducted with other mosquito vectors, including *Aedes* and *Culex*, for both larval and adult life stages [273,278,287,288]. The same is true for previous studies done on *Anopheles* vectors: *An. coluzzii*, *An. gambiae*, and *An. stephensi* [274,290]. Common fungal genera in *Anopheles* adults include *Meyerozyma Rhodotorula*, *Aspergillus*, *Malassezia*, *Cladosporium*, and *Phoma* [274,290]. This study suggests a core gut mycobiome for the larval and adult life stages between the different strains of *An. arabiensis*. Laboratory reared and the F_1 population fourth instar larvae had genera including *Saccharomyces*, *Penicillium*, *Epicoccum*, *Cladosporium*, and *Candida* present. *Candida* and *Cladosporium* were shared between three-day old and fifteen-day old non-blood fed adults. However, to assess whether these fungi are physiologically relevant to *An. arabiensis*, further studies on gut mycobiota-host interactions are needed.

In general, the F_1 population had the highest number of unique genera, followed by SENN DDT. However, in fifteen-day old non-blood adults, SENN DDT had the highest amount of unique fungal genera. This is a marked change from the larvae, where SENN and the F_1 population had the highest number of unique fungal genera. Dietary differences could

potentially account for these differences [241]. This would need to be confirmed by controlled environmental and dietary experiments. A more likely explanation is related to the insecticide resistance phenotype. The F_1 population have a low level of insecticide resistance [31], and their patterns are similar to that of the insecticide resistant strain. Although it may not be due to the presence of insecticide-degrading fungi, it may result from the pleiotropic physiological differences in the gut that exist due to the presence of the insecticide resistant phenotype.

Fungi play important roles in nutrition, as some fungal symbionts have digestive roles [61]. For example, filamentous fungi *Cladosporium* and *Aspergillus* are suggested to have an essential role in fructose digestion in *Aedes albopictus* adults [61]. *Cladosporium* was observed in all strains of *An. arabiensis* used in this study. This mould is, therefore, an essential part of the core mycobiota. Further studies are needed to confirm if this mould is necessary for fructose digestion in *An. arabiensis*. After ingesting a blood meal, *Meyerozyma* and *Cladosporium* were dominant in other mosquito vector species [278,286]. In this study, *Candida*, *Kurtzmaniella*, *Cladosporium*, and *Malassezia* were identified in fifteen-day old blood fed adults. However, the fungal genera *Kurtzmaniella* and *Malassezia* were significantly abundant in fifteen-day old blood fed adults. This suggests that other fungal genera observed in *An. arabiensis* used in this study may also be necessary for blood digestion.

This study identified the entomopathogen *Beauveria bassiana* in the guts of fifteen-day-old laboratory reared SENN and SENN DDT strains. Previous studies have noted that this entomopathogen can successfully invade the gut of field-caught and F_1 *An. arabiensis* and interact with the mosquito, resulting in a reduced lifespan [284,285]. The impact of the presence of this fungus on the life history of these strains remains uncertain. *Penicillium chrysogenum* has been identified as a fungus that increases *Anopheles'* susceptibility to *Plasmodium* infection by interfering with the host immune system [294]. This specific fungal species was found across all life stages of the *An. arabiensis* strains under investigation. Therefore, it is worth investigating the presence of *P. chrysogenum* in other members of the *An. gambiae* complex could provide valuable insights into its potential role in the vector competence of *An. arabiensis*.

Limitations in this study include the inability to identify fungi at the genus or species level, likely due to the primers used in this experiment [273,286]. However, to date, there are no generalised or standardised primers for fungal identification for the mosquito mycobiome. For

example, studies have used ITS2 primers (fITS7 and ITS4), which are primarily efficient in identifying fungi from the phyla Ascomycota and Basidiomycota [273,286]. These phyla alone are insufficient to provide better insight into the dynamics of the fungal mycobiome in vectors. Additionally, studies have also used 18S rRNA genes to identify fungi in *Anopheles*, however, this method requires peptide-nucleic acid oligonucleotides (called PNA blockers) to suppress the mosquito's 18S rRNA gene to allow for the detection of the mosquito's eukaryotic microbiome [299,300]. A standardised procedure for fungal identification in the mosquito is required to develop a more efficient catalogue of the mycobiota [101]. This is important as further studies of the fungal communities are needed to develop microbial-based approaches for vector control.

Furthermore, additional investigations are required to determine the role of fungi in mosquitoes. Currently, the knowledge surrounding the gut mycobiota in vector immunity, pathogen transmission and susceptibility, and mosquito fitness is limited compared to the bacterial component of the gut microbiota [101]. Additionally, culture-dependent studies are needed to obtain culturable fungi for microbial-based vector control strategies.

5.5 Conclusion

In conclusion, these results have provided a broad range of information on the composition and diversity of the fungal communities in the guts of both larval and adult *An. arabiensis*. A diverse spectrum of fungi was identified in the guts of *An. arabiensis*, including entomopathogenic and symbiotic fungi. The diversity of the gut mycobiota of the F_1 population is notably influenced by diet and age. There is less variety in the gut mycobiota between the F_1 population and laboratory reared *An. arabiensis* compared to bacteria. Additionally, the shared fungal genera in the F_1 population are more similar to those found in the insecticide resistant strain rather than the susceptible one. Despite this, there are sufficient common species to suggest the presence of a core mycobiome, which may contribute to various aspects of *An. arabiensis* biology. However, limitations in the identification of fungal species and gaps in understanding mosquito mycobiota physiology suggest that the mycobiome may not be as suitable a target for paratransgenesis as the bacteriome. This is mainly due to changes induced by blood meals, which could reduce the effectiveness of paratransgenesis involving fungal symbionts. Nonetheless, this comprehensive catalogue of fungi has enriched our knowledge of the fungal mycobiota, opening avenues for further research and exploration.

CHAPTER 6

The impact of exposure to variable salt concentrations on larvae and its effects on the life history and gut microbiota of *Anopheles merus*

The data for this chapter was published at https://doi.org/10.3390/insects13121165. This manuscript can be found in **Appendix 2: Manuscripts**.

6.1 Introduction

Mosquito larvae typically inhabit freshwater environments [18]. This is because most mosquito species cannot survive in aquatic environments where the osmotic stress exceeds the osmolarity of the haemolymph (300 osmol/L) [301]. This concentration is approximately equivalent to 30% seawater [301]. However, studies have indicated that mosquito species with a higher osmotolerance can survive in hypersaline conditions [302]. The most notable osmotolerant mosquitoes belong to the genus *Aedes*, including *Ae*. *detritus*, *Ae*. *aegypti*, and *Ae*. *albopictus* [303–306]. Other mosquito vector species, including *Culex quinquefasciatus* and *Cx*. *pipiens* have also been found to be osmotolerant [304]. Within the *Anopheles* genus, some species possess osmotolerance, including *An*. *aquasalis*, *An*. *sundaicu*s, and *An*. *multicolor*[304,307–309]. Some members of the *An*. *gambiae* complex are osmotolerant. This includes *An*. *bwambae*, *An*. *melas*, and *An*. *merus* [18].

In the *An*. *gambiae* complex, *An*. *merus* and *An*. *melas* are considered the dominant vector species in parts of Africa [18,310]. However, these species are not as efficient in malaria transmission as other members of the complex, including *An*. *gambiae* and *An*. *arabiensis* [18]. *Anopheles merus* and *An*. *melas* have achieved dominant vector status as they have been found in high densities and contribute to malaria transmission [177,311–317]. *Anopheles melas* are found in brackish water, saline pools, and mangroves along the western coasts of Africa [318,319]. These mosquitoes have been implicated in malaria transmission in western African countries such as Gambia, Equatorial Guinea, and Senegal [311,313,317]. *Anopheles merus* are found in shallow brackish water, saltwater marshes, or swamps in areas along the eastern and southern coasts of Africa [18]. They have also been found inland in saline pools[320]. *Anopheles merus* has been implicated in malaria transmission in Kenya, Madagascar, Tanzania, and Mozambique [177,314–316].

In South Africa, *An. merus* has been found in all three malaria-endemic provinces and is the most prevalent member of the *An. gambiae* complex in the Ehlanzeni District in Mpumalanga province [179,321]. Despite this, *An. merus* has not yet been implicated in malaria transmission [321]. However, it is possible that in the absence of a dominant vector species, *An. merus* can act as a dominant vector species, as reviewed by Bartilol [175]. More specifically, increased density populations of *An. merus* can potentially contribute to the transmission of malaria [18,316,322]. There is an increased abundance and distribution of *An. merus* in Mpumalanga, South Africa [179]. Therefore, it is a species of concern for vector control in South Africa.

Anopheles merus is primarily outdoor resting, outdoor biting, and predominantly animal biting[18]. However, studies on *An. merus* feeding and resting behaviour have shown that they also feed on humans indoors and will rest either indoors or outdoors [176,177,315,323]. Additionally, in Mpumalanga, South Africa, *An. merus* has been suggested to have low-level resistance to the insecticide DDT due to selective pressure that is potentially driven by the contamination of larval breeding sites [181]. Therefore, similar to *An. arabiensis*, conventional vector control strategies employing insecticides, which primarily target indoor biting and resting vectors, may not be sufficient to control *An. merus* effectively [29].

Many environmental factors contribute to the occurrence of *An. merus* larvae in a particular aquatic habitat, one of the significant determinants includes the salinity of the water in the breeding site [324]. It has been observed that the concentration of salt varies in different seasons. During the dry season, *An. merus* densities have been noted to increase, while freshwater species in the *An. gambiae* complex decrease [181,220]. During the rainy season, the salinity of the breeding water decreases; during the dry season, the salinity of the breeding water increases due to evaporation [325]. This means that *An. merus* can survive in a range of salt concentrations and will have seasonal variations in salt tolerance [325]. This highlights the importance of understanding the effects of *An. merus*' adaptability to varying salinities as it possibly contributes to the ecology and biology of this minor vector species.

The osmotolerance of *An. merus* is a poorly examined characteristic. However, studies have described the effect of salt dosage on *An. merus* development [326]. While the effect of larvae breeding in variable salinities has been studied, the effect on emerging adults has not yet been examined. Although it has been demonstrated that immune genes are predominantly

downregulated in larvae with increasing salt concentration [326], it is not known whether this has a carry-through effect on the adult.

There is limited information regarding *An. merus'* capability to adapt to changes in the salinity of its aquatic habitat and the impact that this would have on the mosquito's life history traits and gut microbiota. This is important in vector biology as it has been established that the larval environment dictates adult fitness [327–330]. The larval environment affects life history traits such as adult longevity, size, fertility, and fecundity, which have been linked to vector competence [327–330]. For example, the presence of organic fertiliser in the larval environment of laboratory-reared *An. merus* resulted in a significant increase in their longevity [331]. Additionally, the larval environment plays a critical role in shaping the microbial community in the gut of *Anopheles* [41,273]. The effect of the larval environment on the adult gut microbiota is an important factor to consider, as the gut microbiota of mosquitoes is essential for development and pupation [41,332] and plays a crucial role in adult life history [67,333]. Furthermore, the gut microbiota plays a vital role in the capacity of *Anopheles* mosquitoes to successfully transmit the *Plasmodium* parasite [43,93]. The effect of changes in the salinity of the larval environment on the gut microbiota of *An. merus* has not yet been examined. This study has provided insight into the effect of changes in the salinity of the larval environment on the life history traits and gut microbiota of laboratory reared *An. merus* adults.

6.2 Materials and Methods

The *An. merus* strain, MAFUS, used in this study was routinely maintained in a solution of 50% seawater concentration (17.5 g of sodium chloride/ 1L of water) as this is the maximum concentration it can tolerate. There have been reports of South African *An. merus* breeding in seawater concentrations as high as 123% [175,320], this laboratory strain could not survive at such elevated salt levels. Although wild *An. merus* populations in South Africa can tolerate higher salt concentrations, the 50% seawater solution was chosen as it was the highest concentration the strain could tolerate and because it effectively excluded other members of the *An. gambiae* complex [326]. Therefore, 50% seawater represented the highest salt concentration used, with all other concentrations being derived from dilutions of this value.

Seawater concentrations were prepared as follows: 50% seawater (17.5g of NaCl/L of H_2O), 25% seawater (8.75g of NaCl/L of H_2O), 12.5% seawater (4.375g of NaCl/L of H_2O), 6.25%

seawater (2.1875g of NaCl/L of H_2O), and 0% seawater which constituted of reverse osmosis water without any salt. For each experiment, except larval development and adult longevity, 200 first-instar larvae under 24 hours old were collected from the existing colony and transferred to a plastic container (214 x 155 x 80 mm) containing 1L of the respective seawater concentrations. The insectary conditions were as described in **General Materials and Methods**. Larvae were fed an equal amount of larval food until pupation, and the emerging adults were allowed *ad libitum* access to a 10% (w/v) sucrose solution.

6.2.1 Larval development

Development time was measured as the time to pupation. Fifty first-instar larvae were set up in seawater concentrations previously described. Larvae were allowed to pupate, and the number of pupae was recorded for each seawater concentration set-up. This experiment was replicated three times using cohorts originating from three separate egg batches for each seawater concentration under investigation. The development time was assessed using a Kaplan-Meier [334] estimator with a Log-rank test [335] as the measure of significance.

6.2.2 Adult longevity

Pupae from the larval development experiment set-ups were collected and allowed to emerge into adults in a cage with *ad libitum* access to 10% (w/v) sucrose solution; the sucrose solution was changed twice a week. The mortality was recorded daily, and cadavers were removed. The experiment was considered complete once all the individuals in the cage were dead. This experiment was replicated three times using cohorts originating from three separate egg batches for each seawater concentration under investigation. The longevity was analysed using a Kaplan-Meier [334] estimator with a log-rank test [335] as the measure of significance.

6.2.3 Fecundity and fertility

Fecundity is the number of eggs laid, and fertility is the percentage of eggs hatched [336]. Larvae originating from the previously described seawater concentrations were allowed to develop into adult mosquitoes. Newly emerged adults (fifty males and twenty-five females) were collected and placed into a cage with *ad libitum* access to a 10% (w/v) sucrose solution. The females were subjected to a human blood meal at the ages of three and seven days. Bloodmeals were provided by a single consenting human volunteer (SVO) per the ethics

waiver (**Appendix 1: Ethics Waiver**). At eleven days, the females were allowed to oviposit for 18 hours. The egg plate was removed, and the eggs were allowed to hatch for seventy-two hours. The number of hatched larvae and remaining unhatched eggs were counted. This experiment was replicated four times using cohorts originating from four separate egg batches for each seawater concentration under investigation. The data was analysed using a Chi-squared test [337].

6.2.4 Insecticide tolerance

Lethal time was measured using a CDC bottle bioassay [338]. Non-blood-fed three-day-old females were exposed to 0.001 mg/mL of deltamethrin for either two, four, eight, sixteen, or thirty-two minutes as described in Samuel *et al*. [339]. Adults were removed from the bottles after the set time and were allowed *ad libitum* access to a 10% (w/v) sucrose solution. Controls included an experiment control where the mosquitoes were not exposed to deltamethrin (zero minutes) and a solvent control where the mosquitoes were exposed to a bottle coated in acetone only. The mortality was recorded 24 hours after exposure to deltamethrin. The lethal times were calculated by probit analysis [340]. The experiment was replicated five times using cohorts originating from five separate egg batches for each seawater concentration under investigation. Data analysis for normality was done using the Shapiro-Wilk test, and the means were compared using a one-way analysis of variance (ANOVA) with an alpha value of 5% [341]. A Tukey HSD test was used as a post-hoc test [342].

6.2.5 Gut microbiota analysis with Next-Generation Sequencing

The effect of larval exposure to varying seawater concentrations on the gut microbiota of laboratory-reared *An. merus* (MAFUS) was conducted as described in **General Materials and Methods.**

The gut microbiota was characterised for fourth instar larvae and three-day old adults for each seawater concentration under investigation (0%, 6.25%, 12.5%, 25%, and 50%). Five replicates for each life stage were prepared. Each replicate consisted of three midguts for each seawater concentration. Total genomic DNA from the mosquito's midgut was extracted using the Qiagen DNeasy Blood & Tissue kit (Cat no. 69506) as described in **2.1.4 Midgut**

dissection and DNA extraction. The gut microbiota of the specimens were then processed and identified as described in **2.2 Next-Generation Sequencing of the gut microbiota**.

6.3 Results

The effects of variable salt concentrations in the larval environment on the life history traits of *An. merus*

6.3.1 Effects of variable salt concentrations in the larval environment on larval development

Larvae reared in 50% (17.5 NaCl g/L) and 25% (8.75 NaCl g/L) seawater took significantly longer to pupate compared to larvae reared in other salinities (log-rank test: $p < 0.01$, $\chi2 = 112.15$, df = 2). There were no significant differences in the time to pupation of the larvae between the 0%, 6.25% (2.188 NaCl g/L), and 12.5 % (4.375 NaCl g/L) seawater concentrations ($p = 0.85$, $\chi2 = 0.31$, df = 2) (**Figure 6.1 A**).

6.3.2 Effects of variable salt concentrations in the larval environment on adult longevity

There was a significant effect on the longevity of females ($p < 0.01$, $\chi2 = 16.81$, df = 4), which was due to the reduced longevity of the females reared from 25% seawater (**Figure 6.1 B**).

6.3.3 Effects of variable salt concentrations in the larval environment on fecundity and fertility

Changes in larval salt exposure affected the fecundity and fertility of *An. merus*. There was a significant increase in the fecundity (number of eggs laid) in MAFUS reared from the 50% seawater ($p = 0.03$, $\chi2 = 124.8$, df = 12). A Tukey post-hoc test showed that the significance was due to the difference between the 0% and 50% seawater treatments (**Figure 6.1 C**). In addition, there was a significant increase in the fertility (percentage of eggs hatched) of MAFUS reared in 12.5% seawater ($p < 0.01$; $\chi2 = 2767$ df = 12). A Tukey post-hoc test showed that the significance was due to the difference between the 0% and 12.5% treatments (**Figure 6.1 D**).

6.3.4 Effects of variable salt concentrations in the larval environment on insecticide tolerance of adults

Adults reared from different salinities impacted and altered their sensitivity to deltamethrin. A Tukey post-hoc test on LT50 values demonstrated that this was due to the differences between 0% and 50% treatments (one-way ANOVA: $p = 0.03$, $F_{(4, 21)} = 3.20$), without any differences observed between the adults reared from 6.25%, 12.5%, and 25% seawater (**Figure 6.1 E**). This was emphasised by the LT99 values, where the adults reared from 50% seawater had the highest lethal time and was significantly different from all other adults reared from the other seawater concentrations ($p < 0.01$, $F_{(4, 21)} = 6.74$) (**Figure 6.1 F**).

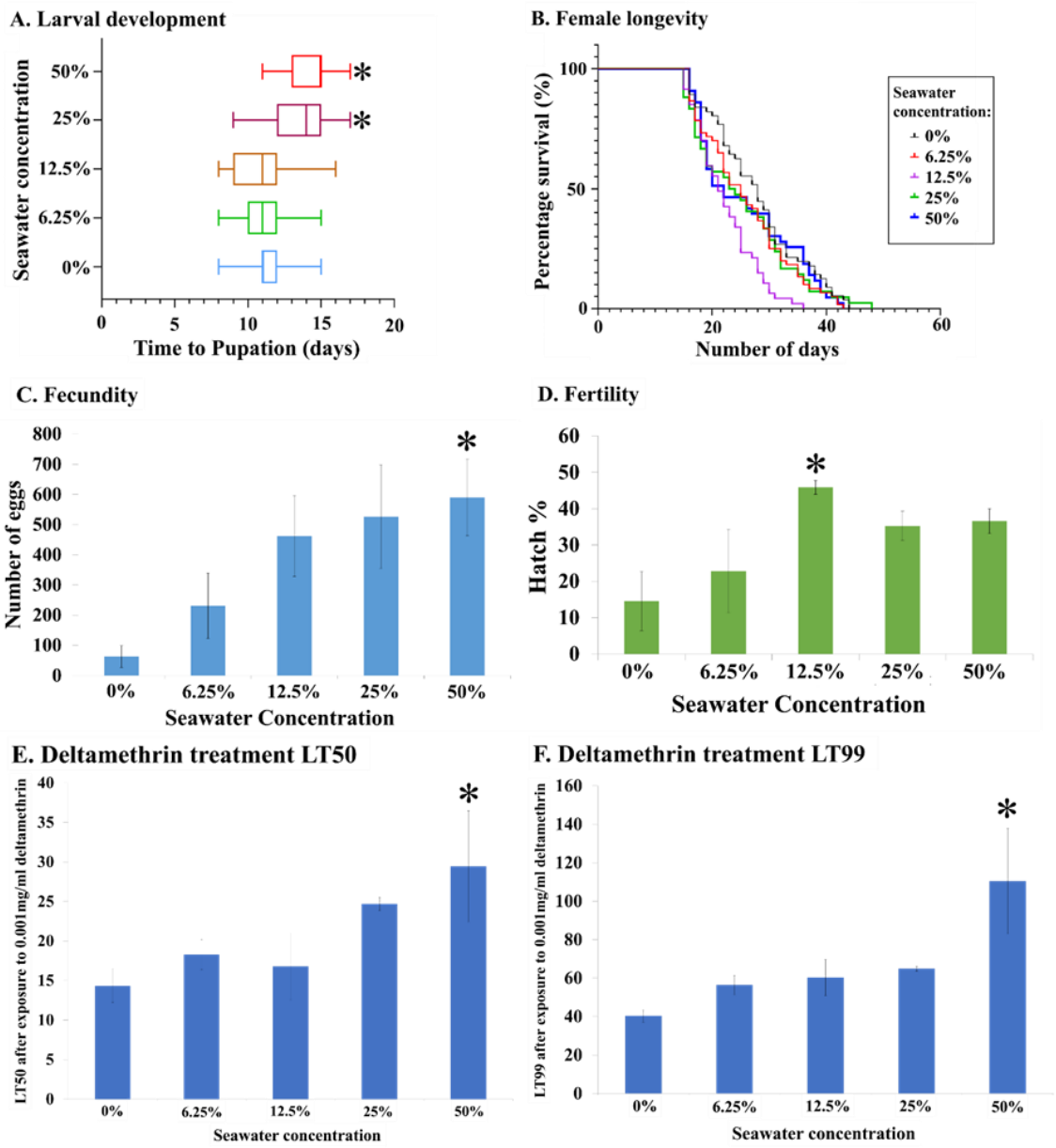

Figure 6.1 The effect of variable salinities on the life history traits of *Anopheles merus*.

(A) Larval development in different seawater concentrations, asterisks (*) indicate a significant difference in larval development time from the untreated control. **(B)** Adult longevity of females reared from different seawater concentrations. **(C)** Fertility of adults reared from the different seawater concentrations, asterisks (*) represent a significantly different number of eggs from the untreated control and **(D)** fecundity of adults reared from different seawater concentrations, asterisks (*) represent a significantly different hatch percentage from the 50% seawater control. **(E)** Deltamethrin lethal time (time to 50% mortality) of adults reared from different seawater concentrations, asterisks (*) indicates a significant difference from the untreated control. **(F)** Deltamethrin lethal time (time to 99% mortality) of adults reared in different seawater concentrations, asterisks (*) indicates a

significant difference from all other treatments. All error bars represent the standard error of the mean.

The effects of variable salt concentrations in the larval environment on the gut microbiota of *An. merus*

6.3.5 α-diversity of the gut microbiota

The α-diversity (within-sample diversity) [147] of the gut microbiota of laboratory reared *An. merus* resulting from the seawater concentrations under investigation was assessed. Boxplots were used to visualise and compare the differences in α-diversity indices of the gut microbiota between the laboratory-read *An. merus* reared in the different seawater concentrations. Significant differences between the indices were assessed using a Kruskal-Wallis rank-sum test [157]. The Chao1 diversity index was used as an estimator of species richness, which considers the number of species present in the gut [148]. The Shannon-Weiner diversity index was used as an estimator of species richness and evenness, which considers both the number of species present in the gut and the relative abundance of each species [153,155,156].

The α-diversity of the gut microbiota differed in *An. merus* larvae and adults across the different seawater treatments. The Chao1 diversity index of larvae reared in 0% seawater was significantly lower compared to those reared in 12.5% seawater ($p = 0.01$), 25% seawater ($p = 0.01$), and 50% seawater ($p = 0.01$) (**Figure 6.2 A**). Additionally, the Chao1 diversity index was significantly higher in larvae reared in 12.5% seawater compared to 6.25% seawater (**Figure 6.2 A**). There were no significant differences in the Chao1 diversity indices between larvae reared in 6.25% seawater when compared to 0% seawater ($p = 0.17$), 25% seawater ($p = 0.06$), and 50% seawater ($p = 0.06$) (**Figure 6.2 A**). The same was true for larvae reared in 12.5% seawater when compared to those reared in 25% seawater ($p = 1.00$) and 50% seawater ($p = 0.21$) (**Figure 6.2 A**). This was also true for the larvae reared in the higher concentrations of 25% seawater and 50% seawater ($p = 0.21$) (**Figure 6.2 A**).

The Shannon-Weiner diversity indices of larvae reared in 0% seawater were significantly lower compared to those reared in 12.5% seawater ($p = 0.01$), 25% seawater ($p = 0.01$), and 50% seawater ($p = 0.01$) (**Figure 6.2 B**). The Shannon-Weiner index was significantly lower for larvae reared in 6.25% seawater compared to those reared in 12.5% seawater ($p = 0.01$), 25% seawater ($p = 0.03$), and 50% seawater ($p = 0.03$) (**Figure 6.2 B**). There were no

significant differences in Shannon-Weiner diversity indices between larvae reared in 0% seawater and 6.25% seawater (p = 0.22) (**Figure 6.2 B**). There were no significant differences in Shannon-Weiner diversity indices between larvae reared in 12.5% seawater when compared to those reared in 25% seawater (p = 0.42) and 50% seawater (p = 0.55) (**Figure 6.2 B**). There were no significant differences in Shannon-Weiner diversity indices between larvae reared in the higher concentrations of 25% seawater and 50% seawater (p = 0.31) (**Figure 6.2 B**).

The adults reared in 25% water had a significantly lower Chao1 diversity index compared to adults reared in 0% seawater (p = 0.03) and 12.5% seawater (p = 0.03) (**Figure 6.2 C**). There were no significant differences between the Chao1 index of adults reared from 0% seawater and 6.25% seawater (p = 0.84), 12.5% seawater (p = 0.92), as well as 50% seawater (p = 0.83) (**Figure 6.2 C**). Additionally, no significant differences were observed between the Chao1 index of adults reared from 6.25% seawater and 12.5% seawater (p = 0.69), 25% seawater (p = 0.69), as well as 50% seawater (p = 0.69) (**Figure 6.2 C**). The same was true for adults reared from 12.5% seawater and 50% seawater (p = 0.75), as well as between 25% seawater and 50% seawater (p = 0.42) (**Figure 6.2 C**).

A similar pattern was observed with the Shannon-Weiner index, though the only significant difference observed was between adults reared in 0% seawater and 25% seawater. The Shannon-Weiner index was significantly lower in adults reared from 25% seawater than 0% seawater (p = 0.01) (**Figure 6.2 D**). There were no significant differences observed in Shannon-Weiner indices between adults reared from 0% seawater and the adults reared from 6.25% seawater (p = 0.69), 12.5% seawater (p = 0.84) and 25% seawater (p = 1.00) (**Figure 6.2 D**). Additionally, no significant differences were observed between adults reared from 6.25% seawater and 12.5% seawater (p = 0.69), 25% seawater (p = 1.00), as well as 50% seawater (p = 0.69) (**Figure 6.2 D**). The same was true for adults reared from 12.5% seawater compared to 25% seawater (p = 0.15) and 50% seawater (p = 1.00) (**Figure 6.2 D**). No significant differences between the adults reared from 25% seawater and 50% seawater (p = 0.22) (**Figure 6.2 D**).

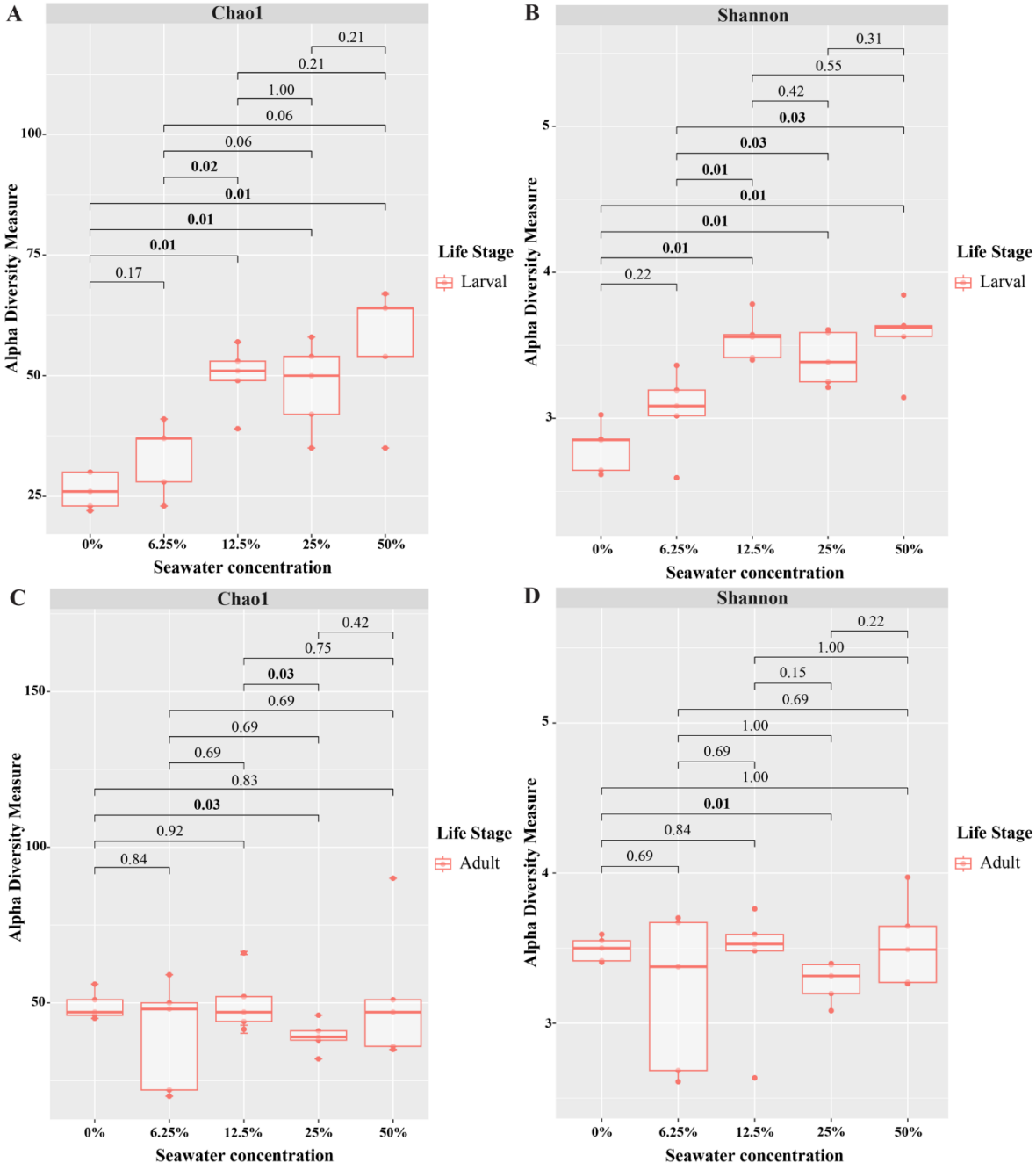

Figure 6.2 Comparison of the α-diversity of the gut microbiota between *Anopheles merus* across all seawater treatments.

(A) Chao1 diversity indices for fourth instar larvae across all seawater treatments. **(B)** Shannon-Weiner diversity indices for fourth instar larvae across all seawater treatments. **(C)** Chao1 diversity indices for three-day old adults across all seawater treatments. **(D)** Shannon-Weiner diversity indices for three-day old adults across all seawater treatments. Comparisons of the diversity indices between the seawater treatments for each life stage were assessed using a Kruskal-Wallis rank-sum test. The dots represent the number of biological replicates used for each group. Significant differences in the diversity indices between samples are indicated by bold print.

6.3.6 β-diversity of the gut microbiota

Non-metric dimensional scaling (NMDS) ordination was used to visualise the β-diversity (between-sample diversity) [147] among *An. merus* reared in various seawater concentrations. The differences in gut bacterial communities were assessed using Bray-Curtis dissimilarity distances. Permutational multivariate analysis (PERMANOVA) determined significant variations in gut microbiota diversity distribution between MAFUS larvae and adults from different seawater concentrations. Notably, similar clustering patterns and overlaps were observed in the diversity of bacterial communities among adults. However, the bacterial diversity observed between the larvae reared in different seawater concentrations exhibited a more dispersed pattern, although the differences were not significantly different. Significant differences were observed in bacterial diversity between the life stages (PERMANOVA; p = 0.01, F = 7.98, stress value = 0.21) (**Figure 6.3**).

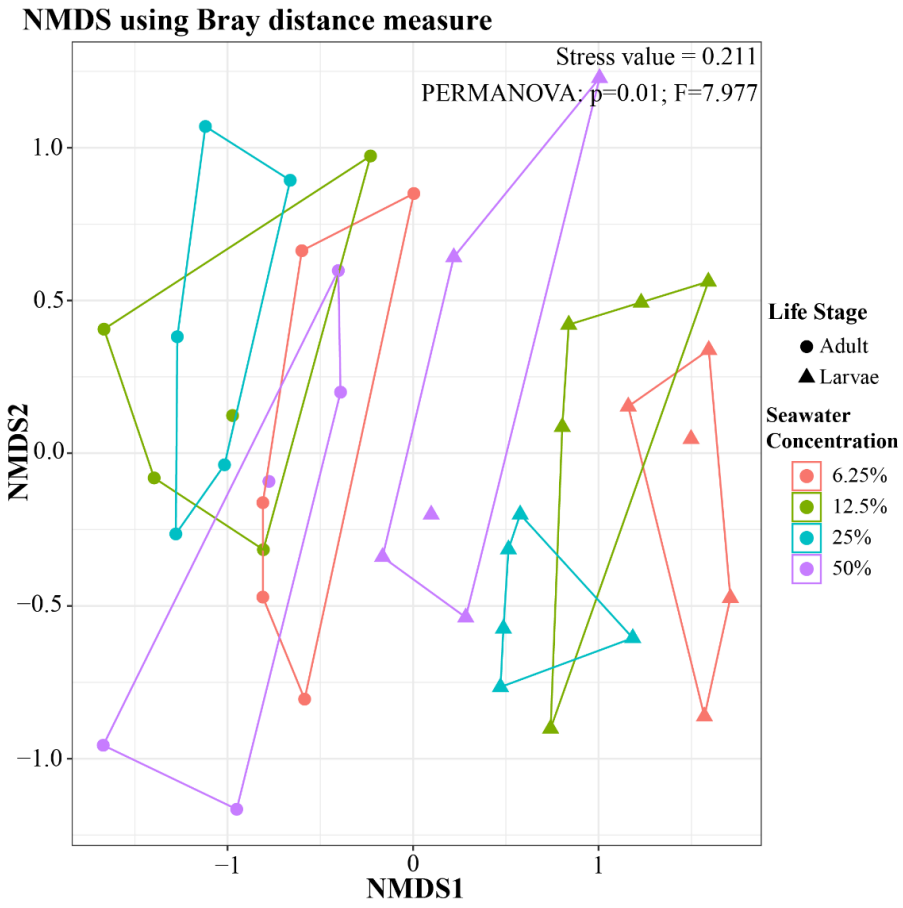

Figure 6.3 Differences in the bacterial diversity between *Anopheles merus* across all seawater treatments.

A visual representation of the β-diversity analysis of the gut bacteria between laboratory reared *An. merus* (MAFUS) larvae and three-day old adults exposed to different seawater concentrations. The following colours indicate seawater concentrations laboratory reared *An. merus* was exposed to red represents 6.25% seawater; green represents 12.5% seawater; blue represents 25% seawater; and purple represents 50% seawater. The shapes correspond to different life stages; circles represent larvae and triangles represent adults. Significant differences in the bacterial diversity between the life stage across all seawater treatments for fourth instar larvae and three-day old laboratory reared *An. merus* were assessed using a PERMANOVA analysis (p = 0.01).

6.3.7 Overlapping bacterial communities

The number of overlapping bacterial families and genera found in laboratory-reared *An. merus* across the different seawater concentrations were analysed and numerically represented on UpSet plots. Bacterial communities that were identified to the genus level are listed below. Note that not all bacterial communities were identified to the respective taxonomic levels, as this is a limitation of 16S rRNA gene sequencing [200–202]. Therefore, the identified bacterial communities may not correspond to the numbers below.

Thirteen bacterial families were shared between both adults and larvae: *Aeromonadaceae, Weeksellaceae, Comamonadaceae, Enterobacteriaceae, Pseudomonadaceae, Rhizobiaceae, Yersiniaceae Sphingobacteriaceae, Xanthobacteraceae,* and *Xanthomonadaceae* (**Figure 6.4 A**). Seventeen bacterial genera were shared between both adults and larvae: *Aeromonas, Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium, Bradyrhizobium, Comamonas, Elizabethkingia, Enterobacter, Enterococcus, Klebsiella, Pseudomonas, Rahnella, Soonwooa, Sphingobacterium,* and *Stenotrophomonas* (**Figure 6.4 B**).

Seventeen bacterial families were shared between all larvae: *Alcaligenaceae, Azospirillaceae, Erwiniaceae, Devosiaceae, Beijerinckiaceae, Carnobacteriaceae, Chromobacteriaceae, Flavobacteriaceae, Kaistiaceae, Listeriaceae, Microbacteriaceae, Saccharimonadaceae, Sphingomonadaceae, Staphylococcaceae,* and phyla from the orders Lactobacillales, and Saccharimonadales (**Figure 6.4 A**). Thirty genera were shared between all larvae: *Achromobacter, Ancylobacter, Aquitalea, Azospirillum, Bosea, Brucella, Chryseobacterium, Devosia, Flavobacterium, Isobaculum, Kaistia, Listeria, Methylobacterium-Methylorubrum, Microbacterium, Ochrobactrum, Pedobacter, Rahnella, Samsonia, Sphingomonas, Sphingopyxis, Staphylococcus,* and *Xanthobacter* (**Figure 6.4 B**).

All adults shared five families, including *Acetobacteraceae, Caulobacteraceae,* and phyla from the order Pseudomonadales (**Figure 6.4 A**). Eleven genera were shared between all adults: *Asaia, Azotobacter, Brevundimonas, Gluconobacter, Mesorhizobium, Serratia,* and *Variovorax* (**Figure 6.4 B**).

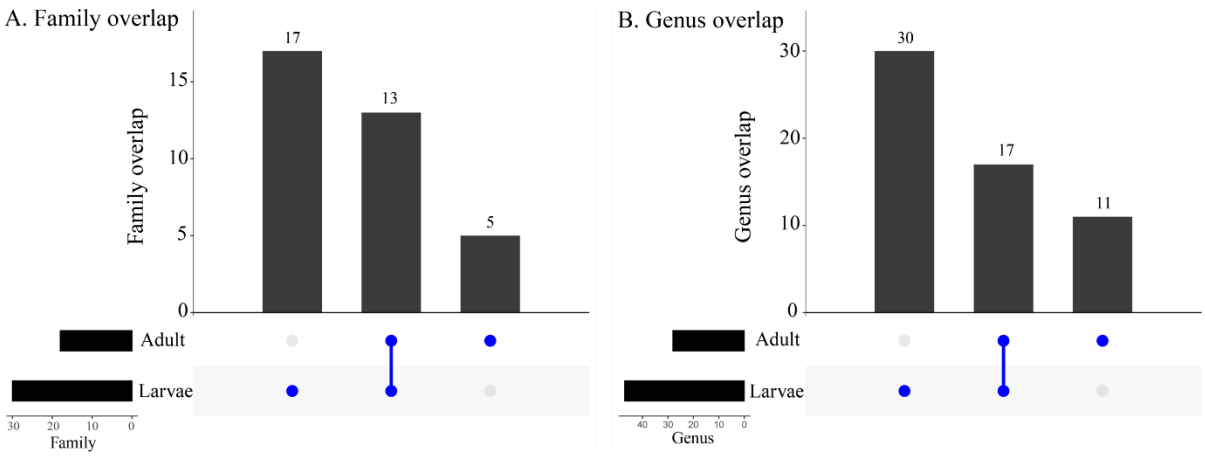

Figure 6.4 Comparison of overlapping gut bacteria between *Anopheles merus* larvae and adults across all seawater treatments.

(A) Comparison of common bacterial families. **(B)** Comparison of common bacterial genera. The blue dots with a joined line represent the overlapping gut bacteria between larvae and adults, with single dots indicating the unique bacteria per life stage. Each numbered column represents the number of bacterial **(A)** families or **(B)** genera that overlap between life stages.

6.3.8 Differential abundance of bacterial genera

Differential abundance of the gut microbiota with pairwise comparisons was assessed between laboratory-reared *An. merus* reared from the different seawater concentrations. Differentially abundant genera were considered significantly different when the assigned log2 Fold Change values were compared and had a *p*-value < 0.01. *Anopheles merus* reared from a 50% seawater concentration was used as a baseline, as this is the standard seawater concentration for rearing this strain.

The phyla most represented in larvae are Acinetobacteriota, Patescibacteria, Firmicutes, Bacteroidota, and Proteobacteria. Fifteen bacterial genera, including *Ochrobactrum*, *Aeromonas*, *Kluyvera*, *Rahnella*, *Elizabethkingia*, *Stenotrophomonas*, *Yersinia*, *Brucella*, *Sphingobacterium*, *Devosia*, *Enterobacter*, *Klebsiella*, *Bosea*, *Enterococcus*, and *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, were significantly abundant in larvae reared from 50% seawater when compared to 0% seawater treatment (**Figure 6.5 A**). Eight bacterial genera, including *Pedobacter*, *Bradyrhizobium*, *Pseudomonas*, *Comamonas*, *Methylobacterium-Methylorubrum Klebsiella*, *Bosea*, *Enterococcus*, and *Allorhizobium-*

Neorhizobium-Pararhizobium-Rhizobium, were significantly abundant in larvae reared from 0% seawater when compared to the 50% seawater treatment (**Figure 6.5 A**).

Fourteen bacterial genera, including *Ochrobactrum*, *Brucella*, *Rahnella*, *Kluyvera*, *Elizabethkingia*, *Stenotrophomonas*, *Sphingomonas*, *Devosia*, *Klebsiella*, *Enterobacter*, *Sphingobacterium*, *Enterococcus*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* and *Microbacterium*, were significantly abundant in larvae reared from 50% seawater when compared to 6.25% seawater treatment (**Figure 6.5 B**). Nine bacterial genera, including *Kaistia*, *Pseudomonas*, *Yersinia*, *Methylobacterium-Methylorubrum*, *Aeromonas*, *Sphingobacterium*, *Enterococcus*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* and *Microbacterium*, were significantly abundant in larvae reared from 6.25% seawater when compared to 50% seawater treatment (**Figure 6.5 B**).

Twelve bacterial genera, including *Kluyvera*, *Rahnella*, *Elizabethkingia*, *Devosia*, *Enterobacter*, *Sphingobacterium*, *Bosea*, *Enterococcus*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Brucella*, *Klebsiella*, and *Microbacterium*, were significantly abundant in larvae reared from 50% seawater when compared to 12.5% seawater treatment (**Figure 6.5 C**). Fifteen bacterial genera, including *Pseudomonas*, *Ancylobacter*, *Aquitalea*, *Chryseobacterium*, *Methylobacterium-Methylorubrum*, *Comamonas*, *Aeromonas*, *Yersinia*, *Sphingobacterium*, *Bosea*, *Enterococcus*, *Brucella*, *Klebsiella*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, and *Microbacterium*, significantly abundant in larvae reared from 12.5% seawater when compared to 50% seawater treatment (**Figure 6.5 C**).

Fifteen bacterial genera, including *Kluyvera*, *Rahnella*, *Elizabethkingia*, *Yersinia*, *Enterobacter*, *Stenotrophomonas*, *Devosia*, *Sphingobacterium*, *Bosea*, *Enterococcus*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Brucella*, *Klebsiella*, and *Microbacterium*, were significantly abundant in larvae reared from 50% seawater when compared to 25% seawater treatment (**Figure 6.5 D**). Twelve bacterial genera, including *Pedobacter*, *Azospirillum*, *Chryseobacterium*, *Bosea*, *Enterococcus*, *Methylobacterium-Methylorubrum*, *Aeromonas*, *Sphingobacterium*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Brucella*, *Klebsiella*, and *Microbacterium*, were significantly abundant in larvae reared from 25% seawater when compared to 50% seawater treatment (**Figure 6.5 D**).

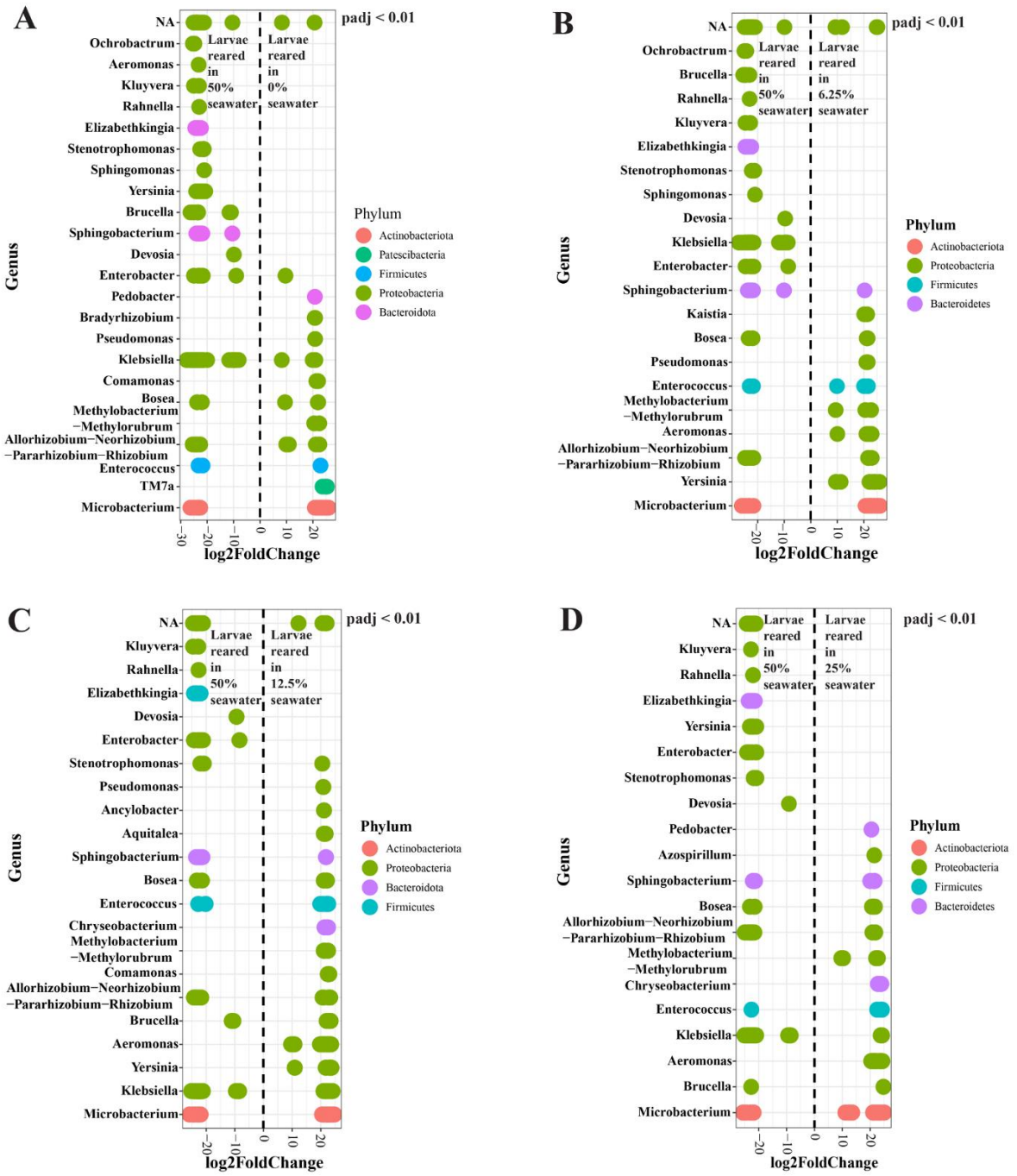

Figure 6.5 Pairwise comparisons of differentially abundant bacterial genera between *Anopheles merus* larvae exposed to different seawater concentrations.

(A) Differentially abundant gut bacteria of larvae reared in 50% seawater compared to 0% seawater. **(B)** Differentially abundant gut bacteria of larvae reared in 50% seawater compared to 6.25% seawater. **(C)** Differentially abundant gut bacteria of larvae reared in 50% seawater compared to 12.5% seawater. **(D)** Differentially abundant gut bacteria of larvae reared in 50% seawater compared to 25% seawater. Differentially abundant genera had a significance threshold of *p*-value < 0.01. Genera to the left of 0 are significantly abundant in the larvae reared in 50% seawater, while genera to the right of 0 are significantly abundant in the larvae from the comparative concentration.

The phyla most represented in the three-day-old adults are Firmicutes, Bacteroidota, and Proteobacteria. Twelve bacterial genera, including *Pseudomonas*, *Variovorax*, *Mesorhizobium*, *Enterobacter*, *Bradyrhizobium*, *Asaia*, *Stenotrophomonas*, *Brevundimonas*, *Rahnella*, *Sphingobacterium*, *Gluconobacter*, and *Klebsiella*, were significantly abundant in adults exposed to 50% seawater as larvae compared to those exposed to 0% seawater (**Figure 6.6 A**). Four bacterial genera, including *Comamonas*, *Enterococcus*, *Yersinia*, and *Elizabethkingia*, were significantly abundant in adults exposed to 0% seawater as larvae compared to 50% seawater (**Figure 6.6 A**).

Fourteen bacterial genera, including *Mesorhizobium*, *Bradyrhizobium*, *Kluyvera*, *Variovorax*, *Stenotrophomonas*, *Brevundimonas*, *Sphingobacterium*, *Asaia*, *Gluconobacter*, *Enterobacter*, *Klebsiella*, *Elizabethkingia*, *Pseudomonas*, and *Rahnella*, were significantly abundant in adults exposed to 50% seawater as larvae compared to those exposed to 6.25% seawater (**Figure 6.6 B**). Eight bacterial genera, including *Gluconobacter*, *Enterobacter*, *Yersinia*, *Klebsiella*, *Elizabethkingia*, *Pseudomonas*, *Rahnella* and *Aeromonas*, were significantly abundant in adults exposed to 6.25% seawater as larvae, compared to those exposed to 50% seawater (**Figure 6.6 B**).

Eleven bacterial genera, including *Pseudomonas*, *Mesorhizobium*, *Variovorax*, *Bradyrhizobium*, *Stenotrophomonas*, *Brevundimonas*, *Sphingobacterium*, *Asaia*, *Gluconobacter*, *Klebsiella*, and *Rahnella*, were significantly abundant in adults exposed to 50% seawater as larvae compared to those exposed to 12.5% seawater (**Figure 6.6 C**). Five bacterial genera, including *Serratia*, *Enterococcus*, *Klebsiella*, *Rahnella*, and *Elizabethkingia*, were significantly abundant in adults exposed to 12.5% seawater as larvae compared to those exposed to 50% seawater (**Figure 6.6 C**).

Twelve bacterial genera, including *Pseudomonas*, *Mesorhizobium*, *Variovorax*, *Bradyrhizobium*, *Stenotrophomonas*, *Brevundimonas*, *Klebsiella*, *Sphingobacterium*, *Asaia*, *Gluconobacter*, *Enterobacter*, and *Rahnella*, were significantly abundant in adults exposed to 50% seawater as larvae compared to those exposed to 25% seawater (**Figure 6.6 D**). Three bacterial genera, including *Enterobacter*, *Rahnella*, and *Elizabethkingia*, were significantly abundant in adults exposed to 25% seawater as larvae compared to those exposed to 50% seawater (**Figure 6.6 D**).

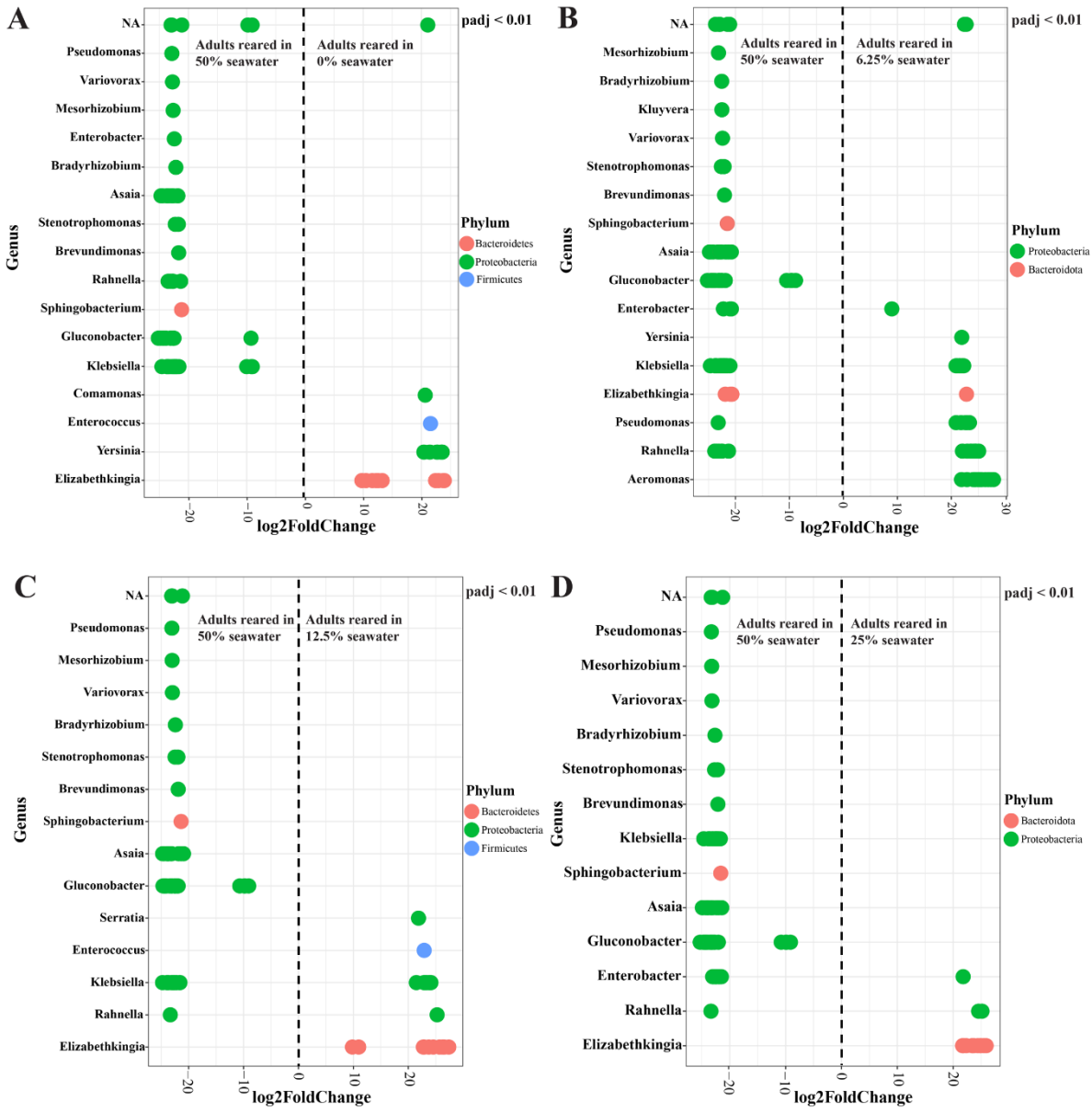

Figure 6.6 Pairwise comparisons of differentially abundant bacterial genera between *Anopheles merus* adults exposed to different seawater concentrations as larvae.

(A) Differentially abundant gut bacteria in adults reared from 50% seawater compared to 0% seawater. **(B)** Differentially abundant gut bacteria in adults reared from 50% seawater compared to 6.25% seawater. **(C)** Differentially abundant gut bacteria in adults reared from 50% seawater compared to 12.5% seawater. **(D)** Differentially abundant gut bacteria in adults reared from 50% seawater compared to 25% seawater. Differentially abundant genera had a significance threshold of *p*-value < 0.01. Genera to the left of 0 are significantly abundant in the adults reared in 50% seawater, while genera to the right of 0 are significantly abundant in the adults from the comparative concentration.

6.4 Discussion

Currently, limited studies characterise the impact of variable salinities in larval breeding sites of *Anopheles* mosquitoes[308,326]. Studies have focused on *Aedes* mosquitoes as this genus of mosquito has more salt-tolerant species[343,344]. As reviewed by Bartilol [175], *An. merus* has been recognised as a secondary dominant vector species capable of transmitting malaria along the Eastern and Southern coasts of Africa [175]. However, this species remains understudied. Despite their known osmotolerance, the effects of developing in variable salt concentration are poorly understood. This study investigated the impact of salt tolerance on the life history and gut microbiota of *An. merus*. The findings demonstrated that the salinity of larval breeding water influences various life history traits and the gut microbiota. Specifically, changes in the larval breeding water salinity significantly impact the developmental time of larvae, as well as the fertility, fecundity, and insecticide tolerance profiles of adult *An. merus*. However, the observed changes in both larvae and adults were dependent upon the salinity levels of the larval breeding water.

In this study, the larvae reared in the higher salt concentrations (25% and 50% seawater) took significantly longer to pupate. A reduced rate of development was observed in this strain of *An. merus* exposed to organic fertiliser [331]. As reviewed by Gondek *et al*. [345], organic fertilisers contain macronutrients such as potassium (K), phosphorous (P), and nitrogen (N), as well as soluble salts, which refer to soluble ions such as calcium ions (Ca^{2+}), potassium ions (K^{+}), magnesium ions (Mg^{2+}), and sodium ions (Na^{+}) [345]. A study conducted by White [326] established a significant shift in the rectal localisation of the ion regulator protein Na^{+}/K^{+}-ATPase. This shift is associated with osmoregulation functions in mosquitoes [301,326,346]. Therefore, unlike organic fertiliser supplementation, which serves as a food source, pure osmotic stress due to increased salt concentration has a negative effect. The changes in the osmotic stress in the larval environment by soluble salts result in a decrease in the development rate of *An. merus* larvae.

The exposure to the higher salt concentrations had no carry-over effect through to the longevity of *An. merus* adults. This is challenging to assess as there is very little data on this species. However, the effect of larval stress on *An. arabiensis* may provide insight into the dynamics of larval stress in *An. merus*. Toxic pollutants like heavy metals tended to reduce longevity [212], while less toxic pollutants such as inorganic and organic fertilisers had neutral to positive effects [331]. Therefore, this suggests that the stress of changes in the

salinity of the larval environment of *An. merus* was not significant enough (either positively or negatively) to alter adult longevity.

Additionally, the salinity of the larval breeding water influenced the number of eggs laid. *Anopheles merus* adults reared from 50% seawater laid a significantly higher number of eggs than those reared in 0% seawater. This finding may reflect that this laboratory strain is routinely reared in 50% seawater. As such, it is not surprising that the routine rearing resulted in the most eggs. However, a study on the effect of salt concentrations on *Ae. albopictus* found that females allowed to oviposit in the highest saltwater concentration had the lowest number of eggs laid [347]. Additionally, the eggs in the highest saltwater concentration did not melanise [347]. In routine mosquito husbandry for the strain used in this study, eggs laid in salt water do not melanise. Therefore, the females in this study were allowed to oviposit in freshwater. This suggests that in fixed breeding sites, which would fluctuate in salinity seasonally [325], this change in osmotic stress may influence fertility and fecundity. This may be due to the changes in salinity having a negative effect on reproduction. This would need to be confirmed with studies on wild mosquitoes.

The percentage of hatchlings from 12.5% seawater was significantly higher than that from 0% seawater. Therefore, 12.5% seawater is the suggested optimal concentration for laboratory-reared MAFUS eggs to hatch. Thus, differences in the salt concentration of the larval environment may result in fluctuations in the population density of wild *An. merus* depending on the salinity of the natural breeding water. Changes in the salinity of larval breeding sites will vary throughout the year due to wet and dry seasons [325]. This is an important factor to consider as it was suggested that in Malahlapanga, Limpopo, during the dry season, there was a change in the salinity of natural breeding habitats that became unfavourable for *An. arabiensis*. This change coincided with an increase in the population of *An. merus* [220]. This suggests that the salinity of larval breeding sites is related to the density populations of *An. merus*. However, further studies on fertility dynamics in wild populations of *An. merus* across varying saltwater concentrations could determine whether the findings from this study are replicated in natural settings. Such studies could also contribute to understanding the factors contributing to the increased population density of *An. merus* in Mpumalanga [179].

Insecticide tolerance response in *An. merus* adults reared from the different salt concentrations were significantly impacted. The 50% seawater treatment had a significantly increased tolerance for deltamethrin compared to the other seawater treatments. This observation is not

unprecedented, as various larval exposures to environmental stressors have resulted in increased insecticide tolerance [331,339]. It was observed that wild *An. merus* in Mpumalanga, South Africa, is susceptible to deltamethrin and has a low-level tolerance to DDT [181]. This low-level tolerance to organochlorine was suggested to be due to selective pressure potentially driven by the contamination of larval breeding sites [181]. Furthermore, in *Ae. aegypti*, various cytochrome P450 genes involved in insecticide resistance and xenobiotic detoxification are differentially expressed in the anal papillae (osmoregulatory organs) in response to brackish water [348]. The increased expression of these genes is congruent with an increased tolerance to pyrethroids [349–352]. As such, there may be an interplay between this capacity and the osmotolerance of the *An. merus* strain used in this study since it has a known insecticide tolerance.

The patterns in α-diversity of gut microbiota between larvae and adults differ. Generally, the larvae reared in untreated water have a significantly lower bacterial richness and evenness than those reared in water with elevated salinities. By contrast, the α-diversity of the gut microbiota in adults is not affected and remains constant between adults regardless of the changes in the salinity of the larval breeding water, except for adults reared in 25% seawater. The difference between the α-diversity of larval and adult gut microbiota is most likely attributed to the osmotic stress encountered in the water at the larval stage. These shifts in the larval gut richness and evenness are necessary for survival.

The species richness of the larval gut microbiota was not significantly impacted after an increase in the salinity of the larval environment beyond 12.5% seawater. The gut microbiota of the larvae was more evenly distributed when they were exposed to higher salinities (12.5% seawater, 25% seawater, and 50% seawater) compared to the lowest salinity examined (6.25% seawater) and untreated water. The significant increase in the species richness and evenness of gut bacteria when exposed to 12.5% seawater suggests that this salt concentration initiates a shift in the gut microbiota in response to osmotic stress. This further indicates that the gut microbiota can potentially modulate osmoregulation in larvae.

There is a noticeable difference in β-diversity among larvae reared in various salt concentrations and the adults emerging from them. The significant reduction in β-diversity in adults is evident from the lower number of unique species compared to larvae. The decrease in bacterial diversity is most likely attributed to the renewal of the gut epithelia from the pupal to the adult life stage [353]. Additionally, the β-diversity indicates that there is a significant

shift in the diversity of the gut microbiota in larvae reared in 50% seawater compared to the overlapping diversity observed for larvae reared in 6.25% seawater, 12.5% seawater, and 25% seawater. This suggests that the shift in bacterial diversity of larvae reared in 50% seawater may be linked to the osmotic stress of highly saline environments. The β-diversity between the adults is similar. This reinforces the notion that the variations in diversity noted among larvae may be linked to their capacity to endure osmotic stress during the aquatic phase. As such, it is possible that the primary role of the changes in gut bacterial diversity in response to osmotic stress was to ensure survival under such conditions. Any other changes seem to be a pleiotropic effect secondary to this effect, hence the lack of changes in diversity in the adult.

It is worth noting that the gut microbiota of *An. merus* used in this study is not dominated by halotolerant bacteria known for their strong salt tolerance, such as *Desulfovibrio*, *Halorubrum*, or *Natronomonas*. However, this does not necessarily imply that microbiota do not contribute to salt tolerance. The relevant halotolerant bacteria may be concentrated in different organs. Research on anophelines and culicine species suggests that the rectum and Malpighian tubules may play a more significant role in salt tolerance mechanisms [326,354]. Therefore, while the gut microbiota may not be dominated by halotolerant bacteria, other organs in mosquitoes could harbour microbes that contribute to their ability to tolerate saline environments. Despite not being dominant, several halotolerant bacterial genera were found in all treatments for both *An. merus* larvae and adults. *Achromobacter* [355], *Ancylobacter* [356], *Azospirillum* [357], *Brucella* [358], *Devosia* [359], *Flavobacterium* [360], *Microbacterium* [361], *Ochrobactrum* [362,363], *Sphingopyxis* [364], and *Staphylococcus* [363,365] are salt-tolerant bacterial genera that are found exclusively in larvae. Salt-tolerant bacterial genera in both larvae and adults include *Aeromonas* [366], *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* [367], *Bradyrhizobium* [368], *Comamonas* [369], *Enterobacter* [363], *Klebsiella* [362], *Pseudomonas* [362], *Rahnella* [370], *Sphingobacterium* [371], *and Stenotrophomonas* [363]. While the specific role of these salt-tolerant bacterial genera in the gut remains unclear, their presence opens opportunities for further investigations into their physiological functions. Future studies can investigate the specific roles of these salt-tolerant genera in the gut environment of *An. merus* larvae. Interestingly, pairwise comparisons of differentially abundant bacterial genera of both larvae and adults from the 50% seawater treatment had a significantly increased abundance of halotolerant genera compared to the 0% and 6.25% treatments. This suggests that the composition of gut microbiota in *An. merus* is adapted for saline habitats, and the abundance of halotolerant bacteria increases significantly in response to the elevated salinity of the larval habitat.

Despite the variability in gut microbiota induced by salt treatment, thirteen bacterial genera consistently appeared in both the larvae and adults across all treatments. These genera include *Aeromonas*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Bradyrhizobium*, *Comamonas*, *Elizabethkingia*, *Enterobacter*, *Enterococcus*, *Klebsiella*, *Pseudomonas*, *Rahnella*, *Soonwooa*, *Sphingobacterium*, and *Stenotrophomonas*. These genera are noteworthy as these are potentially the bacterial genera that represent, at least in part, the core microbiota of *An. merus*. Except for *Elizabethkingia*, *Enterococcus*, and *Soonwooa*, these genera are associated with salt tolerance. As reviewed by Ratcliffe *et al*. [372], *Enterobacter*, *Elizabethkingia*, and *Pseudomonas* are current potential candidates for paratransgenesis [372]. These are ideal paratransgenesis candidates in *An. merus* as they are native symbionts in both the larvae and adults across all treatments.

In this study, several bacterial genera associated with *Plasmodium* protection in other *Anopheles* species were found in *An. merus* adults. *Plasmodium*-protective genera present in the guts of *An. merus* includes *Elizabethkingia*, *Pseudomonas*, *Asaia*, *Serratia*, *Enterococcus*, *Klebsiella*, and *Enterobacter* [43,76,77,126,373]. The diversity of *Plasmodium*-protective genera increases in adults compared to larvae. This suggests a potential trade-off between bacterial genera involved in osmotolerance during the larval stage and those associated with modulating *Plasmodium* infection in adults. These protective bacterial genera were significantly more abundant in the adults reared from the 50% seawater than in other treatments. This suggests that freshwater *An. merus* is potentially more susceptible to *Plasmodium* infection due to the decreased abundance of *Plasmodium*-protective genera. The combination of these findings, coupled with the decrease in immune gene expression[374,375], suggests that the osmotolerant capacity of *An. merus* can influence immune function. Consequently, the modulation of immune functions may have implications for vector competence, potentially altering the ability of *An. merus* to transmit malaria parasites. This suggests an interplay between osmotolerance, immune regulation, and vector competence in *An. merus*.

The strain of *An. merus* used in this study is routinely reared in 50% seawater (17.5 g of salt/1L of water). However, it is important to note that this trait is not fixed, and prolonged breeding in freshwater can reverse salt tolerance (unpublished data). Therefore, in this study, it is expected that the highest salt concentration generally would result in the greatest fitness advantage compared to other treatments. However, it is important to note that although the study highlights statistical differences, they do not necessarily translate into functional effects

in the field. The behaviour of *An. merus* observed in this study may not necessarily reflect that of wild populations breeding in high salt concentrations. As the gut microbiota of laboratory-reared strains can offer initial insights into microbial metagenomics, it is important to understand that they are an underrepresentation of bacterial diversity compared to wild populations[204]. Therefore, to gain a better understanding of the dynamics of wild *An. merus* populations, and to confirm that the salinity of the larval breeding sites has a significant impact, further studies on wild populations of *An. merus* are required. Furthermore, larval environments must be examined, particularly where *An. merus* is prevalent. The definitive way to determine whether our findings are of epidemiological significance is to confirm them through surveillance activities.

6.5 Conclusion

In conclusion, *An. merus* larvae bred in varying salt concentrations exhibit altered physiology, evident in both larval and adult life stages. Significant differences were observed in the life history traits and gut microbiota of *An. merus* across the different salt concentrations. In particular, the larvae were directly affected as higher salt concentrations reduced development time. Varying larval salt exposure significantly impacted adult life history traits such as adult longevity, fertility, and fecundity. Furthermore, changes in the salinity of the larval environment affected the insecticide tolerance of *An. merus* adults, with significant differences observed in lethal time. Evidence suggests the presence of a core microbiota in *An. merus* reared in different salinities. However, alterations in the salt concentration of larval breeding water salinity result in changes in the abundance and diversity of gut microbiota. Where there is a larger variation in salt concentration within the larval environment, a more significant difference is observed in the comparative microbiota between the adults. Adults reared from 50% seawater exhibited a greater bacterial diversity in the gut and the highest number of bacterial genera associated with protection against *Plasmodium*. Overall, changes in the salinity of the larval environment have the potential to significantly impact the life history and immunity modulation of *An. merus* in an epidemiologically relevant manner. These findings highlight the importance of considering the salinity of larval breeding water in surveillance efforts where this dominant vector species is prevalent.

CHAPTER 7

Discussion and Conclusion

This study investigated the gut microbiota of selected members of the *An. gambiae* complex found in South Africa. **CHAPTER 3** compared the gut microbiota of laboratory reared strains of *An. arabiensis*, *An. merus*, and *An. quadriannulatus*. **CHAPTER 4** examined the gut microbiota of adult *An. arabiensis* between insecticide susceptible and insecticide resistant laboratory reared strains, an F_1 population, and a wild population. Additionally, the impact of larval exposure to heavy metals on the adult gut microbiota of insecticide susceptible and insecticide resistant laboratory reared strains and an F_1 population of *An. arabiensis* adult was investigated. **CHAPTER 5** examined the gut mycobiota at different life stages for insecticide susceptible and insecticide resistant laboratory reared strains and an F_1 population of *An. arabiensis* collected from the field. Finally, **CHAPTER 6** investigated the effect of changes in the salt concentration of larval breeding water on the gut microbiota of laboratory reared *An. merus* larvae and adults.

South Africa is a frontline candidate for eliminating malaria in the Southern African Development Community (SADC) region. It is one of the Elimination 8 countries, along with Botswana, Namibia, and eSwatini who are among the frontrunners to eliminate the disease [214,215]. However, this means that South Africa suffers from some of the challenges faced with malaria elimination. Possibly, the greatest challenge to malaria elimination in South Africa is the problem of residual malaria. This is a low-level amount of locally acquired cases that persist despite the full implementation of control methods. In South Africa, this is usually driven by outdoor active and resting vectors [29]. Here, outdoor resting *An. arabiensis* has been implicated as a vector [26], as well as the zoophilic, exophilic and exophagic members of the *An. funestus* group: *An. parensis* and *An. vaneedeni* [376,377]. Controlling these vectors poses significant challenges, particularly since all currently identified vectors in South Africa belong to these challenging species. Therefore, a crucial component of the malaria research agenda in South Africa is to enhance the current understanding of effective methods for controlling these vectors.

Currently, a sterile insect technique (SIT) programme is being trialled to target *An. arabiensis* in the KwaZulu Natal (KZN) province [229]. Although this contends with the problem of outdoor biting by *An. arabiensis*, this does not deal with the issue of potential niche

replacement by other members of the *An. gambiae* complex present in the province, such as *An. merus* [31]. Furthermore, potential vectors present in the province include *An. parensis* and *An. vaneedeni*. As such, further complementary strategies are required.

Ideally, these would be non-insecticidal, or at least non-adulticidal, as low-level insecticide resistance is present in this population [31]. This is why paratransgenesis is an attractive complementary strategy. As reviewed by Wilke and Marrelli [120], biocontrol by symbionts can target outdoor active mosquitoes [120]. Furthermore, there is potential to target more than a single *Anopheles* species. These are qualities required for an entomological strategy to aid the elimination agenda in a country like South Africa, which has multiple exophilic species. However, the lack of information on the diversity and dynamics of the relevant gut microbiota is a challenge for implementing such a strategy. This is a core problem that this research addresses. The data produced in this study forms a baseline for future work on paratransgenesis outside of the major malaria vectors *An. gambiae* and *An. coluzzii* [18,23,24].

One of the first objectives of this study was to characterise the dynamic gut microbiota of members of the *An. gambiae* complex. Ideally, this would have been performed using *An. arabiensis* originating from South Africa. However, due to the importance of considering the insecticide resistant phenotype, this study used genetically matched *An. arabiensis* strains from Sudan that differ in resistance phenotype [104]. Despite the geographical differences, there is enough of a core microbiome to make comparisons. Possibly the most relevant finding of this study is the fact that the gut microbiota of *An. arabiensis* is significantly different from the minor vector *An. merus* and non-vector *An. quadriannulatus*. Specifically, these included a greater relative abundance of *Plasmodium*-protective genera. This highlights that vector competence in these species is not only due to behavioural differences but also differences in immunology, possibly in part due to modulation by the gut microbiota. This could form part of a range of molecular underpinnings that could potentially be exploited for control, for example epigenetic differences [133]. A potential argument against these findings is that this study was performed on laboratory-reared strains, with the SENN strain being in colony since 1980 [128]. Some characteristics of colonised mosquitoes do not conserve well. These include immunity and specific aspects of reproduction [191,192].

Although some characteristics of colonised mosquitoes do not conserve well, the gut microbiota, however, does not entirely fall into this category. As demonstrated in this study, a

core set of microbes becomes less diverse with time in colony. This study reinforces this finding and highlights the dynamics of comparing the gut microbiota of adult mosquitoes directly from the wild (collected as larvae from the field) to the F_1 populations (offspring of adults collected from the field). This study could not compare direct changes in the gut microbiota from wild larvae to their F_1 progeny. This was primarily due to the number of mosquitoes available, as not enough larvae were collected to obtain both F_0 and F_1 individuals. However, as the wild populations were larvae collected in the same area as the adults used to produce the F_1 population, they represent the same population. Notably, at the α-diversity level, there was already a difference in the diversity of the gut microbiota of adults from the wild population compared to the F_1 population. The diversity of the gut microbiota in the F_1 population adults was more evenly distributed after being reared under insectary conditions for only one generation. This rapid change suggests that adaptation to colonisation may begin with the diversity of the gut bacteria becoming more evenly distributed before losing bacterial richness.

Our study also highlights the importance of the environment, particularly the larval environment, in shaping the gut microbiota. The wild population of *An. arabiensis* larvae were reared in the water they were collected from, while the F_1 population of *An. arabiensis* larvae were reared in filtered and ultraviolet-treated (UV-treated) reverse osmosis water. Since they were fed the same diet once coming into the insectary, it is likely that the water, rather than their diet, accounts for these differences. As this distinction in diversity was highly noticeable, it is essential to consider the effect of the larval environment on the dynamics of the gut microbiota. This is particularly true when searching for potential natural, unaltered symbionts to use for paratransgenesis efforts. For practical purposes, direct larval collections will be the best way to collect samples for paratransgenesis studies. However, acquiring wild specimens in South Africa is particularly challenging due to low vector densities in most endemic provinces [214,215]. Furthermore, larval collections do not always correspond to the adult populations encountered. For example, *An. merus* is dominant in Mpumalanga, South Africa. Until recently, it was not possible to collect *An. arabiensis* larvae in the area. Consequently, implicating a vector in the province is challenging. Despite the potential for improved bacterial diversity through larval collections, in scenarios such as South Africa, where multiple members of the *An. gambiae* complex coexists, relying solely on larval collections may overlook relevant mosquito species.

This study has highlighted the effect of two changes in the larval environment and its influence on the gut microbiota. Crucially, this is about two different species of the *An. gambiae* complex. The first relates to the impact of heavy metal stress in the larval environment and its effect on the gut microbiota of the laboratory reared and F_1 population of *An. arabiensis* adults. The second relates to the impact of changes in the salt concentration in the larval environment and its influence on the gut microbiota of laboratory-reared *An. merus* larvae and adult populations.

The carry-over effect of heavy metal exposure on larvae to adult *An. arabiensis* has allowed for some unique insights into the impact of the larval environment on the gut microbiota. Heavy metal exposure altered the number of bacterial genera in the adult gut. However, each strain responded differently to each heavy metal examined. The SENN adults exposed to cadmium had a higher number of bacterial genera associated with heavy metal tolerance and degradation. Furthermore, SENN has more heavy metal degrading genera, while SENN DDT has more heavy metal tolerant genera. Therefore, there is likely an interplay between the gut microbiota and response to heavy metal stress in SENN. However, studies implicating the bacteria found in SENN in heavy metal metabolism must be confirmed. Previous studies [104,232] observed that SENN DDT coped better with heavy metal stress due to upregulated detoxification enzymes and thus already has a defence against heavy metal exposure [232]. This suggests that not only do stressors in the larval environment impact the gut microbiota, but the response to these stressors is also affected by insecticide resistant or susceptible phenotypes.

In contrast to the laboratory reared strains, the F_1 population adults had heavy metal tolerant and degrading bacterial genera in untreated adults and adults exposed to metal pollutants. Although this may be a function of the increased diversity of the F_1 population, there is likely an interplay between the gut microbiota and heavy metal degradation as a result of selective pressure on the gut microbiota. The F_1 population is the offspring of adults collected from an agricultural area. Therefore, exposure of these adults to organic fertilisers and other potential sources of heavy metal pollutants is not unprecedented. Since the effects of metal pollutant exposure are transgenerational and exposure to stressors can alter the microbial communities [103,232], exposure of the previous generations may have resulted in a selective pressure for heavy metal degrading bacteria. Hence, bacterial genera involved in the degradation of heavy metals were present and significantly abundant in both untreated and metal-treated F_1 generation adults. Functional studies of the gut bacteria in the mosquito in response to

environmental stressors are, therefore, a critical aspect of microbiome studies. It is particularly crucial for paratransgenesis vector control strategies as the native bacterial communities are affected.

Heavy metal pollution in larval breeding water is of consequence to mosquito microbiota studies. Members from the *An. gambiae* complex are expanding their breeding sites to polluted water throughout Africa [114,227]. In this study, exposure to heavy metals, specifically copper, had increased bacterial genera that were not present in the untreated adults. Heavy metals, such as zinc, manganese iron, cobalt, nickel, and copper, are micronutrients for microorganisms such as bacteria [257]. These nutrients are essential for the development and growth of microorganisms. Therefore, understanding the role of heavy metals in the larval environment and its impact on the mosquito microbiota is crucial.

The work on the minor vector *An. merus* in this study further highlights the role of the larval environment on gut microbiota. It is essential that *An. merus* is considered a potential vector as it can become a niche-replacing vector in the absence of a dominant vector species [378]. This emphasises the significance of recognising *An. merus* as a vector of concern. In this research, it was observed that laboratory-reared *An. merus* breeding in high salt concentrations may have two potential outcomes. Firstly, it may reduce susceptibility to deltamethrin, a common insecticide used in South Africa [379]. Secondly, it could suggest a potentially lower susceptibility to *Plasmodium*, as an increased presence of *Plasmodium*-protective species is present. To our knowledge, the effect of changes in the salt concentration in the larval environment has never been considered as a factor that may alter vector competence. Although this experiment would need to be replicated with wild mosquitoes, the data from this study suggests this may be happening in the wild. Furthermore, other immunological factors are also at play; changes in the salt concentration of the larval environment also affect the expression of the antimicrobial peptide defensin-1 in adult *An. merus* [375]. As such, this study highlights the potential for the larval environment to alter traits that are of epidemiological importance.

When considering the mining of local microbes for the potential use of native symbionts in paratransgenesis, it's essential to acknowledge that the larval environment plays a significant role in shaping the gut microbiota. The findings in this study suggest that the larval environment dynamics would alter the populations of viable bacteria that could be cultured. Furthermore, paratransgenesis efforts with these specific populations of viable bacteria would

be affected by changes in the gut microbiota of wild mosquitoes that are influenced by larval environments. Therefore, it is crucial that the local breeding sites also be characterised as part of establishing paratransgenesis efforts. This has been highlighted by the work in the Mpumalanga province, South Africa. Different breeding sites within the same region were found to have different pollution profiles, which affected the bacterial diversity of both the water and the guts of the larvae (Noeth *et al*., manuscript in preparation). A recent preprint describes the physicochemical characteristics of the larval breeding sites from which the wild specimens used in this study were collected [380]. Crucially, it did not consider the potential pollutants in the breeding sites, and this study has highlighted the importance of this understanding.

This study not only added to the body of knowledge of *An. gambiae* microbiota, but it also presents one of the first reports of the gut mycobiota of *An. arabiensis*. The *Anopheles* mycobiota has been poorly studied compared to the bacterial component of the microbiota. The search term "*Anopheles* microbiota" returned 188 results on PubMed, compared to 1 for "*Anopheles* mycobiota". For Google Scholar, these terms returned 9,430 and 240 results, respectively. The disparity between these is possible because the Next-Generation Sequencing (NGS) protocols for identifying yeasts and moulds are not as well established as those for bacterial identification. In this study, the V3-V4 region of the 16S rRNA gene was amplified with a single forward and reverse primer. This study amplified the ITS1 region using eight forward primers and seven reverse primers. Furthermore, 18S identification could also be used to identify fungi. It is possibly due to this complexity that the mosquito mycobiota is less examined.

Additionally, working with fungi would be challenging when genetically modifying these organisms. Due to their complex cell walls, yeasts and moulds would not be as easy to transform as bacteria. Therefore, using fungi for paratransgenesis would be more challenging than bacteria. Despite the challenges, there is still a possibility of utilising fungal candidates for paratransgenesis. *Microsporidia MB* is a spore-forming unicellular parasite related to fungi that was first discovered in *An. arabiensis* [121]. It was found to inhibit the transmission of *P. falciparum* and can be transmitted both horizontally and vertically [121,381]. Over and above the capacity to prevent transmission, this strain has several biological effects that make it an excellent paratransgenesis candidate [382]. *Microsporidia* are also a crucial category of candidates that must be considered [383].

The dynamics of the gut mycobiota of three-day old laboratory reared and F_1 population of *An. arabiensis* were similar to the dynamics of the bacteriome of three-day old adults. Like with the bacteriome, there was a far greater diversity in three-day old F_1 population adults than in laboratory-reared strains. As there is a precedent for this in microbiota studies, this was unsurprising [43,216]. Notably, blood-feeding status in older mosquitoes did not affect the richness of the gut mycobiota between laboratory-reared and F_1 population adults. However, there are large-scale differences in the diversity of the gut mycobiota diversity in the fifteen-day old adults, with the F_1 population adults being significantly lower, particularly after multiple bloodmeals. This highlights a difference in the dynamics of the gut mycobiota, which are associated with age and diet.

This study has, therefore, formed some baseline characterisation of gut microbiota in members of the *An. gambiae* complex. However, substantial groundwork must still be performed before a microbial-based strategy for vector control can be implemented. The first is that further baseline characterisation needs to be performed on wild material. This would have to be on a province-by-province level as anopheline diversity differs in endemic provinces. This may suggest that different species are responsible for local transmission in the provinces[321]. Our studies indicate that larval-collected specimens from the field (wild population) would provide the highest diversity. However, in some provinces, a marked disconnect exists between species obtained by larval collection and adult collection (Noeth *et al*., manuscript in production). Therefore, both larval and adult collections would be required.

Identification of species and, ideally, strain level would be needed. Advances in NGS sequencing technology would facilitate this. Shifting from the Illumina platform to the PacBio platform, which generates long reads, could increase the data's resolution. Whole genome sequencing would also provide more information. Furthermore, metagenome sequencing, rather than ITS sequencing, would negate the problems of identifying fungi and possibly *Microsporidia*. It would also provide insight into the role of host-microbe interactions, as specific bacterial and fungal strains are involved in these dynamics. However, even though the price of NGS is coming down, metagenome sequencing is still prohibitively expensive for a large-scale study, even in South Africa. Although this study has categorised and catalogued the gut microbiota of members of the *An. gambiae* complex, more work still needs to be done to understand the functional significance of these species. This study only examined the gut microbiota of females, though understanding microbial dynamics in males would be as insightful [384].

In conclusion, the gut microbiota of the *An. gambiae* complex is a dynamic network consisting of what seems to be a core microbiota that is crucial to its function. There are distinct differences between the diversity and abundance of the gut microbiota of major malaria vectors and the minor and non-vectors. The gut microbiota of the selected members of the *An. gambiae* complex corresponds to their capacity to transmit *Plasmodium*. The larval environment strongly influences the dynamics of the gut microbiota. The gut bacteria may offer protection against metal stress in the absence of xenobiotic detoxification mechanisms. In the minor vector, *An. merus*, larval osmotic stress strongly influences life history traits and could potentially impact the ability to transmit *Plasmodium*. Therefore, the impact of the larval environment on the adult microbiota must be considered for the development and implementation of future paratransgenesis strategies.

REFERENCES

1. Breman JG, Brandling-Bennett AD. The challenge of malaria eradication in the twenty-first century: Research linked to operations is the key. Vol. 29, Vaccine. 2011.29(4):97–103.

2. WHO. World Malaria Report 2013. In: World Health Organisation. 2013;

3. WHO. World Malaria Report 2022. World Health Organization. 2022;

4. Morris N, Frean J, Baker L, Ukpe IS, Barnes KI, Kruger P, *et al*. Re-defining the extent of malaria transmission in South Africa: Implications for chemoprophylaxis. Vol. 103, South African Medical Journal. 2013.103(11):861–4.

5. Crutcher J, Hoffman S. Malaria. In: Medical Microbiology. Galveston (TX): Baron S; 1996; Available from: https://www.ncbi.nlm.nih.gov/books/NBK8584/

6. Votýpka J, Modrý D, Oborník M, Šlapeta J, Lukeš J. Apicomplexa BT - Handbook of the Protists. Archibald JM, Simpson AGB, Slamovits CH, Margulis L, Melkonian M, Chapman DJ, *et al*., editors. Cham: Springer International Publishing; 2017; 1–58 p. Available from: https://doi.org/10.1007/978-3-319-32669-6_20-1

7. Bennink S, Kiesow MJ, Pradel G. The development of malaria parasites in the mosquito midgut. Vol. 18, Cellular microbiology. 2016.18(7):905–18.

8. Sinden RE, Hartley RH. Identification of the meiotic division of malarial parasites. Vol. 32, The Journal of protozoology. 1985.32(4):742–4.

9. Siciliano G, Costa G, Suárez-Cortés P, Valleriani A, Alano P, Levashina EA. Critical Steps of *Plasmodium falciparum* Ookinete Maturation. Vol. 11, Frontiers in microbiology. 2020.11:269.

10. Vaughan JA. Population dynamics of *Plasmodium* sporogony. Vol. 23, Trends in parasitology. 2007.23(2):63–70.

11. Smith RC, Jacobs-Lorena M. *Plasmodium*-Mosquito Interactions: A Tale of

Roadblocks and Detours. Vol. 39, Advances in insect physiology. 2010.39:119–49.

12. Singh M, Suryanshu, Kanika, Singh G, Dubey A, Chaitanya RK. *Plasmodium's* journey through the *Anopheles* mosquito: A comprehensive review. Vol. 181, Biochimie. 2021.181:176–90.

13. Pirahmadi S, Zakeri S, Djadid ND, Mehrizi AA. A review of combination adjuvants for malaria vaccines: a promising approach for vaccine development. Vol. 51, International Journal for Parasitology. 2021.51(9):699–717.

14. Hoffman SL, Vekemans J, Richie TL, Duffy PE. The march toward malaria vaccines. Vol. 33, Vaccine. 2015.33:D13–23.

15. Laurens MB. RTS,S/AS01 vaccine (Mosquirix^{TM}): an overview. Vol. 16, Human vaccines & immunotherapeutics. 2020.16(3):480–9.

16. Agnandji ST, Lell B, Fernandes JF, Abossolo BP, Kabwende AL, Adegnika AA, *et al*. Efficacy and Safety of the RTS,S/AS01 Malaria Vaccine during 18 Months after Vaccination: A Phase 3 Randomized, Controlled Trial in Children and Young Infants at 11 African Sites. Vol. 11, PLoS Medicine. 2014.11(7).

17. El-Moamly AA, El-Sweify MA. Malaria vaccines: the 60-year journey of hope and final success—lessons learned and future prospects. Vol. 51, Tropical Medicine and Health. 2023.51(1).

18. Sinka ME, Bangs MJ, Manguin S, Coetzee M, Mbogo CM, Hemingway J, *et al*. The dominant *Anopheles* vectors of human malaria in Africa, Europe and the Middle East: occurrence data, distribution maps and bionomic précis. Vol. 3, Parasites & Vectors 2010 3:1. 2010.3(1):1–34.

19. Dadzie SK, Brenyah RC, Appawu MA. Role of Species Composition in Malaria Transmission by the *Anopheles funestus* Group (Diptera: Culicidae) in Ghana. Vol. 38, Journal of vector ecology : journal of the Society for Vector Ecology. 2013.38(1):105–10.

20. Russell TL, Beebe NW, Cooper RD, Lobo NF, Burkot TR. Successful malaria

elimination strategies require interventions that target changing vector behaviours. Vol. 12, Malaria Journal. 2013.12(1):1–5.

21. Subbarao SK, Sharma VP. Anopheline species complexes & malaria control. Vol. 106, The Indian journal of medical research. 1997.106:164–73.

22. Fontenille D, Lochouarn L. The complexity of the malaria vectorial system in Africa. Vol. 41, Parassitologia. 1999.41(1–3):267–71.

23. Gillies M.T. and Coetzee M. A supplement to the Anophelinae of Africa South of the Sahara. Vol. 55, South African Institute for Medical Research. 1987.55.

24. Brooke B, Koekemoer L, Kruger P, Urbach J, Misiani E, Coetzee M. Malaria vector control in South Africa. Vol. 103, South African Medical Journal. 2013.103(10):784–8.

25. Dahan-Moss Y, Munhenga G, Kaiser M, Riddin M, Matamba A, Ramashia W, *et al*. Malaria Vector Surveillance Report, South Africa. Vol. 16. 2021.16(May 2021):29–35.

26. Dandalo LC, Brooke BD, Munhenga G, Lobb LN, Zikhali J, Ngxongo SP, *et al*. Population Dynamics and *Plasmodium falciparum* (Haemosporida: Plasmodiidae) Infectivity Rates for the Malaria Vector *Anopheles arabiensis* (Diptera: Culicidae) at Mamfene, KwaZulu-Natal, South Africa. Vol. 54, Journal of Medical Entomology. 2017.54(6):1758–66.

27. Gillies MT, De Meillon B. The Anophelinae of Africa south of the Sahara (Ethiopian Zoogeographical Region). In: The Anophelinae of Africa south of the Sahara (Ethiopian Zoogeographical Region). Johannesburg: South African Institute for Medical Research, P.O. Box 1038, S. Africa.; 1968; p. 343.

28. Killeen GF. Characterizing, controlling and eliminating residual malaria transmission. Vol. 13, Malaria Journal. 2014.13(1):1–22.

29. Kitau J, Oxborough RM, Tungu PK, Matowo J, Malima RC, Magesa SM, *et al*. Species shifts in the *Anopheles gambiae* complex: Do LLINs successfully control *Anopheles arabiensis*? Vol. 7, PLoS ONE. 2012.7(3):1–7.

30. WHO. World Malaria Report: 20 years of global progress and challenges [Internet]. World Health Organization. 2020;

31. Munhenga G, Oliver S V., Lobb LN, Mazarire TT, Sekgele W, Mashatola T, *et al*. Malaria risk and receptivity: Continuing development of insecticide resistance in the major malaria vector *Anopheles arabiensis* in northern KwaZulu-Natal, South Africa. Vol. 118, South African Journal of Science. 2022.118(3–4):1–7.

32. Habtewold T, Groom Z, Christophides GK. Immune resistance and tolerance strategies in malaria vector and non-vector mosquitoes. Vol. 10, Parasites & vectors. 2017.10(1):1–12.

33. Clements A. The Biology of Mosquitoes. Vol. 3, The Biology of Mosquitoes. 2011.3:1–571.

34. Beerntsen BT, James AA, Christensen BM. Genetics of mosquito vector competence. Vol. 64, Microbiology and molecular biology reviews : MMBR. 2000.64(1):115–37.

35. Marchesi JR, Ravel J. The vocabulary of microbiome research: a proposal. Vol. 3, Microbiome. 2015.3(1):1–3.

36. Hou K, Wu ZX, Chen XY, Wang JQ, Zhang D, Xiao C, *et al*. Microbiota in health and diseases. Vol. 7, Signal transduction and targeted therapy. 2022.7(1):135.

37. Chawla M, Gupta R, Das B. Chapter Eight - Gut microbiome dysbiosis in malnutrition. In: Das B, Singh VBTP in MB and TS, editors. Vol. 192, Human Microbiome in Health and Disease - Part B. Academic Press; 2022; p. 205–29. Available from: https://www.sciencedirect.com/science/article/pii/S1877117322001065

38. Berg G, Rybakova D, Fischer D, Cernava T, Vergès MCC, Charles T, *et al*. Correction to: Microbiome definition re-visited: old concepts and new challenges. Vol. 8, Microbiome. 2020.8(1):1–22.

39. Cansado-Utrilla C, Zhao SY, McCall PJ, Coon KL, Hughes GL. The microbiome and mosquito vectorial capacity: rich potential for discovery and translation. Vol. 9, Microbiome. 2021.9(1):1–11.

40. Strand MR. Composition and functional roles of the gut microbiota in mosquitoes. Vol. 28, Current opinion in insect science. 2018.28:59–65.

41. Coon KL, Vogel KJ, Brown MR, Strand MR. Mosquitoes rely on their gut microbiota for development. Vol. 23, Molecular Ecology. 2014.23(11):2727–39.

42. Gimonneau G, Tchioffo M, Abate L, Boissière A, Awono-Ambéné P, SE N, *et al*. Composition of *Anopheles coluzzii* and *Anopheles gambiae* microbiota from larval to adult stages. Vol. 28, Infection, Genetics and Evolution. 2014.28:715–24.

43. Boissière A, Tchioffo MT, Bachar D, Abate L, Marie A, Nsango SE, *et al*. Midgut microbiota of the malaria mosquito vector *Anopheles gambiae* and interactions with *Plasmodium falciparum* infection. Vol. 8, PLoS Pathogens. 2012.8(5):1–12.

44. Segata N, Baldini F, Pompon J, Garrett WS, Truong DT, Dabiré RK, *et al*. The reproductive tracts of two malaria vectors are populated by a core microbiome and by gender swarm-enriched microbial biomarkers. Vol. 6, Scientific reports. 2016.6:24207.

45. Damiani C, Ricci I, Crotti E, Rossi P, Rizzi A, Scuppa P, *et al*. Paternal transmission of symbiotic bacteria in malaria vectors. Vol. 18, Current Biology. 2008.18(23):R1087–8.

46. Hughes GL, Dodson BL, Johnson RM, Murdock CC, Tsujimoto H, Suzuki Y, *et al*. Native microbiome impedes vertical transmission of *Wolbachia* in *Anopheles* mosquitoes. Vol. 111, Proceedings of the National Academy of Sciences of the United States of America. 2014.111(34):12498–503.

47. Favia G, Ricci I, Damiani C, Raddadi N, Crotti E, Marzorati M, *et al*. Bacteria of the genus *Asaia* stably associate with *Anopheles stephensi*, an Asian malarial mosquito vector. Vol. 104, Proceedings of the National Academy of Sciences of the United States of America. 2007.104(21):9047–51.

48. Wang S, Dos-Santos ALA, Huang W, Liu KC, Oshaghi MA, Wei G, *et al*. Driving mosquito refractoriness to *Plasmodium falciparum* with engineered symbiotic bacteria. Vol. 357, Science (New York, N.Y.). 2017.357(6358):1399–402.

49. Chouaia B, Rossi P, Epis S, Mosca M, Ricci I, Damiani C, *et al*. Delayed larval

development in *Anopheles* mosquitoes deprived of *Asaia* bacterial symbionts. Vol. 12, BMC Microbiology 2012 12:1. 2012.12(1):1–8.

50. Lindh JM, Borg-Karlson AK, Faye I. Transstadial and horizontal transfer of bacteria within a colony of *Anopheles gambiae* (Diptera: Culicidae) and oviposition response to bacteria-containing water. Vol. 107, Acta Tropica. 2008.107(3):242–50.

51. Chavshin AR, Oshaghi MA, Vatandoost H, Yakhchali B, Zarenejad F, Terenius O. Malpighian tubules are important determinants of *Pseudomonas* transstadial transmission and longtime persistence in *Anopheles stephensi*. Vol. 8, Parasites and Vectors. 2015.8(1):1–7.

52. Saab SA, Dohna HZ, Nilsson LKJ, Onorati P, Nakhleh J, Terenius O, *et al*. The environment and species affect gut bacteria composition in laboratory co-cultured *Anopheles gambiae* and *Aedes albopictus* mosquitoes. Vol. 10, Scientific Reports. 2020.10(1):1–13.

53. Muturi EJ, Dunlap C, Ramirez JL, Rooney AP, Kim CH. Host blood-meal source has a strong impact on gut microbiota of *Aedes aegypti*. Vol. 95, FEMS Microbiology Ecology. 2018.95(1).

54. Gabrieli P, Caccia S, Varotto-Boccazzi I, Arnoldi I, Barbieri G, Comandatore F, *et al*. Mosquito Trilogy: Microbiota, Immunity and Pathogens, and Their Implications for the Control of Disease Transmission. Vol. 12, Frontiers in Microbiology. 2021.12(April):1–17.

55. Kozlova E V., Hegde S, Roundy CM, Golovko G, Saldaña MA, Hart CE, *et al*. Microbial interactions in the mosquito gut determine *Serratia* colonization and blood-feeding propensity. Vol. 15, ISME Journal. 2021.15(1):93–108.

56. Martinson VG, Strand MR. Diet–Microbiota Interactions Alter Mosquito Development. Vol. 12, Frontiers in Microbiology. 2021.12(June):650743.

57. Muturi EJ, Dunlap C, Smartt CT, Shin D. Resistance to permethrin alters the gut microbiota of *Aedes aegypti*. Vol. 11, Scientific Reports. 2021.11(1):1–8.

58. Muturi EJ, Njoroge TM, Dunlap C, Cáceres CE. Blood meal source and mixed blood-feeding influence gut bacterial community composition in *Aedes aegypti*. Vol. 14, Parasites and Vectors. 2021.14(1):1–8.

59. Clements AN. The biology of mosquitoes. Volume 2: sensory reception and behaviour. :740.

60. Foster WA. Mosquito sugar feeding and reproductive energetics. Vol. 40, Annual review of entomology. 1995.40:443–74.

61. Guégan M, Tran Van V, Martin E, Minard G, Tran FH, Fel B, *et al*. Who is eating fructose within the *Aedes albopictus* gut microbiota? Vol. 22, Environmental microbiology. 2020.22(4):1193–206.

62. Clements A. The biology of mosquitoes. Volume 1: development, nutrition and reproduction. Vol. 1, Chapman & Hall. 1992.1:509.

63. Hansen IA, Attardo GM, Rodriguez SD, Drake LL. Four-way regulation of mosquito yolk protein precursor genes by juvenile hormone-, ecdysone-, nutrient-, and insulin-like peptide signaling pathways. Vol. 5, Frontiers in Physiology. 2014.5(103):1–8.

64. Gaio A de O, Gusmão DS, Santos A V, Berbert-Molina MA, Pimenta PF, Lemos FJ. Contribution of midgut bacteria to blood digestion and egg production in *Aedes aegypti* (Diptera: Culicidae) (L.). Vol. 4, Parasites & Vectors 2011 4:1. 2011.4(1):1–10.

65. Gao H, Cui C, Wang L, Jacobs-Lorena M, Wang S. Mosquito Microbiota and Implications for Disease Control. Vol. 36, Trends in Parasitology. 2020.36(2):98–111.

66. Sharma A, Dhayal D, Singh OP, Adak T, Bhatnagar RK. Gut microbes influence fitness and malaria transmission potential of Asian malaria vector *Anopheles stephensi*. Vol. 128, Acta Tropica. 2013.128(1):41–7.

67. Coon KL, Brown MR, Strand MR. Gut bacteria differentially affect egg production in the anautogenous mosquito *Aedes aegypti* and facultatively autogenous mosquito *Aedes atropalpus* (Diptera: Culicidae). Vol. 9, Parasites and Vectors. 2016.9(1):1–12.

68. Gendrin M, Yerbanga RS, Ouedraogo JB, Lefèvre T, Cohuet A, Christophides GK. Differential effects of azithromycin, doxycycline, and cotrimoxazole in ingested blood on the vectorial capacity of malaria mosquitoes. Vol. 3, Open Forum Infectious Diseases. 2016.3(2):1–8.

69. Debalke S, Habtewold T, Duchateau L, Christophides GK. The effect of silencing immunity related genes on longevity in a naturally occurring *Anopheles arabiensis* mosquito population from southwest Ethiopia. Vol. 12, Parasites and Vectors. 2019.12(1):1–8.

70. Gendrin M, Rodgers FH, Yerbanga RS, Ouédraogo JB, Basáñez MG, Cohuet A, *et al*. Antibiotics in ingested human blood affect the mosquito microbiota and capacity to transmit malaria. Vol. 6, Nature Communications. 2015.6:1–7.

71. Dennison NJ, Saraiva RG, Cirimotich CM, Mlambo G, Mongodin EF, Dimopoulos G. Functional genomic analyses of *Enterobacter*, *Anopheles* and *Plasmodium* reciprocal interactions that impact vector competence. Vol. 15, Malaria Journal. 2016.15(1):1–15.

72. Bahia AC, Dong Y, Blumberg BJ, Mlambo G, Tripathi A, BenMarzouk-Hidalgo OJ, *et al*. Exploring *Anopheles* gut bacteria for *Plasmodium* blocking activity. Vol. 16, Environmental Microbiology. 2014.16(9):2980–94.

73. Garrett-Jones C, Shidrawi GR. Malaria vectorial capacity of a population of *Anopheles gambiae*: an exercise in epidemiological entomology. Vol. 40, Bulletin of the World Health Organization. 1969.40(4):531–45.

74. Ezenwa VO, Ghai RR, McKay AF, Williams AE. Group living and pathogen infection revisited. Vol. 12, Current Opinion in Behavioral Sciences. 2016.12:66–72.

75. Hill CL, Sharma A, Shouche Y, Severson DW. Dynamics of midgut microflora and dengue virus impact on life history traits in *Aedes aegypti*. Vol. 140, Acta Tropica. 2014.140:151–7.

76. Yilmaz B, Portugal S, Tran TM, Gozzelino R, Ramos S, Gomes J, *et al*. Gut microbiota elicits a protective immune response against malaria transmission. Vol. 159, Cell. 2014.159(6):1277–89.

77. Romoli O, Gendrin M. The tripartite interactions between the mosquito, its microbiota and *Plasmodium*. Vol. 11, Parasites and Vectors. 2018.11(1):1–8.

78. Jayakrishnan L, Sudhikumar AV, Aneesh EM. Role of gut inhabitants on vectorial capacity of mosquitoes. Vol. 55, Journal of Vector Borne Diseases. 2018.55(2):69–78.

79. Rodgers FH, Gendrin M, Wyer CAS, Christophides GK. Microbiota-induced peritrophic matrix regulates midgut homeostasis and prevents systemic infection of malaria vector mosquitoes. Vol. 13, PLoS Pathogens. 2017.13(5):1–22.

80. Cirimotich CM, Dong Y, Clayton AM, Sandiford SL, Souza-Neto JA, Mulenga M, *et al*. Natural Microbe-Mediated Refractoriness to *Plasmodium* Infection in *Anopheles* gambiae. Vol. 332, Science. 2011.332(6031):855–8.

81. Shen Z, Jacobs-Lorena M. A type I peritrophic matrix protein from the malaria vector *Anopheles gambiae* binds to chitin. Cloning, expression, and characterization. Vol. 273, The Journal of biological chemistry. 1998.273(28):17665–70.

82. Benoit JB, Lopez-Martinez G, Patrick KR, Phillips ZP, Krause TB, Denlinger DL. Drinking a hot blood meal elicits a protective heat shock response in mosquitoes. Vol. 108, Proceedings of the National Academy of Sciences. 2011.108(19):8026–9.

83. Kumar S, Molina-Cruz A, Gupta L, Rodrigues J, Barillas-Mury C. A Peroxidase/Dual Oxidase System Modulates Midgut Epithelial Immunity in *Anopheles gambiae*. Vol. 327, Science. 2010.327(5973):1644–8.

84. Lazaro JEH, Nitcheu J, Predicala RZ, Mangalindan GC, Nesslany F, Marzin D, *et al*. Heptyl prodigiosin, a bacterial, metabolite, is antimalarial in vivo and non-mutagenic in vitro. Vol. 11, Journal of Natural Toxins. 2002.11(4):367–77.

85. Ramirez JL, Short SM, Bahia AC, Saraiva RG, Dong Y, Kang S, *et al*. *Chromobacterium Csp_P* Reduces Malaria and Dengue Infection in Vector Mosquitoes and Has Entomopathogenic and In Vitro Anti-pathogen Activities. Vol. 10, PLOS Pathogens. 2014.10(10):e1004398.

86. Hillyer JF, Estévez-Lao TY. Nitric oxide is an essential component of the hemocyte-

mediated mosquito immune response against bacteria. Vol. 34, Developmental and comparative immunology. 2010.34(2):141–9.

87. Akorli J, Namaali PA, Ametsi GW, Egyirifa RK, Pels NAP. Generational conservation of composition and diversity of field-acquired midgut microbiota in *Anopheles gambiae* (*sensu lato*) during colonization in the laboratory. Vol. 12, Parasites and Vectors. 2019.12(1):1–9.

88. Dong Y, Das S, Cirimotich C, Souza-Neto JA, McLean KJ, Dimopoulos G. Engineered *Anopheles* immunity to plasmodium infection. Vol. 7, PLoS Pathogens. 2011.7(12):e1002458.

89. Meister S, Agianian B, Turlure F, Relógio A, Morlais I, Kafatos FC, *et al*. *Anopheles gambiae* PGRPLC-mediated defense against bacteria modulates infections with malaria parasites. Vol. 5, PLoS Pathogens. 2009.5(8):e1000542.

90. Meister S, Kanzok SM, Zheng XL, Luna C, Li TR, Hoa NT, *et al*. Immune signaling pathways regulating bacterial and malaria parasite infection of the mosquito *Anopheles gambiae*. Vol. 102, Proceedings of the National Academy of Sciences of the United States of America. 2005.102(32):11420–5.

91. Dennison NJ, Jupatanakul N, Dimopoulos G. The mosquito microbiota influences vector competence for human pathogens. Vol. 3, Current Opinion in Insect Science. 2014.3:6–13.

92. Wang Y, Wang Y, Zhang J, Xu W, Zhang J, Huang FS. Ability of TEP1 in intestinal flora to modulate natural resistance of *Anopheles dirus*. Vol. 134, Experimental Parasitology. 2013.134(4):460–5.

93. Bai L, Wang L, Vega-Rodríguez J, Wang G, Wang S. A Gut Symbiotic Bacterium *Serratia marcescens* Renders Mosquito Resistance to *Plasmodium* Infection Through Activation of Mosquito Immune Responses. Vol. 10, Frontiers in Microbiology. 2019.10(1580):1–11.

94. Rodgers FH, Gendrin M, Christophides GK. Chapter 6 - The Mosquito Immune System and Its Interactions With the Microbiota: Implications for Disease

Transmission. In: Wikel SK, Aksoy S, Dimopoulos G, editors. Arthropod Vector: Controller of Disease Transmission, Volume 1. Academic Press; 2017; p. 101–22.

95. Grieco JP, Achee NL, Chareonviriyaphap T, Suwonkerd W, Chauhan K, Sardelis MR, *et al*. A New Classification System for the Actions of IRS Chemicals Traditionally Used For Malaria Control. Vol. 2, PLOS ONE. 2007.2(8):e716.

96. Kliot A, Ghanim M. Fitness costs associated with insecticide resistance. Vol. 68, Pest management science. 2012.68(11):1431–7.

97. Mack LK, Attardo GM. Heat shock proteins, thermotolerance, and insecticide resistance in mosquitoes. Vol. 4, Frontiers in insect science. 2024.4(309941):1–7.

98. Martinez-Torres D, Chandre F, Williamson MS, Darriet F, Bergé JB, Devonshire AL, *et al*. Molecular characterization of pyrethroid knockdown resistance (kdr) in the major malaria vector *Anopheles gambiae s.s*. Vol. 7, Insect Molecular Biology. 1998.7(2):179–84.

99. Ranson H, Jensen B, Vulule JM, Wang X, Hemingway J, Collins FH. Identification of a point mutation in the voltage-gated sodium channel gene of Kenyan *Anopheles gambiae* associated with resistance to DDT and pyrethroids. Vol. 9, Insect Molecular Biology. 2000.9(5):491–7.

100. Hemingway J, Ranson H. Insecticide resistance in insect vectors of human disease. Vol. 45, Annual review of entomology. 2000.45:371–91.

101. Dada N, Jupatanakul N, Minard G, Short SM, Akorli J, Villegas LM. Considerations for mosquito microbiome research from the Mosquito Microbiome Consortium. Vol. 9, Microbiome. 2021.9(1):1–16.

102. Dada N, Sheth M, Liebman K, Pinto J, Lenhart A. Whole metagenome sequencing reveals links between mosquito microbiota and insecticide resistance in malaria vectors. Vol. 8, Scientific Reports. 2018.8(1):1–13.

103. Dada N, Lol JC, Benedict AC, López F, Sheth M, Dzuris N, *et al*. Pyrethroid exposure alters internal and cuticle surface bacterial communities in *Anopheles albimanus*. Vol.

13, The ISME Journal. 2019.13(10):2447–64.

104. Oliver S V, Brooke BD. The effect of larval nutritional deprivation on the life history and DDT resistance phenotype in laboratory strains of the malaria vector *Anopheles arabiensis*. Vol. 12, Malaria Journal. 2013.12(1):1–9.

105. Oliver S V, Brooke BD. The effect of multiple blood-feeding on the longevity and insecticide resistant phenotype in the major malaria vector *Anopheles arabiensis* (Diptera: Culicidae). Vol. 7, Parasites & Vectors. 2014.7(1):390.

106. Oliver S V., Brooke BD. The Role of Oxidative Stress in the Longevity and Insecticide Resistance Phenotype of the Major Malaria Vectors *Anopheles arabiensis* and *Anopheles funestus*. Vol. 11, PLOS ONE. 2016.11(3):e0151049.

107. Oliver S V, Brooke BD. The effect of elevated temperatures on the life history and insecticide resistance phenotype of the major malaria vector *Anopheles arabiensis* (Diptera: Culicidae). Vol. 16, Malaria Journal. 2017.16(1):1–13.

108. Wang Y, Gilbreath TM, Kukutla P, Yan G, Xu J. Dynamic gut microbiome across life history of the malaria mosquito *Anopheles gambiae* in Kenya. Vol. 6, PLoS ONE. 2011.6(9):1–9.

109. Chen S, Zhang D, Augustinos A, Doudoumis V, Bel Mokhtar N, Maiga H, *et al*. Multiple Factors Determine the Structure of Bacterial Communities Associated With *Aedes albopictus* Under Artificial Rearing Conditions. Vol. 11, Frontiers in Microbiology. 2020.11(605):1–18.

110. Ranasinghe HAK, Amarasinghe LD. Naturally Occurring Microbiota in Dengue Vector Mosquito Breeding Habitats and Their Use as Diet Organisms by Developing Larvae in the Kandy District, Sri Lanka. Vol. 2020, BioMed Research International. 2020.2020:1–12.

111. Antonio-Nkondjio C, Fossog BT, Ndo C, Djantio BM, Togouet SZ, Awono-Ambene P, *et al*. *Anopheles gambiae* distribution and insecticide resistance in the cities of Douala and Yaoundé (Cameroon): Influence of urban agriculture and pollution. Vol. 10, Malaria Journal. 2011.10:1–13.

112. Djouaka RF, Bakare AA, Bankole HS, Doannio JMC, Coulibaly ON, Kossou H, *et al*. Does the spillage of petroleum products in *Anopheles* breeding sites have an impact on the pyrethroid resistance? Vol. 6, Malaria Journal. 2007.6:1–10.

113. Mireji PO, Keating J, Hassanali A, Mbogo CM, Nyambaka H, Kahindi S, *et al*. Heavy metals in mosquito larval habitats in urban Kisumu and Malindi, Kenya, and their impact. Vol. 70, Ecotoxicology and Environmental Safety. 2008.70(1):147–53.

114. Awolola TS, Oduola AO, Obansa JB, Chukwurar NJ, Unyimadu JP. *Anopheles gambiae s.s*. breeding in polluted water bodies in urban Lagos, southwestern Nigeria. Vol. 44, Journal of vector borne diseases. 2007.44(4):241–4.

115. Mwangangi JM, Shililu JI, Muturi EJ, Gu W, Mbogo CM, Kabiru EW, *et al*. Dynamics of immature stages of *Anopheles arabiensis* and other mosquito species (Diptera: Culicidae) in relation to rice cropping in a rice agro-ecosystem in Kenya. Vol. 31, Journal of vector ecology : journal of the Society for Vector Ecology. 2006.31(2):245–51.

116. Mwangangi JM, Muturi EJ, Shililu J, Muriu SM, Jacob B, Kabiru EW, *et al*. Survival of immature *Anopheles arabiensis* (Diptera: Culicidae) in aquatic habitats in Mwea rice irrigation scheme, central Kenya. Vol. 5, Malaria Journal. 2006.5:1–8.

117. Gendrin M, Christophides GK. The Anopheles Mosquito Microbiota and Their Impact on Pathogen Transmission. Manguin S. *Anopheles* mosquitoes - New insights into malaria vectors. InTech; 2013; p. 526–48.

118. Müller GC, Beier JC, Traore SF, Toure MB, Traore MM, Bah S, *et al*. Field experiments of *Anopheles gambiae* attraction to local fruits/seedpods and flowering plants in Mali to optimize strategies for malaria vector control in Africa using attractive toxic sugar bait methods. Vol. 9, Malaria Journal. 2010.9(1):1–11.

119. Lefèvre T, Vantaux A, Dabiré KR, Mouline K, Cohuet A. Non-Genetic Determinants of Mosquito Competence for Malaria Parasites. Vol. 9, PLOS Pathogens. 2013.9(6):e1003365.

120. Wilke ABB, Marrelli MT. Paratransgenesis: A promising new strategy for mosquito

vector control. Vol. 8, Parasites and Vectors. 2015.8(1):1–9.

121. Herren JK, Mbaisi L, Mararo E, Makhulu EE, Mobegi VA, Butungi H, *et al*. A microsporidian impairs *Plasmodium falciparum* transmission in *Anopheles arabiensis* mosquitoes. Vol. 11, Nature Communications. 2020.11(1).

122. Wang S, Ghosh AK, Bongio N, Stebbings KA, Lampe DJ, Jacobs-Lorena M. Fighting malaria with engineered symbiotic bacteria from vector mosquitoes. Vol. 109, Proceedings of the National Academy of Sciences of the United States of America. 2012.109(31):12734–9.

123. Bian G, Joshi D, Dong Y, Lu P, Zhou G, Pan X, *et al*. *Wolbachia* invades *Anopheles stephensi* populations and induces refractoriness to *Plasmodium* infection. Vol. 340, Science (New York, N.Y.). 2013.340(6133):748–51.

124. Baldini F, Segata N, Pompon J, Marcenac P, Robert Shaw W, Dabiré RK, *et al*. Evidence of natural *Wolbachia* infections in field populations of *Anopheles gambiae*. Vol. 5, Nature Communications. 2014.5(1):3985.

125. Rossi P, Ricci I, Cappelli A, Damiani C, Ulissi U, Mancini MV, *et al*. Mutual exclusion of *Asaia* and *Wolbachia* in the reproductive organs of mosquito vectors. Vol. 8, Parasites and Vectors. 2015.8(1):1–10.

126. Sharma P, Rani J, Chauhan C, Kumari S, Tevatiya S, Das De T, *et al*. Altered Gut Microbiota and Immunity Defines *Plasmodium* vivax Survival in *Anopheles stephensi*. Vol. 11, Frontiers in Immunology. 2020.11(May):1–13.

127. Galeano-Castañeda Y, Bascuñán P, Serre D, Correa MM. Trans-stadial fate of the gut bacterial microbiota in *Anopheles albimanus*. Vol. 201, Acta Tropica. 2020.201(June 2019):105204.

128. Hemingway J. Biochemical studies on malathion resistance in *Anopheles arabiensis* from Sudan. Vol. 77, Transactions of the Royal Society of Tropical Medicine and Hygiene. 1983.77(4):477–80.

129. Bass C, Nikou D, Donnelly MJ, Williamson MS, Ranson H, Ball A, *et al*. Detection of

knockdown resistance (kdr) mutations in *Anopheles gambiae*: a comparison of two new high-throughput assays with existing methods. Vol. 6, Malaria Journal 2007 6:1. 2007.6(1):1–14.

130. Matambo TS, Abdalla H, Brooke BD, Koekemoer LL, Mnzava A, Hunt RH, *et al*. Insecticide resistance in the malarial mosquito *Anopheles arabiensis* and association with the kdr mutation. Vol. 21, Medical and veterinary entomology. 2007.21(1):97–102.

131. Davies C, Coetzee M, Lyons CL. Effect of stable and fluctuating temperatures on the life history traits of *Anopheles arabiensis* and *An. quadriannulatus* under conditions of inter- and intra-specific competition. Vol. 9, Parasites & vectors. 2016.9(1):342.

132. Dahan-Moss Y, Hendershot A, Dhoogra M, Julius H, Zawada J, Kaiser M, *et al*. Member species of the *Anopheles gambiae* complex can be misidentified as *Anopheles leesoni*. Vol. 19, Malaria Journal 2020 19:1. 2020.19(1):1–9.

133. Patel NF, Letinić BD, Lobb L, Zawada J, Dlamini DM, Mabaso N, *et al*. Comparison of the effect of bacterial stimulation on the global epigenetic landscape and transcription of immune genes in primarily zoophilic members of the *Anopheles gambiae* complex (Diptera: Culicidae). Vol. 260, Molecular and Biochemical Parasitology. 2024.260:111631.

134. Hunt R, Brooke B, Pillay C, Koekemoer L, Coetzee M. Laboratory selection for and characteristics of pyrethroid resistance in the malaria vector *Anopheles funestus*. Vol. 19, Medical and Veterinary Entomology. 2005.19(3):271–5.

135. Bukin Y, Galachyants Y, Morozov I V, Bukin S, Zakharenko A, Zemskaya T. The effect of 16S rRNA region choice on bacterial community metabarcoding results. Vol. 6, Scientific Data 2019 6:1. 2019.6(1):1–14.

136. Wang Y, Qian P. Conservative Fragments in Bacterial 16S rRNA Genes and Primer Design for 16S Ribosomal DNA Amplicons in Metagenomic Studies. Vol. 4, PLOS ONE. 2009.4(10):e7401.

137. Illumina. 16S Metagenomic Sequencing Library Preparation: Preparing 16S Ribosomal

RNA Gene Amplicons for the Illumina MiSeq System, Part # 15044223 Rev. A. Illumina [Internet]. San Diego, California, USA.: Illumina; 2014; p. 1–28.

138. Khumalo Z, Chan W. NICD Sequencing Core Facility Laboratory Manual (NIC1094v6). Johannesburg, South Africa: NHLS; 2023; p. 1–108.

139. Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, *et al*. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Vol. 41, Nucleic Acids Research. 2013.41(1):e1.

140. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Vol. 13, Nature Methods. 2016.13(7):581–3.

141. Wood DE, Lu J, Langmead B. Improved metagenomic analysis with Kraken 2. Vol. 20, Genome Biology. 2019.20(1):1–13.

142. Ondov BD, Bergman NH, Phillippy AM. Interactive metagenomic visualization in a Web browser. Vol. 12, BMC Bioinformatics. 2011.12(385):1–9.

143. RStudio Team. RStudio: Integrated Development Environment for R. Boston; 2015; Available from: http://www.rstudio.com/

144. McMurdie PJ, Holmes S. phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. Vol. 8, PLOS ONE. 2013.8(4):e61217.

145. Wickham H. ggplot2. Vol. 3, WIREs Computational Statistics. 2011.3(2):180–5.

146. Andersen KS, Kirkegaard RH, Karst SM, Albertsen M. ampvis2: an R package to analyse and visualise 16S rRNA amplicon data. bioRxiv. 2018.:299537.

147. Sepkoski JJ. Alpha, beta, or gamma: where does all the diversity go? Vol. 14, Paleobiology. 1988.14(3):221–34.

148. Chao A. Nonparametric Estimation of the Number of Classes in a Population. Vol. 11,

Scandinavian Journal of Statistics. 1984.11(4):265–70.

149. Chao A, Bunge J. Estimating the Number of Species in a Stochastic Abundance Model. Vol. 58, Biometrics. 2002.58(3):531–9.

150. Chao A, Chazdon RL, Colwell RK, Shen TJ. Abundance-Based Similarity Indices and Their Estimation When There are Unseen Species in Samples. Vol. 62, Biometrics. 2006.62(2):361–71.

151. Chao A, Lee SM. Estimating the Number of Classes via Sample Coverage. Vol. 87, Journal of the American Statistical Association. 1992.87(417):210–7.

152. Chao A, Yang MCK. Stopping Rules and Estimation for Recapture Debugging with Unequal Failure Rates. Vol. 80, Biometrika. 1993.80(1):193–201.

153. Shannon C, Weaver W. The Mathematical Theory of Communication. Urbana: University of Illinois Press; 1949; 1–117 p.

154. Simpson EH. Measurement of Diversity. Vol. 163, Nature. 1949.163(4148):688.

155. Lemos LN, Fulthorpe RR, Triplett EW, Roesch LFW. Rethinking microbial diversity analysis in the high throughput sequencing era. Vol. 86, Journal of Microbiological Methods. 2011.86(1):42–51.

156. Magurran AE. Measuring Biological Diversity. Wiley; 2004;

157. McKight PE, Najab J. Kruskal-Wallis Test. In: The Corsini Encyclopedia of Psychology. 2010 Jan 30; p. 1.

158. Clarke KR. Non-parametric multivariate analyses of changes in community structure. Vol. 18, Australian Journal of Ecology. 1993.18(1):117–43.

159. Anderson MJ. A new method for non-parametric multivariate analysis of variance. Vol. 26, Austral Ecology. 2001.26(1):32–46.

160. Conway JR, Lex A, Gehlenborg N. UpSetR: an R package for the visualization of

intersecting sets and their properties. Vol. 33, Bioinformatics. 2017.33(18):2938–40.

161. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Vol. 15, Genome Biology. 2014.15(12):1–21.

162. Giles GMJ. A Handbook of the Gnats or Mosquitoes: Giving the Anatomy and Life History of the Culicidæ Together with Descriptions of All Species Noticed up to the Present Date. 2nd ed. 2nd ed. London: John Bale, sons & Danielsson; 1902;

163. White GB. *Anopheles bwambae* sp.n., a malaria vector in the Semliki Valley, Uganda, and its relationships with other sibling species of the *An*. gambiae complex (Diptera: Culicidae). Vol. 10, Systematic Entomology. 1985.10(4):501–22.

164. Mattingly PF. Names for the *Anopheles gambiae* complex. Vol. 9, Mosquito Systematics. 1977.9(3):323–8.

165. Coluzzi M. Morphological divergences in the Anopheles gambiae complex. Vol. 43, Rivista di malariologia. 1964.43:197–232.

166. Torre A della, Fanello C, Akogbeto M, Dossou-yovo J, Favia G, Petrarca V, *et al*. Molecular evidence of incipient speciation within *Anopheles gambiae s.s*. in West Africa. Vol. 10, Insect Molecular Biology. 2001.10(1):9–18.

167. Coetzee M, Hunt RH, Wilkerson R, Torre A Della, Coulibaly MB, Besansky NJ. *Anopheles coluzzii* and *Anopheles amharicus*, new members of the *Anopheles gambiae* complex. Vol. 3619, Zootaxa. 2013.3619(3):246–74.

168. Brunhes J, Le Goff G, Geoffroy B. *Anophèles afro-tropicaux*. I. - Descriptions d'espèces nouvelles et changements de statuts taxonomiques (Diptera : Culicidae). Vol. 33, Annales de la Societe Entomologique de France. 1997.33(2):173–83.

169. Hunt RH, Coetzee M, Fettene M. The *Anopheles gambiae* complex: a new species from Ethiopia. Vol. 92, Transactions of the Royal Society of Tropical Medicine and Hygiene. 1998.92(2):231–5.

170. White GB. *Anopheles gambiae* complex and disease transmission in Africa. Vol. 68,

Transactions of the Royal Society of Tropical Medicine and Hygiene. 1974.68(4):278–301.

171. Hunt RH, Mahon RJ. Collection of *Anopheles quadriannulatus* (Diptera: Culicidae) from human habitations in southern Africa. Vol. 49, Journal of the Entomological Society of Southern Africa. 1986.49:390–1.

172. Pates H V., Takken W, Curtis CF, Huisman PW, Akinpelu O, Gill GS. Unexpected anthropophagic behaviour in *Anopheles quadriannulatus*. Vol. 15, Medical and Veterinary Entomology. 2001.15(3):293–8.

173. Takken W, Eling W, Hooghof J, Dekker T, Hunt R, Coetzee M. Susceptibility of *Anopheles quadriannulatus* theobald (Diptera: Culicidae) to *Plasmodium falciparum*. Vol. 93, Transactions of the Royal Society of Tropical Medicine and Hygiene. 1999.93(6):578–80.

174. Hargreaves K, Hunt RH, Brooke BD, Mthembu J, Weeto MM, Awolola TS, *et al*. *Anopheles arabiensis* and *An. quadriannulatus* resistance to DDT in South Africa. Vol. 17, Medical and Veterinary Entomology. 2003.17(4):417–22.

175. Bartilol B, Omedo I, Mbogo C, Mwangangi J, Rono MK. Bionomics and ecology of *Anopheles merus* along the East and Southern Africa coast. Vol. 14, Parasites and Vectors. 2021.14(1):1–11.

176. Mutero CM, Mosha FW, Subra R. Biting activity and resting behaviour of *Anopheles merus* Donitz (Diptera: Culicidae) on the Kenya Coast. Vol. 78, Annals of tropical medicine and parasitology. 1984.78(1):43–7.

177. Kipyab PC, Khaemba BM, Mwangangi JM, Mbogo CM. The bionomics of *Anopheles merus* (Diptera: Culicidae) along the Kenyan coast. Vol. 6, Parasites & Vectors. 2013.6(37):1–6.

178. Mbogo CM, Mwangangi JM, Nzovu J, Gu W, Yan G, Gunter JT, *et al*. Spatial and temporal heterogeneity of *Anopheles* mosquitoes and *Plasmodium falciparum* transmission along the Kenyan coast. Vol. 68, The American journal of tropical medicine and hygiene. 2003.68(6):734–42.

179. Mbokazi F, Coetzee M, Brooke B, Govere J, Reid A, Owiti P, *et al*. Changing distribution and abundance of the malaria vector *Anopheles merus* in Mpumalanga Province, South Africa. Vol. 8, Public Health Action. 2018.8(1):S39–43.

180. Govere J, Durrheim DN, Coetzee M, Hunt RH, La Grange JJ. Captures of mosquitoes of the *Anopheles gambaie* complex (Diptera: Culicidae) in Lowveld Region of Mpumalanga Province, South Africa. Vol. 8, African Entomology. 2000.8(1):91–9.

181. Davies C, Coetzee M, Lyons CL. Characteristics of larval breeding sites and insecticide resistance in the *Anopheles gambiae* complex in Mpumalanga, South Africa. Vol. 24, African Entomology. 2016.24(2):421–31.

182. Mahande A, Mosha F, Mahande J, Kweka E. Feeding and resting behaviour of malaria vector, *Anopheles arabiensis* with reference to zooprophylaxis. Vol. 6, Malaria Journal. 2007.6:1–6.

183. Habtewold T, Prior A, Torr SJ, Gibson G. Could insecticide-treated cattle reduce Afrotropical malaria transmission? Effects of deltamethrin-treated Zebu on *Anopheles arabiensis* behaviour and survival in Ethiopia. Vol. 18, Medical and Veterinary Entomology. 2004.18(4):408–17.

184. Coluzzi M, Sabatini A, Petrarca V, Di Deco MA. Chromosomal differentiation and adaptation to human environments in the *Anopheles gambiae* complex. Vol. 73, Transactions of The Royal Society of Tropical Medicine and Hygiene. 1979.73(5):483–97.

185. Ashine T, Getachew D, Demisse M, Lobo NF, Tadesse FG. *Anopheles arabiensis*. Vol. 40, Trends in Parasitology. 2024.40(1):91–2.

186. Casimiro S, Coleman M, Hemingway J, Sharp B. Insecticide Resistance in *Anopheles arabiensis* and *Anopheles gambiae* from Mozambique . Vol. 43, Journal of Medical Entomology. 2006.43(2):276–82.

187. Orondo PW, Nyanjom SG, Atieli H, Githure J, Ondeto BM, Ochwedo KO, *et al*. Insecticide resistance status of *Anopheles arabiensis* in irrigated and non-irrigated areas in western Kenya. Vol. 14, Parasites and Vectors. 2021.14(1):1–10.

188. Buxton M, Wasserman RJ, Nyamukondiwa C. Spatial *Anopheles arabiensis* (Diptera: Culicidae) insecticide resistance patterns across malaria-endemic regions of Botswana. Vol. 19, Malaria Journal. 2020.19(1):1–9.

189. Mzilahowa T, Chiumia M, Mbewe RB, Uzalili VT, Luka-Banda M, Kutengule A, *et al*. Increasing insecticide resistance in *Anopheles funestus* and *Anopheles arabiensis* in Malawi, 2011-2015. Vol. 15, Malaria Journal. 2016.15(1):1–15.

190. Habtewold T, Povelones M, Blagborough AM, Christophides GK. Transmission Blocking Immunity in the Malaria Non-Vector Mosquito *Anopheles quadriannulatus* Species A. Vol. 4, PLOS Pathogens. 2008.4(5):e1000070.

191. Cornet S, Gandon S, Rivero A. Patterns of phenoloxidase activity in insecticide resistant and susceptible mosquitoes differ between laboratory-selected and wild-caught individuals. Vol. 6, Parasites and Vectors. 2013.6(1):1–11.

192. Vézilier J, Nicot A, Lorgeril J, Gandon S, Rivero A. The impact of insecticide resistance on *Culex pipiens* immunity. Vol. 6, Evolutionary applications. 2013.6(3):497–509.

193. Juache-Villagrana AE, Pando-Robles V, Garcia-Luna SM, Ponce-Garcia G, Fernandez-Salas I, Lopez-Monroy B, *et al*. Assessing the Impact of Insecticide Resistance on Vector Competence: A Review. Vol. 13, Insects. 2022.13(4):1–13.

194. Suh PF, Elanga-Ndille E, Tchouakui M, Sandeu MM, Tagne D, Wondji C, *et al*. Impact of insecticide resistance on malaria vector competence: a literature review. Vol. 22, Malaria Journal. 2023.22(1):1–11.

195. Tchouakui M, Chiang MC, Ndo C, Kuicheu CK, Amvongo-Adjia N, Wondji MJ, *et al*. A marker of glutathione S-transferase-mediated resistance to insecticides is associated with higher *Plasmodium* infection in the African malaria vector *Anopheles funestus*. Vol. 9, Scientific Reports. 2019.9(5772):1–12.

196. Alout H, Yameogo B, Djogbénou LS, Chandre F, Dabiré RK, Corbel V, *et al*. Interplay Between *Plasmodium* Infection and Resistance to Insecticides in Vector Mosquitoes. Vol. 210, The Journal of Infectious Diseases. 2014.210(9):1464–70.

197. Kabula B, Tungu P, Rippon EJ, Steen K, Kisinza W, Magesa S, *et al*. A significant association between deltamethrin resistance, *Plasmodium falciparum* infection and the Vgsc-1014S resistance mutation in *Anopheles gambiae* highlights the epidemiological importance of resistance markers. Vol. 15, Malaria Journal. 2016.15(1):1–5.

198. Omoke D, Kipsum M, Otieno S, Esalimba E, Sheth M, Lenhart A, *et al*. Western Kenyan *Anopheles gambiae* showing intense permethrin resistance harbour distinct microbiota. Vol. 20, Malaria Journal. 2021.20(1):1–14.

199. Barnard K, Jeanrenaud ACSN, Brooke BD, Oliver S V. The contribution of gut bacteria to insecticide resistance and the life histories of the major malaria vector *Anopheles arabiensis* (Diptera: Culicidae). Vol. 9, Scientific Reports. 2019.9(1):1–11.

200. Johnson JS, Spakowicz DJ, Hong BY, Petersen LM, Demkowicz P, Chen L, *et al*. Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. Vol. 10, Nature Communications. 2019.10(1):1–11.

201. Muhamad Rizal NS, Neoh HM, Ramli R, A/L K Periyasamy PR, Hanafiah A, Abdul Samat MN, *et al*. Advantages and Limitations of 16S rRNA Next-Generation Sequencing for Pathogen Identification in the Diagnostic Microbiology Laboratory: Perspectives from a Middle-Income Country. Vol. 10, Diagnostics (Basel, Switzerland). 2020.10(10).

202. Clarridge JE 3rd. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Vol. 17, Clinical microbiology reviews. 2004.17(4):840–62.

203. Mancini M V., Damiani C, Accoti A, Tallarita M, Nunzi E, Cappelli A, *et al*. Estimating bacteria diversity in different organs of nine species of mosquito by next generation sequencing. Vol. 18, BMC Microbiology. 2018.18(1):1–10.

204. Minard G, Mavingui P, Moro CV. Diversity and function of bacterial microbiota in the mosquito holobiont. Vol. 6, Parasites and Vectors. 2013.6(1):1–12.

205. Coon KL, Valzania L, McKinney DA, Vogel KJ, Brown MR, Strand MR. Bacteria-mediated hypoxia functions as a signal for mosquito development. Vol. 114,

Proceedings of the National Academy of Sciences of the United States of America. 2017.114(27):E5362–9.

206. Kim JH, Cho CR, Um TH, Rhu JY, Kim ES, Jeong JW, *et al*. *Morganella morganii* sepsis with massive hemolysis. Vol. 22, Journal of Korean medical science. 2007.22(6):1082–4.

207. Sekowska A, Gospodarek E, Janickca G, Jachna-Sawicka K, Sawicki M. Hydrolytic and haemolytic activity of *Klebsiella pneumoniae* and *Klebsiella oxytoca*. Vol. 58, Medycyna doswiadczalna i mikrobiologia. 2006.58(2):135–41.

208. Roland FP. Interaction of blood with Enterobacteriaceae: hemolysis, hemagglutination, fibrinolysis. Vol. 67, American journal of clinical pathology. 1977.67(3):260–3.

209. Braun V, Günther H, Neuss B, Tautz C. Hemolytic activity of *Serratia marcescens*. Vol. 141, Archives of microbiology. 1985.141(4):371–6.

210. Norris LC, Fornadel CM, Hung WC, Pineda FJ, Norris DE. Frequency of multiple blood meals taken in a single gonotrophic cycle by *Anopheles arabiensis* mosquitoes in Macha, Zambia. Vol. 83, American Journal of Tropical Medicine and Hygiene. 2010.83(1):33–7.

211. Shaw WR, Holmdahl IE, Itoe MA, Werling K, Marquette M, Paton DG, *et al*. Multiple blood feeding in mosquitoes shortens the *Plasmodium falciparum* incubation period and increases malaria transmission potential. Vol. 16, PLOS Pathogens. 2020.16(12):e1009131.

212. Oliver S V., Brooke BD. The effect of metal pollution on the life history and insecticide resistance phenotype of the major malaria vector *Anopheles arabiensis* (Diptera: Culicidae). Vol. 13, PLOS ONE. 2018.13(2):e0192551.

213. Oliver S V, Brooke BD. The effect of commercial herbicide exposure on the life history and insecticide resistance phenotypes of the major malaria vector *Anopheles arabiensis* (Diptera: Culicidae). Vol. 188, Acta tropica. 2018.188:152–60.

214. Sikaala CH, Dlamini B, Lungu A, Fakudze P, Chisenga M. Malaria elimination and the

need for intensive inter - country cooperation . a critical evaluation of regional technical co-operation in Southern Africa. Vol. 23, Malaria Journal. 2024.23(62):1–12.

215. Raman J, Fakudze P, Sikaala CH, Chimumbwa J, Moonasar D. Eliminating malaria from the margins of transmission in Southern Africa through the Elimination 8 Initiative. Vol. 76, Transactions of the Royal Society of South Africa. 2021.76(2):137–45.

216. Osei-Poku J, Mbogo CM, Palmer WJ, Jiggins FM. Deep sequencing reveals extensive variation in the gut microbiota of wild mosquitoes from Kenya. Vol. 21, Molecular ecology. 2012.21(20):5138–50.

217. Dickson LB, Ghozlane A, Volant S, Bouchier C, Ma L, Vega-Rúa A, *et al*. Diverse laboratory colonies of *Aedes aegypti* harbor the same adult midgut bacterial microbiome. Vol. 11, Parasites & Vectors. 2018.11(207):1–8.

218. Santos NAC dos, Carvalho VR de, Souza-Neto JA, Alonso DP, Ribolla PEM, Medeiros JF, *et al*. Bacterial Microbiota from Lab-Reared and Field-Captured *Anopheles darlingi* Midgut and Salivary Gland. Vol. 11, Microorganisms. 2023.11(5):1–17.

219. Linenberg I, Christophides GK, Gendrin M. Larval diet affects mosquito development and permissiveness to *Plasmodium* infection. Vol. 6, Scientific Reports. 2016.6(December):1–10.

220. Munhenga G, Brooke BD, Spillings B, Essop L, Hunt RH, Midzi S, *et al*. Field study site selection, species abundance and monthly distribution of anopheline mosquitoes in the northern Kruger National Park, South Africa. Vol. 13, Malaria Journal. 2014.13(27):1–11.

221. Sarma DK, Kumar M, Dhurve J, Pal N, Sharma P, James MM, *et al*. Influence of Host Blood Meal Source on Gut Microbiota of Wild Caught *Aedes aegypti*, a Dominant Arboviral Disease Vector. Vol. 10, Microorganisms. 2022.10(332):1–17.

222. Vareda JP, Valente AJM, Durães L. Assessment of heavy metal pollution from anthropogenic activities and remediation strategies: A review. Vol. 246, Journal of

Environmental Management. 2019.246:101–18.

223. Jiao W, Chen W, Chang AC, Page AL. Environmental risks of trace elements associated with long-term phosphate fertilizers applications: A review. Vol. 168, Environmental Pollution. 2012.168:44–53.

224. Lekfeldt JDS, Holm PE, Kjærgaard C, Magid J. Heavy Metal Leaching as Affected by Long-Time Organic Waste Fertilizer Application. Vol. 46, Journal of Environmental Quality. 2017.46(4):871–8.

225. Eisler R. Mercury hazards from gold mining to humans, plants, and animals. Vol. 181, Reviews of environmental contamination and toxicology. 2004.181:139–98.

226. Walters CR, Somerset VS, Leaner JJ, Nel JM. A review of mercury pollution in South Africa: current status. Vol. 46, Journal of environmental science and health. Part A, Toxic/hazardous substances & environmental engineering. 2011.46(10):1129–37.

227. Jones CM, Toé HK, Sanou A, Namountougou M, Hughes A, Diabaté A, *et al*. Additional Selection for Insecticide Resistance in Urban Malaria Vectors: DDT Resistance in *Anopheles arabiensis* from Bobo-Dioulasso, Burkina Faso. Vol. 7, PLOS ONE. 2012.7(9):e45995.

228. Kristan M, Fleischmann H, Della Torre A, Stich A, Curtis CF. Pyrethroid resistance/susceptibility and differential urban/rural distribution of *Anopheles arabiensis* and *An. gambiae s.s.* malaria vectors in Nigeria and Ghana. Vol. 17, Medical and Veterinary Entomology. 2003.17(3):326–32.

229. Kaiser ML, Wood OR, Damiens D, Brooke BD, Koekemoer LL, Munhenga G. Estimates of the population size and dispersal range of *Anopheles arabiensis* in Northern KwaZulu-Natal, South Africa: implications for a planned pilot programme to release sterile male mosquitoes. Vol. 14, Parasites and Vectors. 2021.14(1):1–18.

230. Nkya TE, Poupardin R, Laporte F, Akhouayri I, Mosha F, Magesa S, *et al*. Impact of agriculture on the selection of insecticide resistance in the malaria vector *Anopheles gambiae*: A multigenerational study in controlled conditions. Vol. 7, Parasites and Vectors. 2014.7(1):1–12.

231. Mortvedt JJ. Heavy metal contaminants in inorganic and organic fertilizers. Vol. 43, Fertilizer research. 1995.43(1):55–61.

232. Jeanrenaud ACSN, Brooke BD, Oliver S V. Second generation effects of larval metal pollutant exposure on reproduction, longevity and insecticide tolerance in the major malaria vector *Anopheles arabiensis* (Diptera: Culicidae). Vol. 13, Parasites and Vectors. 2020.13(1):1–11.

233. Scott JA, Brogdon WG, Collins FH. Identification of single specimens of the *Anopheles gambiae* complex by the polymerase chain reaction. Vol. 49, The American journal of tropical medicine and hygiene. 1993.49(4):520–9.

234. Musasia FK, Isaac AO, Masiga DK, Omedo IA, Mwakubambanya R, Ochieng R, *et al*. Sex-specific induction of CYP6 cytochrome P450 genes in cadmium and lead tolerant *Anopheles gambiae*. Vol. 12, Malaria journal. 2013.12(97):97.

235. Venter N, Oliver S V., Muleba M, Davies C, Hunt RH, Koekemoer LL, *et al*. Benchmarking insecticide resistance intensity bioassays for *Anopheles* malaria vector species against resistance phenotypes of known epidemiological significance. Vol. 10, Parasites and Vectors. 2017.10(1):1–7.

236. An X, Tian C, Xu J, Dong F, Liu X, Wu X, *et al*. Characterization of hexaconazole-degrading strain *Sphingobacterium multivorum* and analysis of transcriptome for biodegradation mechanism. Vol. 722, Science of The Total Environment. 2020.722(137171):1–9.

237. Warman AJ, Robinson JW, Luciakova D, Lawrence AD, Marshall KR, Warren MJ, *et al*. Characterization of Cupriavidus metallidurans CYP116B1--a thiocarbamate herbicide oxygenating P450-phthalate dioxygenase reductase fusion protein. Vol. 279, The FEBS journal. 2012.279(9):1675–93.

238. Badani H, Fatma Z, AbdElKader E. First Report of Enterobacter hormaechei Isolated from Agricultural Soil in the Biodegradation of Glyphosate. Pollution. 2024.(10):283–98.

239. Andersen SM, Mortensen HS, Bossi R, Jacobsen CS. Isolation and Characterisation of

Rhodococcus erythropolis TA57 able to Degrade the Triazine Amine Product from Hydrolysis of Sulfonylurea Pesticides in Soils. Vol. 24, Systematic and Applied Microbiology. 2001.24(2):262–6.

240. Rani A, Sharma A, Rajagopal R, Adak T, Bhatnagar RK. Bacterial diversity analysis of larvae and adult midgut microflora using culture-dependent and culture-independent methods in lab-reared and field-collected *Anopheles stephensi*-an Asian malarial vector. Vol. 9, BMC Microbiology. 2009.9(96):1–22.

241. MacLeod HJ, Dimopoulos G, Short SM. Larval Diet Abundance Influences Size and Composition of the Midgut Microbiota of *Aedes aegypti* Mosquitoes. Vol. 12, Frontiers in Microbiology. 2021.12(June):1–14.

242. Tandina F, Almeras L, Koné AK, Doumbo OK, Raoult D, Parola P. Use of MALDI-TOF MS and culturomics to identify mosquitoes and their midgut microbiota. Vol. 9, Parasites & Vectors. 2016.9(1):1–11.

243. Tainchum K, Dupont C, Chareonviriyaphap T, Jumas-Bilak E, Bangs MJ, Manguin S. Bacterial Microbiome in Wild-Caught *Anopheles* Mosquitoes in Western Thailand. Vol. 11, Frontiers in Microbiology. 2020.11(965):1–12.

244. Kalappa DM, Subramani PA, Basavanna SK, Ghosh SK, Sundaramurthy V, Uragayala S, *et al*. Influence of midgut microbiota in *Anopheles stephensi* on *Plasmodium berghei* infections. Vol. 17, Malaria Journal. 2018.17(1):1–8.

245. Mitri C, Bischoff E, Belda Cuesta E, Volant S, Ghozlane A, Eiglmeier K, *et al*. Leucine-Rich Immune Factor APL1 Is Associated With Specific Modulation of Enteric Microbiome Taxa in the Asian Malaria Mosquito *Anopheles stephensi*. Vol. 11, Frontiers in Microbiology. 2020.11(306):1–17.

246. Birnberg L, Climent-Sanz E, Codoñer FM, Busquets N. Microbiota Variation Across Life Stages of European Field-Caught *Anopheles atroparvus* and During Laboratory Colonization: New Insights for Malaria Research. Vol. 12, Frontiers in Microbiology. 2021.12(November):1–15.

247. Eappen AG, Smith RC, Jacobs-Lorena M. Enterobacter-Activated Mosquito Immune

Responses to *Plasmodium* Involve Activation of SRPN6 in *Anopheles stephensi*. Vol. 8, PLOS ONE. 2013.8(5):e62937.

248. Dehghan H, Mosa-Kazemi SH, Yakhchali B, Maleki-Ravasan N, Vatandoost H, Oshaghi MA. Evaluation of anti-malaria potency of wild and genetically modified *Enterobacter cloacae* expressing effector proteins in *Anopheles stephensi*. Vol. 15, Parasites and Vectors. 2022.15(1):1–12.

249. Tchioffo MT, Boissière A, Churcher TS, Abate L, Gimonneau G, Nsango SE, *et al*. Modulation of malaria infection in *Anopheles gambiae* mosquitoes exposed to natural midgut bacteria. Vol. 8, PLoS ONE. 2013.8(12):4–12.

250. Cappelli A, Damiani C, Mancini MV, Valzano M, Rossi P, Serrao A, *et al*. Asaia activates immune genes in mosquito eliciting an anti-*Plasmodium* response: Implications in malaria control. Vol. 10, Frontiers in Genetics. 2019.10(SEP):1–11.

251. Grogan C, Bennett M, Moore S, Lampe D. Novel *Asaia bogorensis* Signal Sequences for *Plasmodium* Inhibition in *Anopheles stephensi*. Vol. 12, Frontiers in Microbiology. 2021.12(February):1–14.

252. Maffo CGT, Sandeu MM, Fadel AN, Tchouakui M, Nguete DN, Menze B, *et al*. Molecular detection and maternal transmission of a bacterial symbiont *Asaia* species in field-caught *Anopheles* mosquitoes from Cameroon. Vol. 14, Parasites & vectors. 2021.14(539):1–11.

253. Raman J, Gast L, Balawanth R, Tessema S, Brooke B, Maharaj R, *et al*. High levels of imported asymptomatic malaria but limited local transmission in KwaZulu-Natal, a South African malaria-endemic province nearing malaria elimination. Vol. 19, Malaria Journal. 2020.19(1):1–13.

254. Azrag RS, Mohammed BH. *Anopheles arabiensis* in Sudan: A noticeable tolerance to urban polluted larval habitats associated with resistance to Temephos. Vol. 17, Malaria Journal. 2018.17(1):1–11.

255. Li XD, Xin L, Rong WT, Liu XY, Deng WA, Qin YC, *et al*. Effect of heavy metals pollution on the composition and diversity of the intestinal microbial community of a

pygmy grasshopper (*Eucriotettix oculatus*). Vol. 223, Ecotoxicology and Environmental Safety. 2021.223(112582):1–10.

256. Wu N, Wang X, Xu X, Cai R, Xie S. Effects of heavy metals on the bioaccumulation, excretion and gut microbiome of black soldier fly larvae (*Hermetia illucens*). Vol. 192, Ecotoxicology and Environmental Safety. 2020.192(110323):1–9.

257. Samanovic MI, Ding C, Thiele DJ, Darwin KH. Copper in microbial pathogenesis: meddling with the metal. Vol. 11, Cell host & microbe. 2012.11(2):106–15.

258. Argüello JM, Raimunda D, Padilla-Benavides T. Mechanisms of copper homeostasis in bacteria. Vol. 4, Frontiers in Cellular and Infection Microbiology. 2013.4(NOV):1–14.

259. Ridge PG, Zhang Y, Gladyshev VN. Comparative genomic analyses of copper transporters and cuproproteomes reveal evolutionary dynamics of copper utilization and its link to oxygen. Vol. 3, PLoS ONE. 2008.3(1):e1378.

260. Laviad-Shitrit S, Sharaby Y, Sela R, Thorat L, Nath BB, Halpern M. Copper and chromium exposure affect chironomid larval microbiota composition. Vol. 771, Science of the Total Environment. 2021.771(145330):1–10.

261. González Henao S, Ghneim-Herrera T. Heavy Metals in Soils and the Remediation Potential of Bacteria Associated With the Plant Microbiome. Vol. 9, Frontiers in Environmental Science. 2021.9(April):1–17.

262. Janda JM, Abbott SL. The genus *Hafnia*: from soup to nuts. Vol. 19, Clinical microbiology reviews. 2006.19(1):12–8.

263. Kapahi M, Sachdeva S. Bioremediation Options for Heavy Metal Pollution. Vol. 9, Journal of health & pollution. 2019.9(24):1–20.

264. He LY, Zhang YF, Ma HY, Su LN, Chen ZJ, Wang QY, *et al*. Characterization of copper-resistant bacteria and assessment of bacterial communities in rhizosphere soils of copper-tolerant plants. Vol. 44, Applied Soil Ecology. 2010.44(1):49–55.

265. Vignaroli C, Pasquaroli S, Citterio B, Di Cesare A, Mangiaterra G, Fattorini D, *et al*.

Antibiotic and heavy metal resistance in *enterococci* from coastal marine sediment. Vol. 237, Environmental Pollution. 2018.237:406–13.

266. Wu W, Huang H, Ling Z, Yu Z, Jiang Y, Liu P, *et al*. Genome sequencing reveals mechanisms for heavy metal resistance and polycyclic aromatic hydrocarbon degradation in *Delftia lacustris* strain LZ-C. Vol. 25, Ecotoxicology. 2016.25(1):234–47.

267. Mathivanan K, Chandirika JU, Vinothkanna A, Yin H, Liu X, Meng D. Bacterial adaptive strategies to cope with metal toxicity in the contaminated environment – A review. Vol. 226, Ecotoxicology and Environmental Safety. 2021.226(112863):1–12.

268. Qurbani K, Khdir K, Sidiq A, Hamzah H, Hussein S, Hamad Z, *et al*. *Aeromonas sobria* as a potential candidate for bioremediation of heavy metal from contaminated environments. Vol. 12, Scientific Reports. 2022.12(1):1–8.

269. Gurung K, Wertheim B, Falcao Salles J. The microbiome of pest insects: it is not just bacteria. Vol. 167, Entomologia Experimentalis et Applicata. 2019.167(3):156–70.

270. McGinnis MR, Tyring SK. Introduction to mycology. In: S B, editor. Methods Gen. Mol. Microbiol. Galveston (TX); 1996; p. 925–8.

271. Malassigné S, Moro CV, Luis P. Mosquito mycobiota: An overview of non-entomopathogenic fungal interactions. Vol. 9, Pathogens. 2020.9(7):1–14.

272. Djihinto OY, Medjigbodo AA, Gangbadja ARA, Saizonou HM, Lagnika HO, Nanmede D, *et al*. Malaria-Transmitting Vectors Microbiota : Overview and Interactions With *Anopheles* Mosquito Biology. Vol. 13. 2022.13(May):1–12.

273. Tawidian P, Coon KL, Jumpponen A, Cohnstaedt LW, Michel K. Host-Environment Interplay Shapes Fungal Diversity in Mosquitoes. Vol. 6, mSphere. 2021.6(5):1–14.

274. Bozic J, Capone A, Pediconi D, Mensah P, Cappelli A, Valzano M, *et al*. Mosquitoes can harbour yeasts of clinical significance and contribute to their environmental dissemination. Vol. 9, Environmental Microbiology Reports. 2017.9(5):642–8.

275. Barredo E, DeGennaro M. Not Just from Blood: Mosquito Nutrient Acquisition from Nectar Sources. Vol. 36, Trends in Parasitology. 2020.36(5):473–84.

276. Steyn A, Roets F, Botha A. Yeasts Associated with *Culex pipiens* and *Culex theileri* Mosquito Larvae and the Effect of Selected Yeast Strains on the Ontogeny of Culex pipiens. Vol. 71, Microbial ecology. 2016.71(3):747–60.

277. Díaz-Nieto LM, D'Alessio C, Perotti MA, Berón CM. *Culex pipiens* development is greatly influenced by native bacteria and exogenous yeast. Vol. 11, PLoS ONE. 2016.11(4):e0153133.

278. Muturi EJ, Bara JJ, Rooney AP, Hansen AK. Midgut fungal and bacterial microbiota of *Aedes triseriatus* and *Aedes japonicus* shift in response to La Crosse virus infection. Vol. 25, Molecular ecology. 2016.25(16):4075–90.

279. Asahina S. Food material and feeding procedures for mosquito larvae. Vol. 31, Bulletin of the World Health Organization. 1964.31(4):465–6.

280. Souza RS, Virginio F, Riback TIS, Suesdek L, Barufi JB, Genta FA. Microorganism-Based Larval Diets Affect Mosquito Development, Size and Nutritional Reserves in the Yellow Fever Mosquito *Aedes aegypti* (Diptera: Culicidae). Vol. 10, Frontiers in physiology. 2019.10(152):1–24.

281. Valzania L, Martinson VG, Harrison RE, Boyd BM, Coon KL, Brown MR, *et al*. Both living bacteria and eukaryotes in the mosquito gut promote growth of larvae. Vol. 12, PLoS Neglected Tropical Diseases. 2018.12(7):1–21.

282. Sharma R, Sharma P. Fungal entomopathogens: a systematic review. Vol. 31, Egyptian Journal of Biological Pest Control. 2021.31(57):1–13.

283. Tawidian P, Rhodes VL, Michel K. Mosquito-fungus interactions and antifungal immunity. Vol. 111, Insect biochemistry and molecular biology. 2019.111(103182):1–12.

284. Kikankie CK, Brooke BD, Knols BGJ, Koekemoer LL, Farenhorst M, Hunt RH, *et al*. The infectivity of the entomopathogenic fungus *Beauveria bassiana* to insecticide-

resistant and susceptible *Anopheles arabiensis* mosquitoes at two different temperatures. Vol. 9, Malaria Journal. 2010.9(71):1–9.

285. Mnyone LL, Lyimo IN, Lwetoijera DW, Mpingwa MW, Nchimbi N, Hancock PA, *et al*. Exploiting the behaviour of wild malaria vectors to achieve high infection with fungal biocontrol agents. Vol. 11, Malaria Journal. 2012.11(87):1–11.

286. Flores GAM, Lopez RP, Cerrudo CS, Consolo VF, Berón CM. *Culex quinquefasciatus* Holobiont: A Fungal Metagenomic Approach. Vol. 3, Frontiers in Fungal Biology. 2022.3(August):1–10.

287. Tawidian P, Jumpponen A, Michel K. Patterns of Fungal Community Assembly Across Two *Culex* Mosquito Species. Vol. 10, Frontiers in Ecology and Evolution. 2022.10(July):1–13.

288. Luis P, Vallon L, Tran FH, Hugoni M, Tran-Van V, Mavingui P, *et al*. *Aedes albopictus* mosquitoes host a locally structured mycobiota with evidence of reduced fungal diversity in invasive populations. Vol. 39, Fungal Ecology. 2019.39:257–66.

289. Thongsripong P, Chandler JA, Green AB, Kittayapong P, Wilcox BA, Kapan DD, *et al*. Mosquito vector-associated microbiota: Metabarcoding bacteria and eukaryotic symbionts across habitat types in Thailand endemic for dengue and other arthropod-borne diseases. Vol. 8, Ecology and evolution. 2018.8(2):1352–68.

290. Krajacich BJ, Huestis DL, Dao A, Yaro AS, Diallo M, Krishna A, *et al*. Investigation of the seasonal microbiome of *Anopheles coluzzii* mosquitoes in Mali. Vol. 13, PLoS ONE. 2018.13(3):e0194899.

291. Nattoh G, Bargul JL, Magoma G, Mbaisi L, Butungi H, Mararo E, *et al*. The fungus *Leptosphaerulina* persists in *Anopheles gambiae* and induces melanization. Vol. 16, PLoS ONE. 2021.16(2):e0246452.

292. Thomas MB, Read AF. Can fungal biopesticides control malaria? Vol. 5, Nature Reviews Microbiology. 2007.5(5):377–83.

293. Bargielowski I, Koella JC. A possible mechanism for the suppression of *Plasmodium*

berghei development in the mosquito *Anopheles gambiae* by the Microsporidian *Vavraia culicis*. Vol. 4, PLoS ONE. 2009.4(3):e4676.

294. Angleró-Rodríguez YI, Blumberg BJ, Dong Y, Sandiford SL, Pike A, Clayton AM, *et al*. A natural *Anopheles*-associated *Penicillium chrysogenum* enhances mosquito susceptibility to *Plasmodium* infection. Vol. 6, Scientific Reports. 2016.6(June):1–10.

295. Valzano M, Cecarini V, Cappelli A, Capone A, Bozic J, Cuccioloni M, *et al*. A yeast strain associated to *Anopheles* mosquitoes produces a toxin able to kill malaria parasites. Vol. 15, Malaria Journal. 2016.15(1):1–9.

296. Bokulich NA, Mills DA. Improved selection of internal transcribed spacer-specific primers enables quantitative, ultra-high-throughput profiling of fungal communities. Vol. 79, Applied and Environmental Microbiology. 2013.79(8):2519–26.

297. Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, *et al*. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Vol. 109, Proceedings of the National Academy of Sciences of the United States of America. 2012.109(16):6241–6.

298. Hoggard M, Vesty A, Wong G, Montgomery JM, Fourie C, Douglas RG, *et al*. Characterizing the human mycobiota: A comparison of small subunit rRNA, ITS1, ITS2, and large subunit rRNA genomic targets. Vol. 9, Frontiers in Microbiology. 2018.9(2208):1–14.

299. Belda E, Coulibaly B, Fofana A, Beavogui AH, Traore SF, Gohl DM, *et al*. Preferential suppression of *Anopheles gambiae* host sequences allows detection of the mosquito eukaryotic microbiome. Vol. 7, Scientific Reports. 2017.7(1):1–13.

300. Cuesta EB, Coulibaly B, Bukhari T, Eiglmeier K, Kone R, Coulibaly MB, *et al*. Comprehensive Ecological and Geographic Characterization of Eukaryotic and Prokaryotic Microbiomes in African Anopheles. Vol. 12, Frontiers in Microbiology. 2021.12(635772):1–18.

301. Bradley TJ. Physiology of osmoregulation in mosquitoes. Vol. 32, Annual review of entomology. 1987.32:439–62.

302. O'Meara G. Saltmarsh mosquitoes (Diptera: Culicidae). Cheng L, editor. Marine insects. Amsterdam: North-Holland Publishing; 1976; 303-333. p.

303. Bradley TJ. Saline-water insects: ecology, physiology and evolution. In: Royal Entomological Society. CABI; 2008; p. 20–35.

304. Kengne P, Charmantier G, Blondeau-Bidet E, Costantini C, Ayala D. Tolerance of disease-vector mosquitoes to brackish water and their osmoregulatory ability. Vol. 10, Ecosphere. 2019.10(10):1–14.

305. Blagrove MSC, Sherlock K, Chapman GE, Impoinvil DE, McCall PJ, Medlock JM, *et al*. Evaluation of the vector competence of a native UK mosquito *Ochlerotatus detritus* (*Aedes detritus*) for dengue, chikungunya and West Nile viruses. Vol. 9, Parasites & vectors. 2016.9(452):1–6.

306. Ramasamy R, Surendran SN, Jude PJ, Dharshini S, Vinobaba M. Larval Development of *Aedes aegypti* and *Aedes albopictus* in Peri-Urban Brackish Water and Its Implications for Transmission of Arboviral Diseases. Vol. 5, PLOS Neglected Tropical Diseases. 2011.5(11):e1369.

307. Ramasamy R, Surendran SN. Global climate change and its potential impact on disease transmission by salinity-tolerant mosquito vectors in coastal zones. Vol. 3, Frontiers in physiology. 2012.3(198):1–14.

308. Surendran SN, Jude PJ, Ramasamy R. Variations in salinity tolerance of malaria vectors of the *Anopheles subpictus* complex in Sri Lanka and the implications for malaria transmission. Vol. 4, Parasites & vectors. 2011.4(117):9.

309. Ould Lemrabott MA, Le Goff G, Kengne P, Ndiaye O, Costantini C, Mint Lekweiry K, *et al*. First report of *Anopheles* (Cellia) *multicolor* during a study of tolerance to salinity of Anopheles arabiensis larvae in Nouakchott, Mauritania. Vol. 13, Parasites & vectors. 2020.13(522):1–9.

310. Harbach RE. The classification of genus *Anopheles* (Diptera: Culicidae): a working hypothesis of phylogenetic relationships. Vol. 94, Bulletin of entomological research. 2004.94(6):537–53.

311. Bryan JH. *Anopheles gambiae* and *A. melas* at Brefet, The Gambia, and their role in malaria transmission. Vol. 77, Annals of tropical medicine and parasitology. 1983.77(1):1–12.

312. Tuno N, Kjaerandsen J, Badu K, Kruppa T. Blood-Feeding Behavior of *Anopheles gambiae* and *Anopheles melas* in Ghana, Western Africa . Vol. 47, Journal of Medical Entomology. 2010.47(1):28–31.

313. Moreno M, Cano J, Nzambo S, Bobuakasi L, Buatiche JN, Ondo M, *et al*. Malaria Panel Assay versus PCR: Detection of naturally infected *Anopheles melas* in a coastal village of Equatorial Guinea. Vol. 3, Malaria Journal. 2004.3:1–6.

314. Pock Tsy JML, Duchemin JB, Marrama L, Rabarison P, Le Goff G, Rajaonarivelo V, *et al*. Distribution of the species of the *Anopheles gambiae* complex and first evidence of *Anopheles merus* as a malaria vector in Madagascar. Vol. 2, Malaria Journal. 2003.2(33):1–7.

315. Thomson RCM. Studies on Salt-Water and Fresh-Water *Anopheles gambiae* on the East African Coast. Vol. 41, Bulletin of Entomological Research. 1951.41(3):487–502.

316. Cuamba N, Mendis C. The role of *Anopheles merus* in malaria transmission in an area of southern Mozambique. Vol. 46, Journal of vector borne diseases. 2009.46(2):157–9.

317. Sy O, Sarr PC, Assogba BS, Nourdine MA, Ndiaye A, Konaté L, *et al*. Residual malaria transmission and the role of *Anopheles arabiensis* and *Anopheles melas* in central Senegal. Vol. 60, Journal of Medical Entomology. 2023.60(3):546–53.

318. Caputo B, Nwakanma D, Jawara M, Adiamoh M, Dia I, Konate L, *et al*. *Anopheles gambiae* complex along the Gambia river, with particular reference to the molecular forms of *An. gambiae s.s.* Vol. 7, Malaria Journal. 2008.7(182):1–17.

319. Bøgh C, Bøgh C, Clarke SE, Jawara M, Thomas CJ, Lindsay SW. Localized breeding of the *Anopheles gambiae* complex (Diptera: Culicidae) along the River Gambia, West Africa. Vol. 93, Bulletin of entomological research. 2003.93(4):279–87.

320. Paterson HE, Paterson JS. Records of the Breeding of 'Saltwater *Anopheles gambiae*'

at Inland Localities in Southern Africa. Vol. 201, Nature. 1964.201:524–5.

321. Braack L, Bornman R, Kruger T, Dahan-Moss Y, Gilbert A, Kaiser M, *et al*. Malaria Vectors and Vector Surveillance in Limpopo Province (South Africa): 1927 to 2018. Vol. 17, International journal of environmental research and public health. 2020.17(4125):1–22.

322. Temu EA, Minjas JN, Coetzee M, Hunt RH, Shiff CJ. The role of four anopheline species (Diptera: Culicidae) in malaria transmission in coastal Tanzania. Vol. 92, Transactions of The Royal Society of Tropical Medicine and Hygiene. 1998.92(2):152–8.

323. Iyengar R. The bionomics of salt-water *Anopheles gambiae* in East Africa. Vol. 27, Bulletin of the World Health Organization. 1962.27(2):223–9.

324. Kipyab PC, Khaemba BM, Mwangangi JM, Mbogo CM. The physicochemical and environmental factors affecting the distribution of *Anopheles merus* along the Kenyan coast. Vol. 8, Parasites & vectors. 2015.8(221):1–8.

325. Mosha FW, Mutero CM. The influence of salinity on larval development and population dynamics of *Anopheles merus* Dönitz (Diptera: Culicidae). Vol. 72, Bulletin of Entomological Research. 1982.72(1):119–28.

326. White BJ, Kundert PN, Turissini DA, Van Ekeris L, Linser PJ, Besansky NJ. Dose and developmental responses of *Anopheles merus* larvae to salinity. Vol. 216, Journal of Experimental Biology. 2013.216(18):3433–41.

327. Barreaux AMG, Stone CM, Barreaux P, Koella JC. The relationship between size and longevity of the malaria vector *Anopheles gambiae* (*s.s.*) depends on the larval environment. Vol. 11, Parasites & vectors. 2018.11(485):1–9.

328. Jannat KNE, Roitberg BD. Effects of larval density and feeding rates on larval life history traits in *Anopheles gambiae s.s.* (Diptera: Culicidae). Vol. 38, Journal of vector ecology : journal of the Society for Vector Ecology. 2013.38(1):120–6.

329. Moller-Jacobs LL, Murdock CC, Thomas MB. Capacity of mosquitoes to transmit

malaria depends on larval environment. Vol. 7, Parasites & vectors. 2014.7(593):1–12.

330. Okech BA, Gouagna LC, Yan G, Githure JI, Beier JC. Larval habitats of *Anopheles gambiae s.s.* (Diptera: Culicidae) influences vector competence to *Plasmodium falciparum* parasites. Vol. 6, Malaria journal. 2007.6(50):1–7.

331. Jeanrenaud ACSN, Brooke BD, Oliver S V. The effects of larval organic fertiliser exposure on the larval development, adult longevity and insecticide tolerance of zoophilic members of the *Anopheles gambiae* complex (Diptera: Culicidae). Vol. 14, PLOS ONE. 2019.14(4):e0215552.

332. Coon KL, Valzania L, Brown MR, Strand MR. Predaceous Toxorhynchites mosquitoes require a living gut microbiota to develop. Vol. 287, Proc. R. Soc. B. 2020.287(20192705):1–9.

333. Coon KL, Hegde S, Hughes GL. Interspecies microbiome transplantation recapitulates microbial acquisition in mosquitoes. Vol. 10, Microbiome. 2022.10(58):1–13.

334. Kaplan EL, Meier P. Nonparametric Estimation from Incomplete Observations. In: Kotz S, Johnson NL, editors. Breakthroughs in Statistics: Methodology and Distribution. New York: Springer New York; 1992; p. 319–37.

335. Harrington DP, Fleming TR. A class of rank test procedures for censored survival data. Vol. 69, Biometrika. 1982.69(3):553–66.

336. Buck Louis GM. Fecundity and Fertility. Buck Louis GM, Platt RW, editors. Reproductive and Perinatal Epidemiology. Oxford University Press; 2011 Feb 23; p. 0.

337. McHugh ML. The chi-square test of independence. Vol. 23, Biochemia medica. 2013.23(2):143–9.

338. Brogdon W, Chan A. Guideline for evaluating insecticide resistance in vectors using the CDC bottle bioassay. USA: CDC Atlanta. 2010.

339. Samuel M, Brooke BD, Oliver S V. Effects of inorganic fertilizer on larval development, adult longevity and insecticide susceptibility in the malaria vector

Anopheles arabiensis (Diptera: Culicidae). Vol. 76, Pest management science. 2020.76(4):1560–8.

340. Finney D. Probit Analysis. In: Cambridge University Press. Oxford; 1952;

341. Shapiro SS, Wilk MB. An Analysis of Variance Test for Normality (Complete Samples). Vol. 52, Biometrika. 1965.52(3/4):591–611.

342. Tukey JW. Comparing Individual Means in the Analysis of Variance. Vol. 5, Biometrics. 1949.5(2):99–114.

343. Ramasamy R, Thiruchenthooran V, Jayadas TTP, Eswaramohan T, Santhirasegaram S, Sivabalakrishnan K, *et al*. Transcriptomic, proteomic and ultrastructural studies on salinity-tolerant *Aedes aegypti* in the context of rising sea levels and arboviral disease epidemiology. Vol. 22, BMC Genomics. 2021.22(253):1–16.

344. Van Schie C, Spafford H, Carver S, Weinstein P. Salinity tolerance of *Aedes camptorhynchus* (Diptera: Culicidae) from two regions in southwestern Australia. Vol. 48, Australian Journal of Entomology. 2009.48(4):293–9.

345. Gondek M, Weindorf DC, Thiel C, Kleinheinz G. Soluble Salts in Compost and Their Effects on Soil and Plants: A Review. Vol. 28, Compost Science and Utilization. 2020.28(2):59–75.

346. Patrick ML, Aimanova K, Sanders HR, Gill SS. P-type Na+/K+-ATPase and V-type H+-ATPase expression patterns in the osmoregulatory organs of larval and adult mosquito *Aedes aegypti*. Vol. 209, Journal of Experimental Biology. 2006.209(23):4638–51.

347. Guo X, Zhou S, Wu J, Zhang X, Wang Y, Li Z, *et al*. An Experimental Evaluation of Toxicity Effects of Sodium Chloride on Oviposition, Hatching and Larval Development of *Aedes albopictus*. Vol. 11, Pathogens. 2022.11(262):1–9.

348. Durant AC, Grieco Guardian E, Kolosov D, Donini A. The transcriptome of anal papillae of *Aedes aegypti* reveals their importance in xenobiotic detoxification and adds significant knowledge on ion, water and ammonia transport mechanisms. Vol. 132,

Journal of Insect Physiology. 2021.132(104269):1–16.

349. Ibrahim SS, Riveron JM, Stott R, Irving H, Wondji CS. The cytochrome P450 CYP6P4 is responsible for the high pyrethroid resistance in knockdown resistance-free *Anopheles arabiensis*. Vol. 68, Insect biochemistry and molecular biology. 2016.68:23–32.

350. Moyes CL, Lees RS, Yunta C, Walker KJ, Hemmings K, Oladepo F, *et al*. Assessing cross-resistance within the pyrethroids in terms of their interactions with key cytochrome P450 enzymes and resistance in vector populations. Vol. 14, Parasites & vectors. 2021.14(115):1–13.

351. Weedall GD, Mugenzi LMJ, Menze BD, Tchouakui M, Ibrahim SS, Amvongo-Adjia N, *et al*. A cytochrome P450 allele confers pyrethroid resistance on a major African malaria vector, reducing insecticide-treated bednet efficacy. Vol. 11, Science translational medicine. 2019.11(484):73–86.

352. Wondji CS, Hearn J, Irving H, Wondji MJ, Weedall G. RNAseq-based gene expression profiling of the *Anopheles funestus* pyrethroid-resistant strain FUMOZ highlights the predominant role of the duplicated CYP6P9a/b cytochrome P450s. Vol. 12, G3. 2022.12(1):1–13.

353. Alfano N, Tagliapietra V, Rosso F, Manica M, Arnoldi D, Pindo M, *et al*. Changes in Microbiota Across Developmental Stages of *Aedes koreicus,* an Invasive Mosquito Vector in Europe: Indications for Microbiota-Based Control Strategies. Vol. 10, Frontiers in Microbiology. 2019.10(December):1–15.

354. Xiang MA, Linser PJ, Price DA, Harvey WR. Localization of two Na+- or K+-H+ antiporters, AgNHA1 and AgNHA2, in *Anopheles gambiae* larval Malpighian tubules and the functional expression of AgNHA2 in yeast. Vol. 58, Journal of Insect Physiology. 2012.58(4):570–9.

355. Mainka T, Weirathmüller D, Herwig C, Pflügl S. Potential applications of halophilic microorganisms for biological treatment of industrial process brines contaminated with aromatics. Vol. 48, Journal of industrial microbiology & biotechnology. 2021.48(1):1–19.

356. Poroshina MN, Doronina N V, Kaparullina EN, Kovalevskaya NP, Trotsenko YA. Halophilic and halotolerant aerobic methylobacteria from the technogenic Solikamsk biotopes. Vol. 82, Microbiology. 2013.82(4):490–8.

357. Nabti E, Sahnoune M, Adjrad S, Van Dommelen A, Ghoul M, Schmid M, *et al*. A Halophilic and Osmotolerant *Azospirillum brasilense* Strain from Algerian Soil Restores Wheat Growth under Saline Conditions. Vol. 7, Engineering in Life Sciences. 2007.7(4):354–60.

358. Guo J, Zhu J, Zhao T, Sun Z, Song S, Zhang Y, *et al*. Survival characteristics and transcriptome profiling reveal the adaptive response of the *Brucella melitensis* 16M biofilm to osmotic stress. Vol. 13, Frontiers in microbiology. 2022.13(968592):1–17.

359. Talwar C, Nagar S, Kumar R, Scaria J, Lal R, Negi RK. Defining the Environmental Adaptations of Genus *Devosia*: Insights into its Expansive Short Peptide Transport System and Positively Selected Genes. Vol. 10, Scientific Reports. 2020.10(1151):1–18.

360. Waśkiewicz A, Irzykowska L. Flavobacterium spp. – Characteristics, Occurrence, and Toxicity. In: Batt CA, Tortorello MLBTE, editors. Oxford: Academic Press; 2014; p. 938–42.

361. Oren A. Microbial life at high salt concentrations: phylogenetic and metabolic diversity. Vol. 4, Saline systems. 2008.4(2):1–13.

362. Egamberdieva D, Wirth S, Bellingrath-Kimura SD, Mishra J, Arora NK. Salt-Tolerant Plant Growth Promoting Rhizobacteria for Enhancing Crop Productivity of Saline Soils. Vol. 10, Frontiers in microbiology. 2019.10(2791):1–18.

363. Rathakrishnan D, Gopalan AK. Isolation and characterization of halophilic isolates from Indian salterns and their screening for production of hydrolytic enzymes. Vol. 6, Environmental Challenges. 2022.6(100426):1–10.

364. Yoon JH, Lee CH, Yeo SH, Oh TK. *Sphingopyxis baekryungensis* sp. nov., an orange-pigmented bacterium isolated from sea water of the Yellow Sea in Korea. Vol. 55, International journal of systematic and evolutionary microbiology. 2005.55(3):1223–7.

365. Foster T. Staphylococcus. In: Baron S, editor. Galveston: Medical Microbiology; 1996; p. Chapter 12.

366. Batra P, Mathur P, Misra MC. *Aeromonas* spp.: An Emerging Nosocomial Pathogen. Vol. 8, Journal of Laboratory Physicians. 2016.8(1):1–4.

367. Zheng Y, Xu Z, Liu H, Liu Y, Zhou Y, Meng C, *et al*. Patterns in the Microbial Community of Salt-Tolerant Plants and the Functional Genes Associated with Salt Stress Alleviation. Vol. 9, Microbiology spectrum. 2021.9(2):e0076721.

368. Jenkins MB. Rhizobial and bradyrhizobial symbionts of mesquite from the Sonoran Desert: Salt tolerance, facultative halophily and nitrate respiration. Vol. 35, Soil Biology and Biochemistry. 2003.35(12):1675–82.

369. Ghanbarinia F, Kheirbadi M, Mollania N. *Comamonas* sp. halotolerant bacterium from industrial zone of Jovein of Sabzevar introduced as good candidate to remove industrial pollution. Vol. 7, Iranian journal of microbiology. 2015.7(5):273–80.

370. Novello G, Gamalero E, Massa N, Cesaro P, Lingua G, Todeschini V, *et al*. Proteome and Physiological Characterization of Halotolerant Nodule Endophytes: The Case of *Rahnella aquatilis* and *Serratia plymuthica*. Vol. 10, Microorganisms. 2022.10(890):1–21.

371. Tóth Á, Bata-Vidács I, Kosztik J, Máté R, Kutasi J, Tóth E, *et al*. *Sphingobacterium pedocola* sp. nov. a novel halotolerant bacterium isolated from agricultural soil. Vol. 114, Antonie van Leeuwenhoek. 2021.114(10):1575–84.

372. Ratcliffe NA, Furtado Pacheco JP, Dyson P, Castro HC, Gonzalez MS, Azambuja P, *et al*. Overview of paratransgenesis as a strategy to control pathogen transmission by insect vectors. Vol. 15, Parasites and Vectors. 2022.15(1):1–31.

373. Feng Y, Peng Y, Song X, Wen H, An Y, Tang H, *et al*. Anopheline mosquitoes are protected against parasite infection by tryptophan catabolism in gut microbiota. Vol. 7, Nature microbiology. 2022.7(5):707–15.

374. Uyhelji HA, Cheng C, Besansky NJ. Transcriptomic differences between euryhaline

and stenohaline malaria vector sibling species in response to salinity stress. Vol. 25, Molecular ecology. 2016.25(10):2210–25.

375. Singh A, Patel NF, Allam M, Chan WY, Mohale T, Ismail A, *et al*. Marked Effects of Larval Salt Exposure on the Life History and Gut Microbiota of the Malaria Vector *Anopheles merus* (Diptera: Culicidae). Vol. 13, Insects. 2022.13(12):1–20.

376. Burke A, Dahan-Moss Y, Duncan F, Qwabe B, Coetzee M, Koekemoer L, *et al*. *Anopheles parensis* contributes to residual malaria transmission in South Africa. Vol. 18, Malaria Journal. 2019.18(1):1–7.

377. Burke A, Dandalo L, Munhenga G, Dahan-Moss Y, Mbokazi F, Ngxongo S, *et al*. A new malaria vector mosquito in South Africa. Vol. 7, Scientific reports. 2017.7(43779):1–5.

378. Moffett A, Shackelford N, Sarkar S. Malaria in Africa: Vector species' niche models and relative risk maps. Vol. 2, PLoS ONE. 2007.2(9):e824.

379. Hlongwana KW, Mavundza EJ, Mohapi EP, Kruger P, Urbach J, Mukaratirwa S, *et al*. Vector-control personnel's knowledge, perceptions and practices towards insecticides used for indoor residual spraying in Limpopo Province, South Africa. Vol. 6, Parasites and Vectors. 2013.6(1):1–9.

380. Marubini EE, Musekiwa A, Mazarire T, Sekgele W. Anopheles arabiensis larval habitats characterization and *Anopheles* species diversity in water bodies from Jozini, KwaZulu-Natal Province. 2024.

381. Nattoh G, Maina T, Makhulu EE, Mbaisi L, Mararo E, Otieno FG, *et al*. Horizontal Transmission of the Symbiont *Microsporidia MB* in *Anopheles arabiensis*. Vol. 12, Frontiers in Microbiology. 2021.12(647183):1–13.

382. Makhulu E, Onchuru T, Joseph G, Otieno F, Wairimu A, Joseph M, *et al*. Localization and tissue tropism of the symbiont *Microsporidia MB* in the germ line and somatic tissues of *Anopheles arabiensis*. Vol. 15, mBio. 2023.15(1):e02192-23.

383. Bukhari T, Pevsner R, Herren JK. *Microsporidia*: a promising vector control tool for

residual malaria transmission. Vol. 3, Frontiers in Tropical Diseases. 2022.3(July):1–18.

384. Egyirifa RK, Akorli J. Two promising candidates for paratransgenesis, *Elizabethkingia* and *Asaia*, increase in both sexes of *Anopheles gambiae* mosquitoes after feeding. Vol. 23, Malaria Journal. 2024.23(1):1–8.

APPENDIX

Appendix 1: Ethics Waiver

ANIMAL RESEARCH ETHICS COMMITTEE
Registration number: AREC-101210-002

Date: 29/02/2024

Certificate reference: Waiver 29-02-2024-O
Category: O
Applicant: Dr Shüné Oliver
Department: Vector control Reference Laboratory, National Institutes for Communicable Diseases, Sandringham, Johannesburg, South Africa.
Tel: 011 386 6483 **Email**: Shuneo@nicd.ac.za

Re: Waiver from the Animal Ethics Research Committee of the University of the Witwatersrand

This letter is to confirm that following studies to be undertaken in the Vector Control Reference Laboratory at the National Institutes for communicable Diseases, do not require a full Animal Ethics Research Committee clearance.

A. Singh_29-02-2024-O
Student Details: Ashmika Singh, PhD Candidate (859507)
Project Title: Characterisation of the dynamic gut microbiota of members of the An. gambiae complex.

K. Noeth_29-02-2024-O
Student Details: Kayla Noeth, PhD Candidate (2525673)
Project Title: Bionomics of Anopheles mosquitoes in Ehlanzeni District Municipality, Mpumalanga

S. Misser_29-02-2024-O
Student details: Shristi Misseer, MSc Candidate (1388499)
Project Title: The effect of larval exposure to plastic pollution on the major malaria vector, Anopheles arabiensis Patton (Diptera: Culicidae)

W. Rasikhanya_29-02-2024-O
Student details: Wendy Rasikhanya, MSc Canidate (1884976)
Project Title: The effect of rural and urban pollutant exposure on the major malaria vector Anopheles arabiensis.

Reason for waiver
These projects entail metagenomic data collection from invertebrate species, of laboratory reared and wild *Anopheles arabensis* mosquitoes and thus do not require a full animal ethics clearance.

Details of the study
These projects aim to generate metagenomic data on the Anopheles gambiae complex (including An. arabiensis species) gut microflora using next generation sequencing (NGS) technology. Changes in microflora will determined in response to the exposure of heavy metals and salt (A. Singh), organosulphate larvicides (K. Noeth), plastic pollutants (S. Misser) and nitrate, nitrites, sulphides and sulphates (W. Rasikhanya) and the subsequent effect on larval development, adult longevity and insecticide resistance.

The individuals covered by this waiver include students; Ashmika Singh, Kayla Noeth, Shristi misser Wendy Rasikhanya and supervisors Dr Shüné Oliver (A002517), Dr Givemore Munhenga (A002589) and Dr Chia-yu Chen (A0048530) in the Vector control Reference Laboratory, NICD and WITS joint staff.

Please contact me should you require further information.

Yours sincerely,

Dr Mark Killick
Deputy Chair: Animal Research Ethics Committee
University of the Witwatersrand.
Mail: Mark.Killick@wits.ac.za Office number: 011 717 2362

Appendix 2: Manuscripts

Marked Effects of Larval Salt Exposure on the Life History and Gut Microbiota of the Malaria Vector *Anopheles merus*

insects

MDPI

Article

Marked Effects of Larval Salt Exposure on the Life History and Gut Microbiota of the Malaria Vector *Anopheles merus* (Diptera: Culicidae)

Ashmika Singh [1,2], Nashrin F. Patel [1,2], Mushal Allam [3], Wai-Yin Chan [4,5], Thabo Mohale [4], Arshad Ismail [4,6] and Shüné V. Oliver [1,2,*]

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Simple Summary: *Anopheles merus* is a malaria-transmitting mosquito with the unusual ability to breed in a range of saltwater concentrations. Although previous studies have looked at the consequences of this capacity on the immature mosquitoes present in the saltwater, what this means for the adults that emerge from this water is unknown. In this study, we look at the consequences of breeding in a range of saltwater concentrations on laboratory-bred *An. merus*. We found that high salt concentrations delayed the time to pupation but does not affect adult longevity. Therefore, although the larvae are at risk for a longer period, it does not affect the lifespan of the adult. Increased salt exposure also results in increased insecticide tolerance, which makes them harder to control. Finally, the salt concentration affects the composition of the mosquito's gut microbiota. The significance of this is that the gut bacteria composition affects the capacity to transmit the malaria parasite. This study suggests that due to changes in the gut bacteria, mosquitoes that breed in very salty water are less likely to carry the malaria parasite in their gut. Therefore, the saltiness of the water that these mosquitoes breed in may affect how well they transmit malaria.

Abstract: *Anopheles merus* can breed in a range of saltwater concentrations. The consequences of this ability on the life history of adult *An. merus* are poorly understood. This study examined the effects of exposure to 0, 2.1875, 4.375, 8.75, and 17.5 g/L of sodium chloride on *An. merus*. The effects on larval development, adult longevity, fertility, and fecundity, as well as deltamethrin tolerance were examined. The effect of larval salt exposure on the expression of *defensin-1* in adults was examined by quantitative Real-Time PCR. Finally, the effect of the larval salt concentration on microbial dynamics was assessed by 16S Next Generation Sequencing. High concentrations of saltwater increased larval development time and number of eggs laid, as well as deltamethrin tolerance. Larval exposure to salt also reduced the expression of *defensin-1*. The exposure also had a significant effect on microbial diversity in larvae and adults. The diversity of larvae decreased once adults emerged. Salt-tolerant bacterial genera predominated in larvae but were absent in adults. High salt concentrations resulted in greater abundance of *Plasmodium*-protective genera in adults. Although this study was conducted on a laboratory strain of *An. merus*, these data suggest that osmoregulation has a significant effect on the life history of the species with potential epidemiological consequences.

Keywords: osmoregulation; development; diversity; bacterial abundance

Citation: Singh, A.; Patel, N.F.; Allam, M.; Chan, W.-Y.; Mohale, T.; Ismail, A.; Oliver, S.V. Marked Effects of Larval Salt Exposure on the Life History and Gut Microbiota of the Malaria Vector *Anopheles merus* (Diptera: Culicidae). *Insects* **2022**, *13*, 1165. https://doi.org/10.3390/insects13121165

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1. Introduction

Insects are one of the most prolific organisms in terrestrial and fresh-water environments. By contrast, their success in marine environments is more limited. As such, to the best of our knowledge, the hemipteran genus *Halobates* is the only genus of insects that are strictly marine insects. Other insects that are considered marine insects are associated with coastal habitats such as estuaries and saltmarshes, rather than relying solely on the ocean [1]. This pattern is also true for many species of mosquitoes, where the larval stage of the mosquito occurs in fresh water 95% of the time, owing to the fact that majority of mosquito species cannot tolerate osmotic stress that exceeds the osmolarity of the haemolymph. This critical concentration is approximately 300 osmol/L, which corresponds to roughly 30% seawater [2]. However, approximately 5% of mosquito species are more osmotolerant and are capable of surviving hypersaline conditions [3].

The most well-known osmotolerant mosquitoes belong to the genus *Aedes*. This includes the saltmarsh mosquito *Aedes detritus*. The New Zealand salt pool mosquito *Opifex fuscus* is another example of a marine mosquito [4]. Furthermore, while *Aedes* mosquitoes are generally considered obligate freshwater mosquitoes [5], there have been reports of salt-tolerance in *Aedes detritus* [6]. Salt tolerance has also been reported in other significant vector species, where *Aedes albopictus* was found to be the most salt tolerant, followed by *Anopheles coluzzii*, *Aedes aegypti*, *Culex quinquefasciatus*, and *Anopheles gambiae*, with *Culex. pipiens* being the least salt tolerant [5]. Another study examined the long-term effects of salt exposure on the competition between *Ae. aegypti* and *Ae. albopictus*. This study found that *Ae. aegypti* was more tolerant to long-term exposure, but that salinity did not affect the competition between the two species. The preference for saline larval breeding conditions may be due to reduced competition and predation in such conditions. Furthermore, such breeding conditions are high in nutrients, providing a competitive advantage to mosquitoes that can inhabit these niches [4].

Apart from *An. coluzzi*, other salt-tolerant *Anopheles* mosquitoes include *Anopheles aquasalis* [7], *Anopheles sundaicus* [8], and *Anopheles multicolor* [9]. Furthermore, within the *An. gambiae* complex, *Anopheles bwambae*, *Anopheles melas*, and *Anopheles merus* are salt-tolerant species [10]. It is also notable that *Anopheles arabiensis* in Mauritania showed variable saline tolerance, with a 75% survival in 17.5 g/L saltwater [9]. The salt tolerance of the *An. gambiae* complex has significant implications for vector control in Africa because while *Anopheles bwambae* is limited in distribution, *An. melas* and *An. merus* have wider distribution. *Anopheles melas* is found on the west coast of Africa, while *An. merus* is found on the east coast of Africa and is found as far south as South Africa [10]. Thus, *An. merus* is a secondary dominant vector species in Africa and is commonly found in southern Africa where it has been found to be a vector of bancroftian filariasis in Tanzania [11]. *An. merus* also tends to dominate in the dry season [11]. In South Africa, this species is found in all three malaria-endemic provinces and is the most prevalent member of the *An. gambiae* complex in the Mpumalanga province [12]. Although *An. merus* has been implicated in malaria transmission in Tanzania and Madagascar, it has not been implicated in malaria transmission in South Africa yet [12]. Despite this, there is a possibility that the species could contribute to the low-level outdoor transmission of malaria, which is known as residual malaria [13].

The osmotolerance in *An. merus* is an unusual characteristic and is thus poorly examined. The effect of salt dosage on *An. merus* development has previously been described [14]. The transcriptomic response to osmotic stress in euryhaline (salt tolerant) and stenohaline (freshwater) malaria-vector sibling species has also been described with], the transcriptome of larvae reared in salt water were investigated [15]. However, while the effect of breeding in variable salt concentrations on mosquito larvae has been examined a few times, the effect on the emerging adults has not been examined. This is true for *An. merus* as well. Therefore, although it has been demonstrated that immune genes are largely downregulated in larvae with increasing salt concentration [14], it is not known whether this has a carry-through effect to the adult. Therefore, in the current study, the effect of variable

larval salt concentrations on the antimicrobial peptide defensin-1 was examined. Like most defensins, the antimicrobial peptide defensin-1 is a cysteine-rich peptide, with activity against Gram-positive bacteria and fungi [16]. Defensin-1 is also expressed in the gut [17]. This peptide was chosen as it has been associated with saline tolerance in plants [18]. The paucity of information on this topic is problematic, as it is well known that the larval environment dictates adult fitness, because larval environment affects factors such as adult longevity, size, fertility, and fecundity [19–22]. Yet, the effect of osmoregulation on mosquito life history and malaria transmission is poorly understood. This is particularly true for solutes in the water ranging from innocuous pollutants such as organic fertiliser [23], to toxic pollutants such as herbicides and heavy metals that may contribute to the need for mosquito species to be adapted to higher salt concentrations in breeding waters [24,25].

Another factor that is not often considered is the effect of the larval environment on the adult gut microbiota. The gut microbiota of mosquitoes is essential for development and pupation [26,27] and plays a critical role in adult life history [28,29]. Crucially, gut microbiota plays an important role in the capacity to transmit the *Plasmodium* parasite in *Anopheles* mosquitoes [30,31]. There has been a range of studies that have demonstrated direct effects on both the bacteria on the parasite [32]. There has also been demonstration of the indirect effects of gut bacteria on *Plasmodium* through modulation of the immune system [33]. Gut microbiota are therefore a potential target for vector control interventions [34,35], and as such, understanding the composition of the gut microbiome is an important consideration for vector biologists. As the adult gut microbiome is impacted by the larval environment [36–38], it is possible that variable salt concentrations could differentially affect the gut microbiota of salt-tolerant species. This is likely, therefore, to have epidemiological consequences.

This study therefore aims to address this lack of information of the effects of breeding in variable salt concentrations on adult *An. merus*. This will be accomplished by examining the effect of larval salt concentration on larval development, adult longevity, fertility and fecundity, insecticide tolerance, and gut microbiota.

The *An. merus* strain, MAFUS, used in this study is routinely reared in 17.5 g or 50% seawater. Although there are reports of South African *An. merus* breeding in water as salty as 123% seawater, this laboratory strain was not capable of surviving in higher salt concentrations. Therefore, although there may be wild *An. merus* in South Africa capable of surviving higher salt concentrations, this concentration was high enough to exclude other members of the *An. gambiae* complex [14], as well as being the highest concentration that the strain could tolerate (unpublished data). Therefore the 17.5 g (50% seawater) represented the highest salt concentration used, and all concentrations were based on dilutions of this value. As such, the concentrations used in this study are substantially higher than a previous study on the effects of salt on *Ae. albopictus* larvae [39] and are similar to the previous study of the effects of salt concentration on *An. gambiae* larvae [14]. It is hypothesised that variable salt concentrations will differentially affect a range of life history traits and expression of the antimicrobial peptide defensin-1, as well as the composition of the gut microbiome. As the strain is routinely reared in 17.5 g salt, it is hypothesised that the most marked effects will be in adults emerging from larvae reared in this concentration.

2. Materials and Methods

2.1. Strain and Larval Treatment

MAFUS is an *An. merus* strain colonised from Mafayeni, Kruger National Park, Limpopo, South Africa in 2012. The strain has low-level tolerance to insecticides. In the colonisation period, the strain was reared in 17.5 g/L of sodium chloride. This concentration represents the 50% seawater concentration of sea water. As such 8.75 g/L represented the 25% seawater concentration, 4.375 g/L represented the 12.5% seawater concentration, 2.1875 g/L represented the 6.25% seawater concentration, and the water without salt added constituted the 0% salt concentration.

For each experiment, 200 first-instar larvae (less than 24 h old) were collected. These were transferred to 1000 mL of each concentration of water in a plastic container (214 × 155 × 80 mm). The larvae were fed an equal amount of larval food until pupation and were reared under the conditions and diet described in [40]. In brief, mosquitoes were reared at 25 °C (±2 °C) and 80% humidity (±5%). The mosquitoes were subjected to a 12:12 h light-dark cycle with a 30 min dusk/dawn cycle. Adults were maintained on a 10% sucrose solution.

2.2. The Effects of Variable Salt Concentration on Life History

2.2.1. Development Time

Fifty first-instar larvae were set up as described earlier. Development time was measured as the time to pupation. This experiment was replicated three times using cohorts originating from three separate egg batches. The development time was assessed using a Kaplan–Meier [41] estimator with a log-rank test [42] as the measure of significance.

2.2.2. Adult Longevity

From each concentration, 25 males and 25 females were kept in a cage with ad libitum access to 10% sucrose. The sucrose solution was changed twice a week. The adults were collected upon emergence and placed in a cage. Throughout the duration of the experiment, the females were not allowed access to blood. Mortality was recorded daily, and cadavers were removed once a day. The experiment was considered complete once all the individuals were dead. This experiment was replicated three times. Longevity was analysed using a Kaplan–Meier [41] estimator with a log-rank test [42] as the measure of significance.

2.2.3. Fertility and Fecundity

From each concentration, 50 males and 25 females were collected and allowed a blood meal at the ages of 3 and 7 days. At 11 days, the cage was allowed to oviposit for 18 h. The egg plate was removed, and the eggs were allowed to hatch for 72 h. The number of larvae and remaining unhatched eggs were counted. This experiment was replicated four times using adults that were set up from independent larval preparations. Bloodmeals were provided by a single consenting human volunteer (SVO) as per the ethics waiver S-Oliver 03-01-2018-O from the University of the Witwatersrand. Data were analysed using a Chi-squared test.

2.2.4. Insecticide Tolerance

At the age of 3 days, non-blood-fed females were used to determine lethal time using a CDC bottle bioassay. The adults were exposed to 0.001 mg/mL of deltamethrin for either 2, 4, 8, 16, or 32 min as per [43]. Adults were removed from the bottles after the set time and were allowed ad libitum access to sucrose. Mortality was scored 24 h after exposure. Lethal times were calculated by probit analysis [44]. Controls consisted of the unexposed mosquitoes (0 min—environmental control) and mosquitoes exposed to a bottle coated in acetone only (solvent control). The experiment was replicated five times. Data were analysed for normality using the Shapiro–Wilk test and the means were compared using a One-Way Analysis of Variance (ANOVA) with an alpha value of 5% [45]. A Tukey test was used as a post-hoc test [46].

2.3. The Effect of Larval Salt Concentration on the Expression of Defensin-1

2.3.1. RNA Extraction and Quality Control

RNA was extracted from females from each of the different concentrations. Five replicates of 3 non-blood-fed 3-day-old females were used in the extraction. RNA was extracted using a column-based Quick-RNA™ MiniPrep kit (Zymo Research: R1054). Samples were mechanically homogenised using a Gene® 2 vortex according to manufacturer's instructions. Purity and concentration of the extracted RNA were assessed using a Nanodrop™ 2000 C and integrity was confirmed by TapeStation™ 4200 system analysis with RNA ScreenTape® (Agilent Technologies, Santa Clara, CA, USA). RNA integrity was also

assessed using 1% 0.5× Tris-Borate-EDTA (TBE) agarose gel electrophoresis at 70 V, 90 mA for 30 min. Samples were sized using a RiboRuler™ High Range RNA Ladder (Thermo Scientific™: SM1821).

2.3.2. Analysis of Transcript Levels

The extracted RNA was used to generate 1000 nM of complementary DNA (cDNA) in replicates of three, using the iScript™ cDNA Synthesis Kit (Bio-Rad, Rosebank, South Africa: 1708891) according to manufacturer's instructions. The cDNA (100 nM) was used as a template for the quantitative Real-Time Polymerase Chain reaction (qRT-PCR) reaction in SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad: 1725270) according to the manufacturer's instructions with the following cycling conditions: DNA denaturation at 98 °C (3 min) followed by 40 cycles at 95 °C (10 s) with a final extension at 60 °C (30 s). The sequences of the primers and reference genes used in this study are listed in Supplementary Table S3. Differences in transcripts levels were assessed by the Pfaffl method [47] by One-Way ANOVA using Bio-Rad CFX maestro™ software (version 4.0.2325.0418: 2017)

2.4. The Effect of Larval Exposure to Variable Salt Concentration on An. merus Gut Microbiota

2.4.1. Midgut Resection and DNA Extraction

Larval activity was minimised by chilling at 4 °C. The adult female mosquitoes were cold killed at −70 °C before their midguts were resected. The dissection procedure took place under sterile conditions. All equipment as well as the mosquitoes were sterilised with 70% ethanol and 3% hydrogen peroxide. The midguts from the respective samples were dissected using an Olympus SZ2-ILST stereomicroscope at a magnification of 40×. Five replicates of 3 midguts each in 200 µL of 0.1 M Phosphate Buffered Saline (PBS) at pH 7.2 were prepared.

The DNeasy Blood & Tissue Kit® (Qiagen: 69506) was used to extract total genomic DNA from the midgut of the mosquito. The midguts were centrifuged at 5000× *g* and approximately 150 µL of PBS was discarded; the remaining PBS and midguts were briefly vortexed and added to new tubes. The protocol was then followed as per manufacturer's instructions.

2.4.2. Amplification of the V3–V4 Hypervariable Region of the 16S rRNA Gene

Universal bacterial primers were used to amplify the V3–V4 hypervariable region of the bacterial 16S rRNA gene. The primers used were 4 nmol Ultramer® DNA Oligo 55 bases (Integrated DNA Technologies, Coralville, IA, USA) [48]. Primer sequences are detailed in Supplementary Table S1. Library preparation was performed according to the standard instructions of the 16S Metagenomic Sequencing Library Preparation protocol (IlluminaTM, Inc., San Diego, CA, USA). The Qubit® High Sensitivity dsDNA Assay Kit (Thermo Fisher Scientific: Waltham, MA, USA) was used to quantify indexed amplicons. The amplicons were then visualised and sized using the 4200 TapeStation™ (Agilent Technologies: Santa Clara, CA, USA).

Normalized libraries were pooled and denatured using 0.2N NaOH. Libraries were subsequently sequenced on the MiSeq platform using the MiSeq Reagent kit v3 (Illumina) and paired-end 2 × 300 bp sequencing was performed at the Sequencing Core Facility, National Institute for Communicable Diseases, South Africa.

2.4.3. Bioinformatic Analysis

Several quality control steps were taken to ensure clean data were used for clustering. Sequences were filtered and trimmed. Raw reads were trimmed with a 0.05 quality limit. The SILVA 16S v132 database was used to cluster data at a 99% similarity threshold. The OTUs were assigned to sequences based on 97% identity. The OTU table was then used to prepare a bar plot to visually represent the midgut bacteria of each respective sample.

Raw reads were used for bacterial diversity analysis. The raw reads were quality-controlled and filtered (Q > 20 and length > 50 bp) using the programmes fastqc (v0.11.8)

and Trim Galore (v0.6.4_dev; https://github.com/FelixKrueger/TrimGalore, accessed on 18 May 2022), respectively [49,50]. Trim Galore was also used for adapter removal.

All the downstream analyses were performed in R (v3.6.1). This included classification, abundance estimations, statistical analysis, and visualization. The dada2 package (v1.12.1) [51] was used to pre-process clean reads. This included quality inspection, trimming, dereplication, merging paired-end reads, and removal of chimeric sequences.

The SILVA reference database (v138; https://zenodo.org/record/1172783#.XvCmtkUzY2w: accessed on 18 May 2022) was used to assign taxonomy to the amplicon sequence variants (ASVs). The ASV abundance estimates were determined using training sequence sets based on the SILVA database.

The phyloseq package (v1.28.0) [52], ggplot2 (v3.2.1), and AmpVis2 package (v2.6.4) [53] were used to determine ordinations for beta diversity, abundance bar plots, alpha diversity, and richness estimates, and heatmaps. Data generated using clustering in Non-metric Multidimensional Scaling (NMDS) were assessed using PERMANOVA (permutation test with pseudo-F ratios) as implemented in the adonis function in the vegan package (https://github.com/vegandevs/vegan, accessed on 18 May 2022).

Alpha diversity between groups was compared using Kruskal–Wallis rank-sum tests. Overlapping species diagrams were generated using Venn Diagram (v1.6.20) and UpsetR (v1.4.0) [54]. DESeq2 (v1.24.0) [55] was used to determine differential abundance analysis between sample groups.

3. Results

3.1. The Effect of Larval Salt Concentration on Life History

3.1.1. Development Time

Larvae that developed in the 50% (17.5 g/L) and 25% (8.75 g/L) salt concentration conditions took significantly longer to pupate than the other treatments (log-rank test: $p < 0.01$, $\chi^2 = 112.15$, df = 2). There was no significant difference in development time of the larvae between the 0%, 6.25% (2.188 g/L), and 12.5 % (4.375 g/L) salt concentrations ($p = 0.85$, $\chi^2 = 0.31$, df = 2) (Figure 1A).

3.1.2. Adult Longevity

There was no significant difference in the male adult longevity regardless of treatment (log-rank test: $p = 0.22$, $\chi^2 = 5.79$ df = 4). For females, there was a significant effect on longevity ($p < 0.01$, $\chi^2 = 16.81$ df = 4), but the effect was only due to the reduced longevity of the 25% treatment (Figure 1B).

3.1.3. Fertility and Fecundity

Larval salt exposure affected adult fertility and fecundity. There was a significant difference in fecundity (number of eggs laid) (Chi-squared: $p < 0.01$; $\chi^2 = 2767$ df = 12). A Tukey post-hoc test showed that the significance was due to the difference between the 0% and 50% treatments. There was also a significant difference in fertility (percentage of eggs hatched) ($p = 0.03$ $\chi^2 = 124.8$ df = 12) (Figure 1C).

3.1.4. Insecticide Tolerance

Larval salt treatment altered sensitivity to deltamethrin when comparing LT50 values (one-way ANOVA: $p = 0.03$, $F_{(4, 21)} = 3.20$). A Tukey post-hoc test demonstrated that this was due to the differences between 0% and 50% treatments, without any difference between the 6.25, 12.5, and 25% treatments (Figure 1D). This was emphasised by the LT99 values, where the 50% treatment had the highest lethal time ($p < 0.01$, $F_{(4, 21)} = 6.74$).

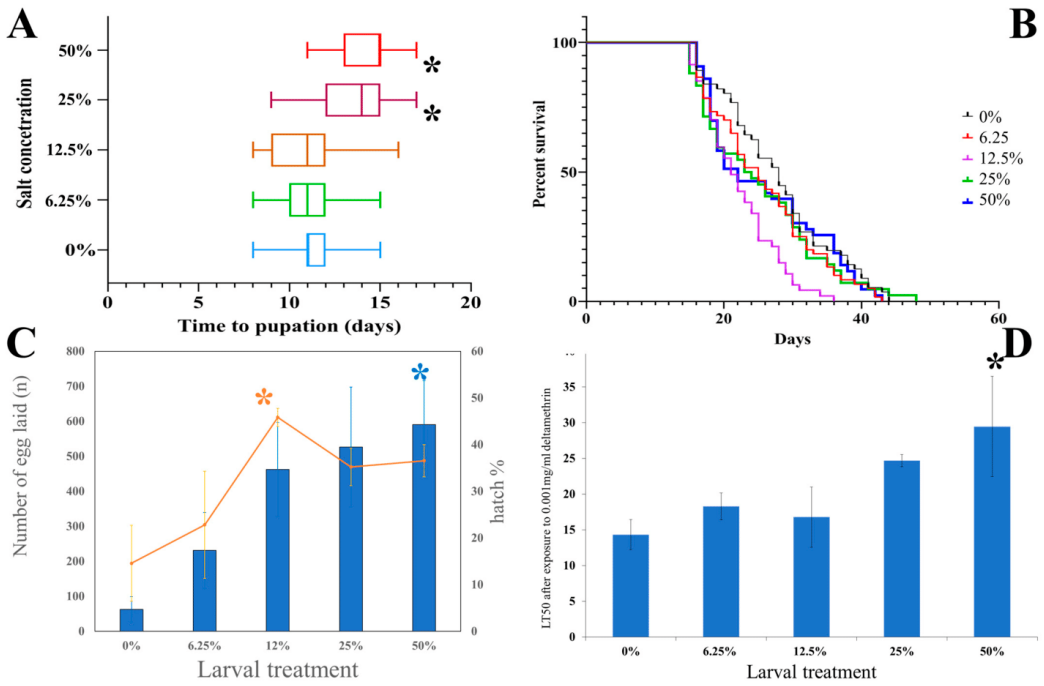

Figure 1. The effect of variable larval salt concentration on life history traits of Anopheles merus. (**A**) Time-to-pupation in different larval salt concentrations. Asterisks (*) indicate a significant difference from the untreated control. (**B**) Adult longevity of female *An. merus* after rearing in different larval salt exposures. (**C**) Fertility and fecundity of adults reared in different larval salt concentrations. Blue bars represent number of eggs, and the orange line represents hatch percentage. Blue asterisks represent a significantly different number of eggs from the control. Orange asterisks represent a significantly different hatch percentage from the untreated control. (**D**) Deltamethrin lethal time of adults reared in different larval salt concentrations. Asterisks indicate a significant difference from the untreated control.

3.2. The Effect of the Larval Salt Concentration on Defensin-1 Expression

There was no effect of the treatment on the expression of the 18S transcript (one-way ANOVA: $p = 0.73$, $F_{(4, 40)} = 0.50$) or the 26S transcript ($p = 0.97$, $F_{(4, 40)} = 0.13$). The treatment, however, did result in a significant change in defensin-1 transcript level compared to the 0% treatment ($p < 0.01$, $F_{(4, 40)} = 21.31$). A Tukey post-hoc test showed that all the treatments had significantly lower defensin-1 transcript levels than the 0% treatment. Furthermore, the 6.25% treatment had a significantly higher transcript level than the 12.5% treatment (Holm–Bonferroni corrected *p*-value: $p < 0.01$) and the 50% treatment (corrected *p*-value: $p < 0.01$). The 50% treatment also had a significantly higher transcript level than the 25% treatment (corrected *p*-value: $p = 0.03$), but not the 6.25% or 12.5% treatment (corrected *p*-values: $p = 0.52$ and $p = 0.23$ respectively) (Figure 2).

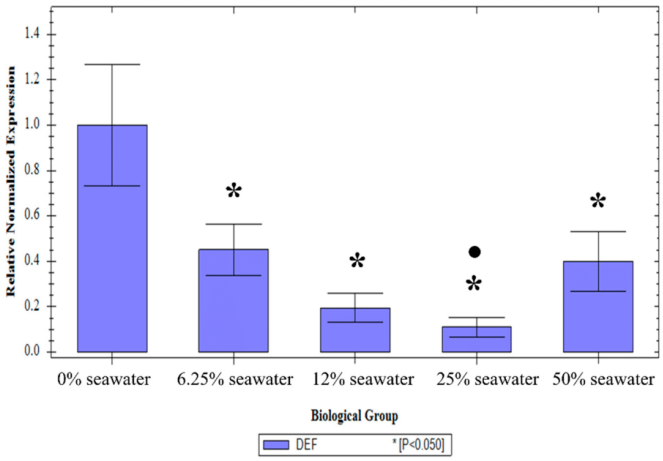

Figure 2. Relative normalised expression of defensin-1 transcripts from *An. Merus* adults reared in different larval salt concentrations. Asterisks (*) denote a significant difference from the untreated control. Circles (●) indicate a significantly lower expression in the treated groups.

3.3. The Effect of Larval Salt Concentration on An. merus Microbiota

Bar plots summarising the species found in the Next Generation Sequencing study, as well as the rarefaction, plots can be found in Supplementary Figures S1–S4, while the OTU tables can be found in Supplementary Tables S1–S4.

3.3.1. Alpha and Beta Diversity

Alpha diversity is a measure of diversity within the sample. As such, α-diversity is a measure of the microbial diversity within each treatment. Alpha diversity can be described in terms of species richness, which is a measure of the number of species present, or the absolute abundance. This is described by the ACE and Chao1 index. It can also be described in terms of species evenness, or relative abundance within a community. This can be described by the Shannon and Simpson indices. This analysis gives an indication of the direct effect of the treatment on the local microbiota.

When examining α-diversity, there was a marked difference in the effect of larval salt exposure on species richness and evenness. There was no significant difference in species richness, as assessed by the Chao1 index (Figure 3A) and ACE index. By contrast, there were significant differences in species evenness. When examining the Shannon index, there were significant differences between the 0% and 50% treatments (Kruskal–Wallis ANOVA: $p = 0.02$), the 6.25% and 12.5% treatments ($p = 0.04$), the 6.25% and 50% treatments ($p = 0.04$), and the 12.5% and 25% treatments ($p = 0.04$) (Figure 3B). A similar pattern was observed for the Simpson index. There was a significant difference between 0% and 50% ($p = 0.03$), and 6.25% and 50% ($p = 0.04$).

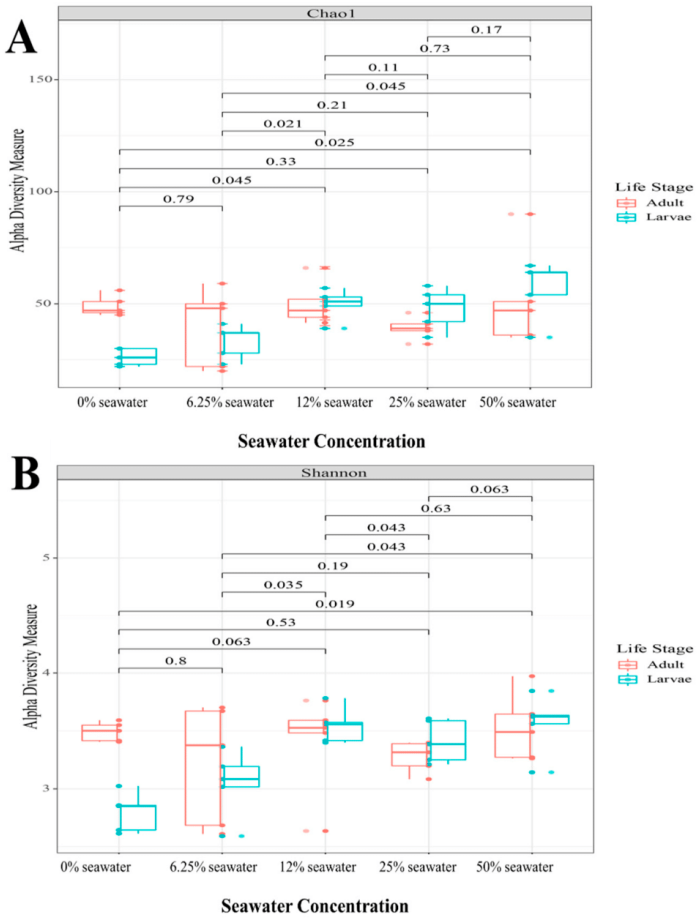

Figure 3. Alpha diversity of adult *An. merus* gut microbiota. (**A**) Chao1 index (species richness). (**B**) Simpson index (species evenness). Values indicate *p*-values from Kruskall–Wallis ANOVA.

Beta diversity refers to the change in species between communities. For microbiome studies, β-diversity involves a quantification of the similarity and dissimilarity between communities. In this study, β-diversity was measured by the Bray–Curtis index and the ordination method used was the non-metric multi-dimensional scaling (NMDS) method. This analysis allows a comparison of the effect of different larval salt exposures on microbiota.

There was a significant difference in the β-diversity of the larvae and the adults (PERMANOVA: $p = 0.01$, $F = 7.98$, stress value = 0.21). While the β-diversity of the larvae differed from each other, the diversity of the adults overlapped (Figure 4).

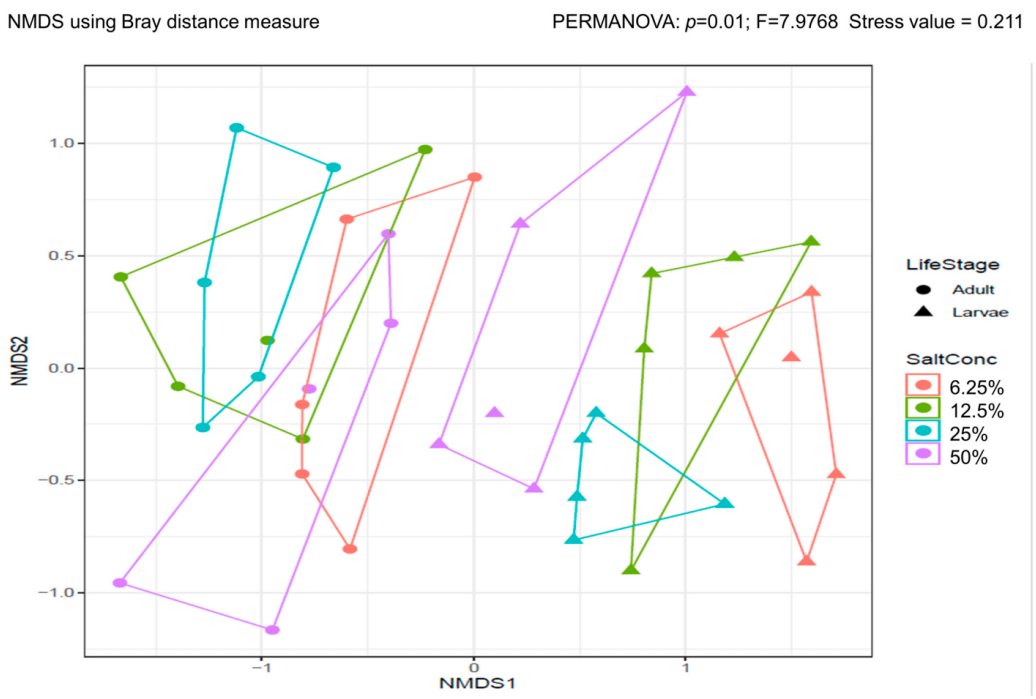

Figure 4. Beta diversity of 4th instar larvae and adult *An. merus* reared in differing larval salt concentration. Non-Metric Multidimensional Scaling plot of β-diversity. Larvae (triangles) from different salt concentrations cluster separately from the adults (circles) and are diverse from each other. Adults are also less diverse and their diversities overlap.

3.3.2. Overlapping Species

In order to understand how many species the treatments had in common, the number of overlapping species was analysed. All treatments had eight families in common, as well as 10 genera and 10 species. The 50% treatment had the most unique families, genera, and species (six, nine, and nine respectively). By contrast, the 25% treatment was the least unique, with only a single unique family and no unique genera or species. This was followed by the 0% treatment, which had only four unique genera and species, and only two unique families (Figure 5).

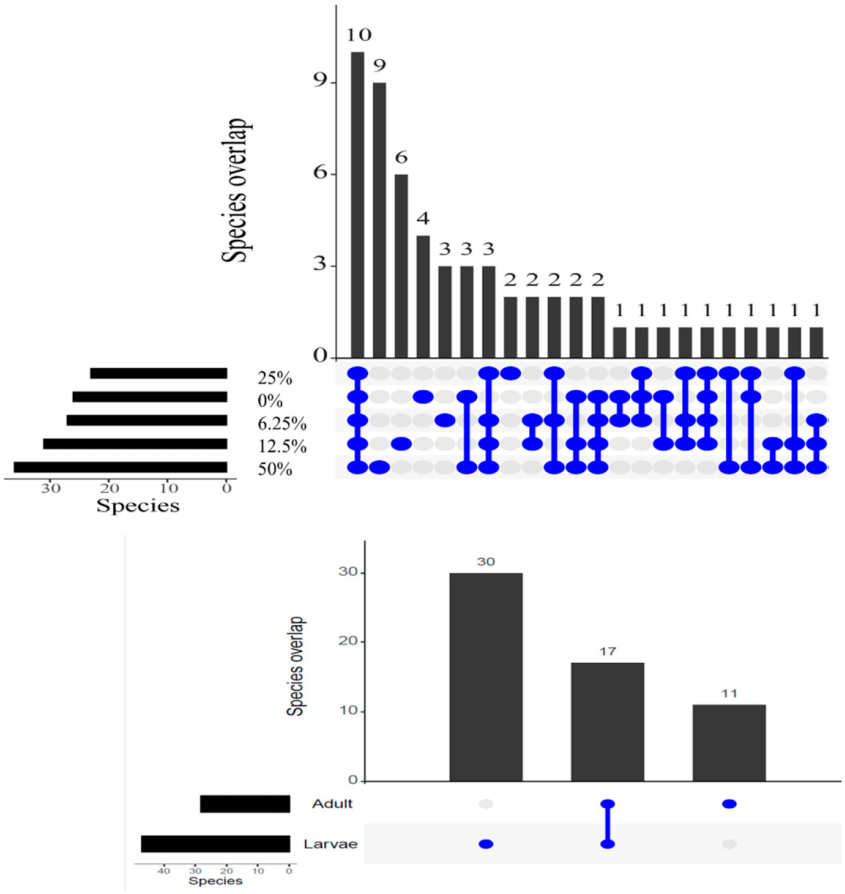

Figure 5. Overlapping gut microbiota in *An. merus* reared in varying larval salt concentrations. (**A**) Overlapping species in adults originating from larval different treatments. (**B**) Overlapping species in adults and larvae. Individual dots indicate numbers of individual species. Joined dots indicate numbers of shared species between the relevant concentrations.

When looking at life stages, larvae were always more diverse. This was true for unique families (17:5, with 13 shared), genera, and species (30:11, with 17 shared).

3.3.3. Relative Abundance

In order to understand which species are relevant in each treatment, it is important to understand which species predominate in each treatment. This can be achieved by calculating the relative abundance of species in each treatment. In this analysis, the species abundant in each treatment were compared (either positively or negatively) to the 50% seawater treatment, as this is the concentration that the strain is bred in routinely. The species found are summarised in Figure 6.

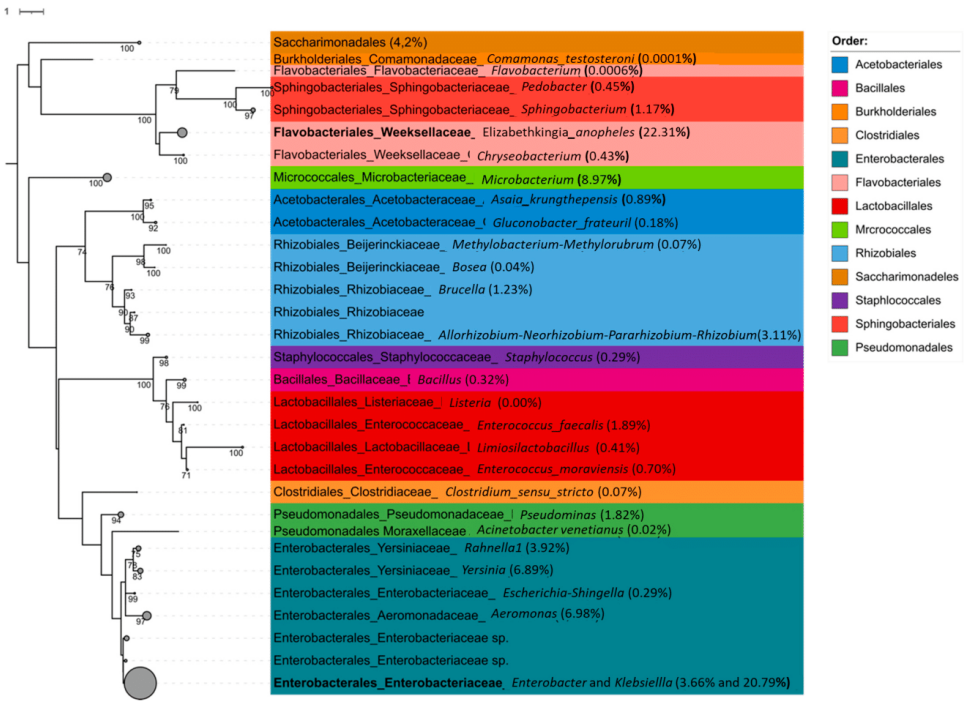

Figure 6. Phylogenetic tree of 16S rRNA sequences from *Anopheles merus* adults and larvae from different salt concentrations. Using the alignment of classified OTU sequences, the maximum likelihood tree was constructed using RAxML with the GTRGAMMAIX model and 1000 bootstraps. Taxa under the same genus were collapsed and their overall OTU abundance percentages are provided within the parentheses.

The phyla most represented in larvae are Acinetobacteriota, Patescibacteria, Firmicutes, Bacteroidota, and (the most dominant) Proteobacteria. The 50% treatment was used as a baseline, as this is the standard concentration the strain is reared in. For the larvae, the 50% treatment generally had more genera differentially expressed. This was true when compared to the 0% (12 genera vs. 18), 6.25% (10 genera vs. 15), and 25% treatments (12 genera vs. 14). The exception was the 12.5% treatment, in which the treatment had 16 genera represented, compared to 13 in the baseline (See Supplementary Figure S4).

This is even more marked in adults. When comparing the 50% treatment to the 0% treatment, the former had twelve genera more abundantly represented, compared to the four in the 0% group (Figure 7A). For the 50% and 6.25% treatments, there were fourteen genera compared to seven, respectively (Figure 7B). For the 50% and 12.5% treatments, there were eleven genera compared to five, respectively (Figure 7C). Finally, for the 50% compared to 25% treatment, there were twelve genera compared to three (Figure 7D). All the genera considered abundantly represented were significant an alpha value of 0.01.

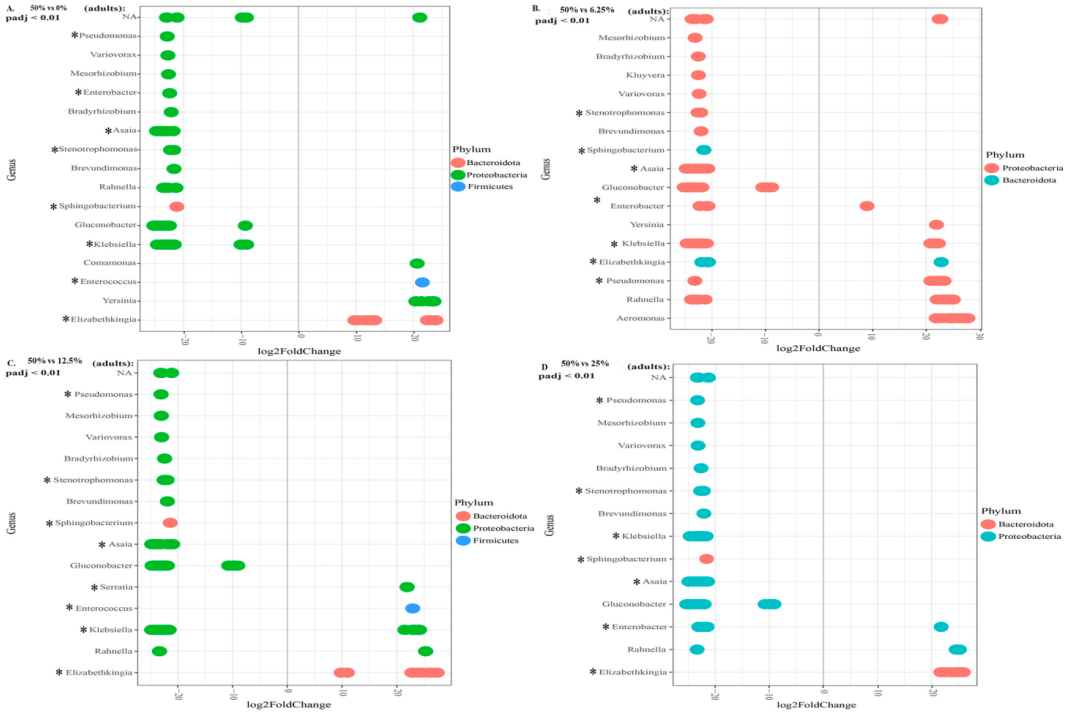

Figure 7. Differential abundance of microbiota in treatments compared to a 50% seawater treatment. Genera to the left of 0 are abundant in the 50% treatment, while genera to the right of 0 are abundant in the comparative concentration. (**A**) Adult 50% compared to 0% treatment. (**B**) Adult 50% compared to 6.25% treatment. (**C**) Adult 50% compared to 12.5% treatment. (**D**) Adult 50% compared to 25% treatment. Genera with asterisks have been associated with protection against *Plasmodium*.

4. Discussion

Despite *An. merus*' status as a secondary vector species, there is remarkably little information on this species. It is worth noting that this study is based on a laboratory strain of *An. merus*, which is not a commonly colonised strain. In White et al. [14], one of the few comprehensive studies on salinity tolerance in the species, the MAF strain was used. This strain (MRA-1156) originated in the same region as the MAFUS strain but is routinely reared in 17.56 g/L rather than 15.85 g/L. This suggests that the findings in MAFUS may be similar to that of MAF.

The two higher concentrations of salt resulted in slower time-to-pupation. This reduced rate of development has been seen when *An. arabiensis* was exposed to metal salts [24]. Although this can be attributed to the toxicity of the heavy metal salts, high salt concentrations are toxic to *An. merus* as well. As *An. merus* coped better with salt exposure under chronic rather than acute exposure, this suggests that osmotic stress is a challenge that this euryhaline species needs to adapt to [14]. However, the lack of effects on longevity suggests that although challenging, exposure to high salt concentrations is not as toxic as exposure to metals or herbicides [24,25], but also not as much of an advantage as exposure to fertiliser, which increased longevity [23,43]. Although, to the best of the authors' knowledge, there is no record of a similar study in mosquitoes, there is a striking similarity in the response to sodium chloride by the Fall armyworm, *Spodoptera frugiperda*.

Namely, the salt exposure increased larval development but did not affect adult viability or reproductive capacity in this species either [56].

There are very few studies on the effects of larval sodium chloride exposure on mosquitoes. Typically, the research has tended to be focussed on *Aedes* species, as this genus has more saline-tolerant species. Furthermore, where saline effects were examined in *Anopheles* mosquitoes, usually *An. merus*, it tended to be focussed on the effects on the larvae. This study, therefore, adds to a very small body examining the effect of larval salt concentration on adult life history. A study of the effect of salt concentrations on Aedes albopictus found that oviposition decreased with increasing salt concentration [39]. This was not examined in this study, as all larvae were allowed to oviposit in fresh water, which the aforementioned study suggests would have resulted in the greatest number of eggs produced. In the Guo et al. [39] study, wild *Ae. albopictus* demonstrated a marked pattern of reduced hatching beyond 3% salt concentration, while hatching from 0 to 2% salt was relatively consistent. Although the concentrations tolerated by this An. merus strain were substantially higher, the hatch percentage peaked at 25% salt and decreased with higher concentrations. The increased number of eggs laid by the offspring exposed to higher concentrations of salt is a reflection of the laboratory strain being selected for high saltwater tolerance. This is therefore unlikely to be reflective of patterns in wild *An. merus* breeding in fresh water.

The effect of high salt concentration on insecticide tolerance is not unprecedented. Various larval exposures to stressors in the environment have increased insecticide tolerance [23,43]. A previous study also indicated the upregulation of various cytochrome P450 genes in *An. merus* in response to osmotic stress [15]. This is congruent with an increased tolerance to pyrethroids [57–60]. What is notable is that this effect is dose dependent, and that a high salt concentration is needed to alter insecticide tolerance in the strain. Therefore, this is likely only to be of epidemiological concern under exceptional circumstances.

In plants, defensins play a role in defence against osmotic stress [18]. This was the motivation for examining the effect of larval salt exposure on the expression of this antimicrobial peptide. There was a marked effect of the salt exposure on defensin expression, with all treatments resulting in a significant decrease in expression. This is not unprecedented. An examination of the effect of larval salt concentration on the transcriptome indicated that although several immune-related genes in *An. merus* were upregulated, the majority were downregulated in a dose-dependent manner [15]. Therefore, defensin-1 does not contribute to osmotolerance in this strain, but it does suggest that larval salt exposure would modulate the immune system, usually by reducing the expression of immune-related genes.

The effect of larval salt exposure on gut microbiota is largely unexamined. There are few studies on the species in general, and even fewer on the subject of the microbiota of this species. To the knowledge of the authors, there are two descriptions of microbiota in *An. merus* [61,62].

When examining α-diversity, variable larval salt concentrations resulted in a differential response in species richness and evenness. Species richness was not significantly affected by the treatments, but there were effects on species evenness. The most consistent differences were between low salt concentrations (0% and 6.25%) and the 50% treatment. Notably the diversity between larvae and adults were more similar in the high concentration than in the low concentrations. The lack of difference in species richness is not unexpected because the diversity was being compared in the same strain. Therefore, the intrinsic number of species does not differ, but the larval salt concentration does affect the distribution of those species.

There is a clear difference in β-diversity of the larvae reared in different salt concentrations and the adults that emerge from them. The marked reduction in β-diversity in adults is confirmed by the lower number of unique species in adults compared to larvae. This is due to the reduction in diversity associated with the renewal of the gut epithelia between pupae and adults [34]. However, examining β-diversity shows that not only is there a reduction in diversity from larvae to adult, but also that the diversity of the adults

overlap, while that of the larvae does not (except for 50 and 100%). This suggests that the wide bacterial diversity in larvae may be associated with their surviving in the salty environment, and that the bacterial composition of the gut may play a role in this ability.

It is notable that the gut microbiome is not dominated by strong halophilic bacteria such as *Desulfovibrio*, *Halorubrum*, or *Natronomonas*. This does not mean that microbiota do not play a role in salt tolerance. The relevant halophiles may be concentrated in a different organ. The rectum and malphigian tubules play a more significant role in salt tolerance [14,63], and therefore, more halophilic bacteria may be concentrated there. However, *Klebsiella*, *Pseudomonas*, and *Ochrobacterum* are genera that have been found to be salt tolerant in plants [64]. It is therefore notable that when comparing the 50% seawater treatment to the 0% and 6.25% treatments, the high concentration had a significantly increased abundance of *Ochrobactrum*. By contrast, *Ochrobactrum* is not present in adults, nor was it represented in larvae reared in water without salt. This differed from the ubiquitous presence of *Klebsiella* or *Pseudomonas*. This suggests a role for *Ochrobactrum* in tolerating salt in *An. merus* larvae.

Despite the variability in gut microbiota due to salt treatment, there were 10 genera consistently represented in all treatments. *Bosea*, *Elizabethkingia*, *Enterobacter*, *Enterococcus*, *Klebsiella*, *Microbacterium*, *Rahnella*, *Sphingobacterium*, *Enterobacteriaceae*, and representatives of the *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* group were present in all treatments. The latter represents *Agrobacterium* genera with a moderate salt tolerance [65]. Although not as highly overabundant in high salt treatment as *Ochrobacterum*, it is worth noting that they are not represented in adults, possibly representing another group of salt tolerant bacteria contributing to the osmotic tolerance of *An. merus*.

The ubiquitously representative genera, with the exception of *Bosea*, are represented in the guts of various other anopheline species (see, e.g., [66–68]). This adds to the body of evidence suggesting the existence of a core microbiome in the genus. *Elizabethkingia*, *Enterobacter*, *Enterococcus*, and *Klebsiella* have all been associated with various aspects of life history [69], potentially making them paratransgenesis candidates. It is also worth noting that *Rahnella* has been represented substantially in recent studies [35,62,70]. The role of this genera in life history is, however, poorly understood.

There is a range of bacterial genera associated with protection against *Plasmodium* species (see [31,32,71–73]). It can be expected that this is introduced to the mosquito gut during the aquatic life stage. This can be seen in this study where genera such as *Elizabethkingia*, *Pseudomonas*, and *Enterobacter* are present in the larvae. The variety of these *Plasmodium*-protective genera increases in adults. This suggests a trade-off between bacterial genera that may play a role in osmotolerance in the larvae, and genera that may play a role in *Plasmodium* modulation in the adult. It has been hypothesised that the change in diversity between larvae and adults may either be due to selection of particular species between pupation and emergence, or that some species are capable of colonising the newly emerged adult more rapidly [74]. The shift from genera that may assist surviving larval osmotic stress to genera associated with *Plasmodium* modulation suggests that the change is due to the change in mosquito biology rather than improved opportunistic colonisation.

There are various examples of increased bacterial diversity in the 50% seawater treatment. This is particularly notable in the relative abundance data, where adults from the 50% seawater treatment have significantly more genera represented. Notably, the number of *Plasmodium*-protective genera in these adults is far higher than in all the lower-salt counterparts. This may be a protective mechanism to compensate for the reduced immune function associated with breeding in highly saline waters [15].

In a previous study, the microbiota of the MAFUS strain used in this study were compared to those of laboratory *An. arabiensis* strains. It was demonstrated that this *An. merus* strain as well as an *Anopheles quadriannulatus* strain had significantly different bacterial diversity than the major vector *An. arabiensis*. Furthermore, it also had more *Plasmodium*-protective genera than the major vector [62]. This finding suggested a potential role for gut microbiota in vector competence. The findings of this study reinforced the findings of

Singh et al., (2022), as similar patterns of microbiota were found. The combination of these findings along with the reduced immune gene expression (both in this study and in [15]) suggests that the osmotolerant capacity of *An. merus* may regulate immune function in this species and as such, potentially alter vector competence.

It is worth considering the real-life implications of this study. Although the strain is routinely reared in 17.5 g of salt, this trait is not fixed, and the tolerance can be reversed with prolonged breeding in fresh water (unpublished data). It is therefore not surprising that the highest salt concentration generally resulted in the greatest fitness advantage, with the notable exception of defensin expression. This does not necessarily mean that high salt concentrations in the field will result in greater fitness. The most likely scenario is that a change in the salinity between generations may result in decreased fitness unless a generational selection procedure is possible. What is important is that the larval saline exposure can alter the life history of this experience, and this makes it a consideration for surveillance where *An. merus* can be a potential vector. To fully understand the significance, particularly at the local level, the study would have to be repeated with wild local mosquitoes. The most practical suggestion arising from this study, therefore, is that the salinity of larval breeding sites where *An. merus* is found must be considered in surveillance exercises.

There are several limitations to this study. The most important is that this study was performed on a laboratory strain of *An. merus*. While some life history traits are comparable in wild and laboratory-reared strains, immunity is not one of them [75]. While the laboratory study may provide initial information of the microbial metagenomics, they will be an underrepresentation due to the reduced bacterial diversity in wild populations [76]. Additionally, this study used mRNA expression of a single antimicrobial peptide to examine the role of these peptides in osmotolerance and potentially immunity. To properly disentangle this effect, this must be examined at the protein level, as well as including more AMPs and other immune effectors, ideally from wild or F1 *An. merus*. Finally, it is important to note that although the study highlights statistical differences, which do not necessarily translate into functional effects in the field. This highlights the importance of incorporating the examination of larval environment, particularly where *An. merus* is present. The definitive way to determine whether our findings are of epidemiological significance is by confirmation from surveillance activities.

5. Conclusions

In conclusion, larval exposure to different salt concentrations alters the life history traits and gut microbiota of *An. merus*. High salt concentrations reduced the development time of the larvae but did not have a significant effect on adult longevity, fertility, or fecundity. Exposure to high salt concentrations increased deltamethrin tolerance and all larval salt exposure reduced the expression of *defensin-1*. Although there was a core microbiome, larval salt exposure altered the gut microbiota of both the larvae and the adults. The greater the difference in larval salt exposure, the greater the difference in comparative microbiota. Notably, the higher salt concentration had greater bacterial diversity as well as more bacterial genera associated with protection against *Plasmodium* parasites. As such, it is possible that the salinity of the breeding water of *An. merus* larvae may have the capacity to modulate the life history of this species in an epidemiologically significant manner. These findings suggest that the salinity of larval breeding water should be considered in surveillance efforts where *An. merus* is present

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/insects13121165/s1, Figure S1: Bar plot of all specimens. Figure S2: rarefaction curves of larval specimens. Figure S3: rarefaction curves of adult specimens. Figure S4: Differential abundance in larvae. Table S1: Operational Taxonomic Unit (OTU) table of sequencing data; Table S2: Summary of metagenomic data as per the Mosquito Microbiome Consortium. Table S3: Primers used in this study. Table S4: Deltamethrin lethal times in the MAFUS strain.

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References

1. Dobson, T. Seaweed flies (Diptera: Coleopidae). In *Marine Insects*; Cheng, L., Ed.; North Holland Publishers: Amsterdam, The Netherlands, 1976.
2. Bradley, T.J. Physiology of osmoregulation in mosquitoes. *Annu. Rev. Entomol.* **1987**, *32*, 439–462. [CrossRef] [PubMed]
3. O'Meara, G.F. Saltmarsh mosquitoes (Diptera: Culicidae). In *Marine Insects*; Cheng, L., Ed.; North Holland Publishing Company: Amsterdam, The Netherlands, 1976; pp. 300–330.
4. Bradley, T.J. Saline-water insects: Ecology, physiology and evolution. In *Aquatic Insects: Challenges to Populations*; Lancaster, J., Briers, R.A., Eds.; CAB International: Wallingford, UK, 2008; pp. 20–35.
5. Kengne, P.; Charmantier, G.; Blondeau-Bidet, E.; Costantini, C.; Ayala, D. Tolerance of disease-vector mosquitoes to brackish water and their osmoregulatory ability. *Ecosphere* **2019**, *10*, e02783. [CrossRef]
6. Blagrove, M.S.; Sherlock, K.; Chapman, G.E.; Impoinvil, D.E.; McCall, P.J.; Medlock, J.M.; Lycett, G.; Solomon, T.; Baylis, M. Evaluation of the vector competence of a native UK mosquito Ochlerotatus detritus (Aedes detritus) for dengue, chikungunya and West Nile viruses. *Parasites Vectors* **2016**, *9*, 452. [CrossRef]
7. Ramasamy, R.; Surendran, S.N. Global climate change and its potential impact on disease transmission by salinity-tolerant mosquito vectors in coastal zones. *Front. Physiol.* **2012**, *3*, 198. [CrossRef]
8. Surendran, S.N.; Jude, P.J.; Ramasamy, R. Variations in salinity tolerance of malaria vectors of the Anopheles subpictus complex in Sri Lanka and the implications for malaria transmission. *Parasites Vectors* **2011**, *4*, 117. [CrossRef] [PubMed]
9. Ould Lemrabott, M.A.; Le Goff, G.; Kengne, P.; Ndiaye, O.; Costantini, C.; Mint Lekweiry, K.; Ould Ahmedou Salem, M.S.; Robert, V.; Basco, L.; Simard, F.; et al. First report of Anopheles (Cellia) multicolor during a study of tolerance to salinity of Anopheles arabiensis larvae in Nouakchott, Mauritania. *Parasites Vectors* **2020**, *13*, 522. [CrossRef]
10. 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.; et al. The dominant Anopheles vectors of human malaria in Africa, Europe and the Middle East: Occurrence data, distribution maps and bionomic précis. *Parasites Vectors* **2010**, *3*, 117. [CrossRef]
11. Bartilol, B.; Omedo, I.; Mbogo, C.; Mwangangi, J.; Rono, M.K. Bionomics and ecology of Anopheles merus along the East and Southern Africa coast. *Parasites Vectors* **2021**, *14*, 84. [CrossRef]
12. Braack, L.; Bornman, R.; Kruger, T.; Dahan-Moss, Y.; Gilbert, A.; Kaiser, M.; Oliver, S.V.; Cornel, A.J.; Lee, Y.; Norris, D.E.; et al. Malaria Vectors and Vector Surveillance in Limpopo Province (South Africa): 1927 to 2018. *Int. J. Environ. Res. Public Health* **2020**, *17*, 4125. [CrossRef]

13. Mbokazi, F.; Coetzee, M.; Brooke, B.; Govere, J.; Reid, A.; Owiti, P.; Kosgei, R.; Zhou, S.; Magagula, R.; Kok, G.; et al. Changing distribution and abundance of the malaria vector Anopheles merus in Mpumalanga Province, South Africa. *Public Health Action* **2018**, *8*, S39–S43. [CrossRef]
14. White, B.J.; Kundert, P.N.; Turissini, D.A.; Van Ekeris, L.; Linser, P.J.; Besansky, N.J. Dose and developmental responses of Anopheles merus larvae to salinity. *J. Exp. Biol.* **2013**, *216*, 3433–3441. [CrossRef] [PubMed]
15. Uyhelji, H.A.; Cheng, C.; Besansky, N.J. Transcriptomic differences between euryhaline and stenohaline malaria vector sibling species in response to salinity stress. *Mol. Ecol.* **2016**, *25*, 2210–2225. [CrossRef] [PubMed]
16. Vizioli, J.; Richman, A.M.; Uttenweiler-Joseph, S.; Blass, C.; Bulet, P. The defensin peptide of the malaria vector mosquito Anopheles gambiae: Antimicrobial activities and expression in adult mosquitoes. *Insect Biochem. Mol. Biol.* **2001**, *31*, 241–248. [CrossRef] [PubMed]
17. Richman, A.M.; Dimopoulos, G.; Seeley, D.; Kafatos, F.C. Plasmodium activates the innate immune response of Anopheles gambiae mosquitoes. *EMBO J.* **1997**, *16*, 6114–6119. [CrossRef]
18. Kido, E.A.; Pandolfi, V.; Houllou-Kido, L.M.; Andrade, P.P.; Marcelino, F.C.; Nepomuceno, A.L.; Abdelnoor, R.V.; Burnquist, W.L.; Benko-Iseppon, A.M. Plant antimicrobial peptides: An overview of SuperSAGE transcriptional profile and a functional review. *Curr. Protein Pept. Sci.* **2010**, *11*, 220–230. [CrossRef] [PubMed]
19. Barreaux, A.M.G.; Stone, C.M.; Barreaux, P.; Koella, J.C. The relationship between size and longevity of the malaria vector Anopheles gambiae (s.s.) depends on the larval environment. *Parasites Vectors* **2018**, *11*, 485. [CrossRef]
20. Jannat, K.N.; Roitberg, B.D. Effects of larval density and feeding rates on larval life history traits in Anopheles gambiae s.s. (Diptera: Culicidae). *J. Vector Ecol. J. Soc. Vector Ecol.* **2013**, *38*, 120–126. [CrossRef]
21. Moller-Jacobs, L.L.; Murdock, C.C.; Thomas, M.B. Capacity of mosquitoes to transmit malaria depends on larval environment. *Parasites Vectors* **2014**, *7*, 593. [CrossRef]
22. Okech, B.A.; Gouagna, L.C.; Yan, G.; Githure, J.I.; Beier, J.C. Larval habitats of Anopheles gambiae s.s. (Diptera: Culicidae) influences vector competence to Plasmodium falciparum parasites. *Malar. J.* **2007**, *6*, 50. [CrossRef]
23. Jeanrenaud, A.; Brooke, B.D.; Oliver, S.V. The effects of larval organic fertiliser exposure on the larval development, adult longevity and insecticide tolerance of zoophilic members of the Anopheles gambiae complex (Diptera: Culicidae). *PLoS ONE* **2019**, *14*, e0215552. [CrossRef]
24. Oliver, S.V.; Brooke, B.D. The effect of metal pollution on the life history and insecticide resistance phenotype of the major malaria vector Anopheles arabiensis (Diptera: Culicidae). *PLoS ONE* **2018**, *13*, e0192551. [CrossRef]
25. Oliver, S.V.; Brooke, B.D. The effect of commercial herbicide exposure on the life history and insecticide resistance phenotypes of the major malaria vector Anopheles arabiensis (Diptera: Culicidae). *Acta Trop.* **2018**, *188*, 152–160. [CrossRef] [PubMed]
26. Coon, K.L.; Valzania, L.; Brown, M.R.; Strand, M.R. Predaceous Toxorhynchites mosquitoes require a living gut microbiota to develop. *Proc. Biol. Sci.* **2020**, *287*, 20192705. [CrossRef] [PubMed]
27. Coon, K.L.; Vogel, K.J.; Brown, M.R.; Strand, M.R. Mosquitoes rely on their gut microbiota for development. *Mol. Ecol.* **2014**, *23*, 2727–2739. [CrossRef] [PubMed]
28. Coon, K.L.; Brown, M.R.; Strand, M.R. Gut bacteria differentially affect egg production in the anautogenous mosquito Aedes aegypti and facultatively autogenous mosquito Aedes atropalpus (Diptera: Culicidae). *Parasites Vectors* **2016**, *9*, 375. [CrossRef]
29. Coon, K.L.; Hegde, S.; Hughes, G.L. Interspecies microbiome transplantation recapitulates microbial acquisition in mosquitoes. *Microbiome* **2022**, *10*, 58. [CrossRef]
30. Bai, L.; Wang, L.; Vega-Rodríguez, J.; Wang, G.; Wang, S. A Gut Symbiotic Bacterium Serratia marcescens Renders Mosquito Resistance to Plasmodium Infection through Activation of Mosquito Immune Responses. *Front. Microbiol.* **2019**, *10*, 1580. [CrossRef]
31. 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.; et al. Midgut microbiota of the malaria mosquito vector Anopheles gambiae and interactions with Plasmodium falciparum infection. *PLoS Pathog.* **2012**, *8*, e1002742. [CrossRef]
32. Yilmaz, B.; Portugal, S.; Tran, T.M.; Gozzelino, R.; Ramos, S.; Gomes, J.; Regalado, A.; Cowan, P.J.; d'Apice, A.J.; Chong, A.S.; et al. Gut microbiota elicits a protective immune response against malaria transmission. *Cell* **2014**, *159*, 1277–1289. [CrossRef]
33. Tchioffo, M.T.; Boissière, A.; Churcher, T.S.; Abate, L.; Gimonneau, G.; Nsango, S.E.; Awono-Ambéné, P.H.; Christen, R.; Berry, A.; Morlais, I. Modulation of malaria infection in Anopheles gambiae mosquitoes exposed to natural midgut bacteria. *PLoS ONE* **2013**, *8*, e81663. [CrossRef]
34. Alfano, N.; Tagliapietra, V.; Rosso, F.; Manica, M.; Arnoldi, D.; Pindo, M.; Rizzoli, A. Changes in Microbiota Across Developmental Stages of Aedes koreicus, an Invasive Mosquito Vector in Europe: Indications for Microbiota-Based Control Strategies. *Front. Microbiol.* **2019**, *10*, 2832. [CrossRef] [PubMed]
35. Dinparast Djadid, N.; Jazayeri, H.; Raz, A.; Favia, G.; Ricci, I.; Zakeri, S. Identification of the midgut microbiota of *An. stephensi* and *An. maculipennis* for their application as a paratransgenic tool against malaria. *PLoS ONE* **2011**, *6*, e28484. [CrossRef] [PubMed]
36. Duguma, D.; Hall, M.W.; Smartt, C.T.; Neufeld, J.D. Effects of Organic Amendments on Microbiota Associated with the Culex nigripalpus Mosquito Vector of the Saint Louis Encephalitis and West Nile Viruses. *Msphere* **2017**, *2*, e00387-16. [CrossRef] [PubMed]

37. Krajacich, B.J.; Huestis, D.L.; Dao, A.; Yaro, A.S.; Diallo, M.; Krishna, A.; Xu, J.; Lehmann, T. Investigation of the seasonal microbiome of Anopheles coluzzii mosquitoes in Mali. *PLoS ONE* **2018**, *13*, e0194899. [CrossRef] [PubMed]
38. Louie, W.; Coffey, L.L. Microbial Composition in Larval Water Enhances Aedes aegypti Development but Reduces Transmissibility of Zika Virus. *Msphere* **2021**, *6*, e0068721. [CrossRef] [PubMed]
39. Guo, X.; Zhou, S.; Wu, J.; Zhang, X.; Wang, Y.; Li, Z.; Chen, X.G.; Zhou, X. An Experimental Evaluation of Toxicity Effects of Sodium Chloride on Oviposition, Hatching and Larval Development of Aedes albopictus. *Pathogens* **2022**, *11*, 262. [CrossRef]
40. Hunt, R.H.; Brooke, B.D.; Pillay, C.; Koekemoer, L.L.; Coetzee, M. Laboratory selection for and characteristics of pyrethroid resistance in the malaria vector Anopheles funestus. *Med. Vet. Entomol.* **2005**, *19*, 271–275. [CrossRef]
41. Kaplan, E.L.; Meier, P. Nonparametric Estimation from Incomplete Observations. *J. Am. Stat. Assoc.* **1958**, *53*, 457–481. [CrossRef]
42. Harrington, D.P.; Fleming, T.R. A class of rank test procedures for censored survival data. *Biometrika* **1982**, *69*, 553–566. [CrossRef]
43. Samuel, M.; Brooke, B.D.; Oliver, S.V. Effects of inorganic fertilizer on larval development, adult longevity and insecticide susceptibility in the malaria vector Anopheles arabiensis (Diptera: Culicidae). *Pest Manag. Sci.* **2020**, *76*, 1560–1568. [CrossRef]
44. Finney, D.J. *Probit Analysis*, 2nd ed.; Cambridge University Press: New York, NY, USA, 1952.
45. Shapiro, S.S.; Wilk, M.B. An Analysis of Variance Test for Normality (Complete Samples). *Biometrika* **1965**, *52*, 591–611. [CrossRef]
46. Tukey, J.W. Comparing individual means in the analysis of variance. *Biometrics* **1949**, *5*, 99–114. [CrossRef] [PubMed]
47. Pfaffl, M.W.; Horgan, G.W.; Dempfle, L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res.* **2002**, *30*, e36. [CrossRef] [PubMed]
48. Klindworth, A.; Pruesse, E.; Schweer, T.; Peplies, J.; Quast, C.; Horn, M.; Glöckner, F.O. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* **2013**, *41*, e1. [CrossRef] [PubMed]
49. Ondov, B.D.; Bergman, N.H.; Phillippy, A.M. Interactive metagenomic visualization in a Web browser. *BMC Bioinform.* **2011**, *12*, 385. [CrossRef]
50. Wood, D.E.; Lu, J.; Langmead, B. Improved metagenomic analysis with Kraken 2. *Genome Biol.* **2019**, *20*, 257. [CrossRef]
51. Callahan, B.J.; McMurdie, P.J.; Rosen, M.J.; Han, A.W.; Johnson, A.J.; Holmes, S.P. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* **2016**, *13*, 581–583. [CrossRef]
52. McMurdie, P.J.; Holmes, S. phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE* **2013**, *8*, e61217. [CrossRef]
53. Andersen, K.S.; Kirkegaard, R.H.; Karst, S.M.; Albertsen, M.J.B. ampvis2: An R package to analyse and visualise 16S rRNA amplicon data. *BioRxiv* **2018**, 299537.
54. Conway, J.R.; Lex, A.; Gehlenborg, N. UpSetR: An R package for the visualization of intersecting sets and their properties. *Bioinformatics* **2017**, *33*, 2938–2940. [CrossRef]
55. Love, M.I.; Huber, W.; Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **2014**, *15*, 550. [CrossRef] [PubMed]
56. dos Santos Albuquerque, F.M.; Freitas, E.C.B.F.; do Nascimento, D.V.; de Almeida, W.A.; Nova, I.C.V.; Paiva, P.M.G.; Napoleão, T.H.; Barros, R.; Pontual, E.V.J.R. Sodium chloride reduces the viability of eggs, larvae, and pupae of Spodoptera frugiperda, as well as delays its development. *Res. Soc. Dev.* **2022**, *11*, e29311427232. [CrossRef]
57. Ibrahim, S.S.; Riveron, J.M.; Stott, R.; Irving, H.; Wondji, C.S. The cytochrome P450 CYP6P4 is responsible for the high pyrethroid resistance in knockdown resistance-free Anopheles arabiensis. *Insect Biochem. Mol. Biol.* **2016**, *68*, 23–32. [CrossRef] [PubMed]
58. Moyes, C.L.; Lees, R.S.; Yunta, C.; Walker, K.J.; Hemmings, K.; Oladepo, F.; Hancock, P.A.; Weetman, D.; Paine, M.J.I.; Ismail, H.M. Assessing cross-resistance within the pyrethroids in terms of their interactions with key cytochrome P450 enzymes and resistance in vector populations. *Parasites Vectors* **2021**, *14*, 115. [CrossRef] [PubMed]
59. Weedall, G.D.; Mugenzi, L.M.J.; Menze, B.D.; Tchouakui, M.; Ibrahim, S.S.; Amvongo-Adjia, N.; Irving, H.; Wondji, M.J.; Tchoupo, M.; Djouaka, R.; et al. A cytochrome P450 allele confers pyrethroid resistance on a major African malaria vector, reducing insecticide-treated bednet efficacy. *Sci. Transl. Med.* **2019**, *11*, eaat7386. [CrossRef]
60. Wondji, C.S.; Hearn, J.; Irving, H.; Wondji, M.J.; Weedall, G. RNAseq-based gene expression profiling of the Anopheles funestus pyrethroid-resistant strain FUMOZ highlights the predominant role of the duplicated CYP6P9a/b cytochrome P450s. *G3* **2022**, *12*, jkab352. [CrossRef] [PubMed]
61. Mancini, M.V.; Damiani, C.; Accoti, A.; Tallarita, M.; Nunzi, E.; Cappelli, A.; Bozic, J.; Catanzani, R.; Rossi, P.; Valzano, M.; et al. Estimating bacteria diversity in different organs of nine species of mosquito by next generation sequencing. *BMC Microbiol.* **2018**, *18*, 126. [CrossRef]
62. Singh, A.; Allam, M.; Kwenda, S.; Khumalo, Z.T.H.; Ismail, A.; Oliver, S.V. The dynamic gut microbiota of zoophilic members of the Anopheles gambiae complex (Diptera: Culicidae). *Sci. Rep.* **2022**, *12*, 1495. [CrossRef]
63. Xiang, M.A.; Linser, P.J.; Price, D.A.; Harvey, W.R. Localization of two Na+- or K+-H+ antiporters, AgNHA1 and AgNHA2, in Anopheles gambiae larval Malpighian tubules and the functional expression of AgNHA2 in yeast. *J. Insect Physiol.* **2012**, *58*, 570–579. [CrossRef]
64. Egamberdieva, D.; Wirth, S.; Bellingrath-Kimura, S.D.; Mishra, J.; Arora, N.K. Salt-Tolerant Plant Growth Promoting Rhizobacteria for Enhancing Crop Productivity of Saline Soils. *Front. Microbiol.* **2019**, *10*, 2791. [CrossRef]

65. Zheng, Y.; Xu, Z.; Liu, H.; Liu, Y.; Zhou, Y.; Meng, C.; Ma, S.; Xie, Z.; Li, Y.; Zhang, C.S. Patterns in the Microbial Community of Salt-Tolerant Plants and the Functional Genes Associated with Salt Stress Alleviation. *Microbiol. Spectr.* **2021**, *9*, e0076721. [CrossRef] [PubMed]
66. Dada, N.; Lol, J.C.; Benedict, A.C.; López, F.; Sheth, M.; Dzuris, N.; Padilla, N.; Lenhart, A. Pyrethroid exposure alters internal and cuticle surface bacterial communities in Anopheles albimanus. *ISME J.* **2019**, *13*, 2447–2464. [CrossRef] [PubMed]
67. Dickson, L.B.; Jiolle, D.; Minard, G.; Moltini-Conclois, I.; Volant, S.; Ghozlane, A.; Bouchier, C.; Ayala, D.; Paupy, C.; Moro, C.V.; et al. Carryover effects of larval exposure to different environmental bacteria drive adult trait variation in a mosquito vector. *Sci. Adv.* **2017**, *3*, e1700585. [CrossRef] [PubMed]
68. Wang, Y.; Gilbreath, T.M., 3rd; Kukutla, P.; Yan, G.; Xu, J. Dynamic gut microbiome across life history of the malaria mosquito Anopheles gambiae in Kenya. *PLoS ONE* **2011**, *6*, e24767. [CrossRef]
69. Terenius, O.; Lindh, J.M.; Eriksson-Gonzales, K.; Bussière, L.; Laugen, A.T.; Bergquist, H.; Titanji, K.; Faye, I. Midgut bacterial dynamics in Aedes aegypti. *FEMS Microbiol. Ecol.* **2012**, *80*, 556–565. [CrossRef]
70. E Silva, B.; Matsena Zingoni, Z.; Koekemoer, L.L.; Dahan-Moss, Y.L. Microbiota identified from preserved Anopheles. *Malar. J.* **2021**, *20*, 230. [CrossRef]
71. Feng, Y.; Peng, Y.; Song, X.; Wen, H.; An, Y.; Tang, H.; Wang, J. Anopheline mosquitoes are protected against parasite infection by tryptophan catabolism in gut microbiota. *Nat. Microbiol.* **2022**, *7*, 707–715. [CrossRef]
72. Romoli, O.; Gendrin, M. The tripartite interactions between the mosquito, its microbiota and Plasmodium. *Parasites Vectors* **2018**, *11*, 200. [CrossRef]
73. Sharma, P.; Rani, J.; Chauhan, C.; Kumari, S.; Tevatiya, S.; Das De, T.; Savargaonkar, D.; Pandey, K.C.; Dixit, R. Altered Gut Microbiota and Immunity Defines Plasmodium vivax Survival in Anopheles stephensi. *Front. Immunol.* **2020**, *11*, 609. [CrossRef]
74. Zouache, K.; Martin, E.; Rahola, N.; Gangue, M.F.; Minard, G.; Dubost, A.; Van, V.T.; Dickson, L.; Ayala, D.; Lambrechts, L.; et al. Larval habitat determines the bacterial and fungal microbiota of the mosquito vector Aedes aegypti. *FEMS Microbiol. Ecol.* **2022**, *98*, fiac016. [CrossRef]
75. Vézilier, J.; Nicot, A.; Lorgeril, J.; Gandon, S.; Rivero, A. The impact of insecticide resistance on Culex pipiens immunity. *Evol. Appl.* **2013**, *6*, 497–509. [CrossRef] [PubMed]
76. Minard, G.; Mavingui, P.; Moro, C.V. Diversity and function of bacterial microbiota in the mosquito holobiont. *Parasites Vectors* **2013**, *6*, 146. [CrossRef] [PubMed]

Characterisation of the dynamic gut microbiota of members of the *Anopheles gambiae* complex (Diptera: Culicidae)

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The dynamic gut microbiota of zoophilic members of the *Anopheles gambiae* complex (Diptera: Culicidae)

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The gut microbiota of mosquitoes plays a critical role in the life history of the animal. There is a growing body of research characterising the gut microbiota of a range of mosquito species, but there is still a paucity of information on some members of the *Anopheles gambiae* complex. In this study, the gut microbiota of four laboratory strains were characterised. SENN (*Anopheles arabiensis*—insecticide susceptible major vector), SENN DDT (*Anopheles arabiensis*—insecticide resistant major vector), MAFUS (*Anopheles merus*—minor vector) and SANGWE (*Anopheles quadriannulatus*—non-vector) were used in this study. The microbiota of fourth instar larvae, 3-day old, 15-day old non-blood fed and 15-day old blood fed females were characterised by MALDI-TOF mass spectroscopy and 16 s rRNA gene sequencing by next generation sequencing. The four strains differed in species richness but not diversity. The major vectors differ in β-diversity from that of the minor and non-vectors. There was no difference in α- or β-diversity in 15 non-blood fed females and 15-day old females that had 3 blood meals before day 15. These differences may be related to a mixture of the effect of insecticide resistance phenotype as well as a potential relationship to vector competence to a limited extent. Bacterial diversity is affected by species and age. There is also a potential relationship between the differences in gut microbiota and capacity to transmit parasites. This genetic background of the mosquitoes, however, play a major role, and must be considered in this relationship.

The *Anopheles gambiae* complex is a group of morphologically identical mosquitoes that differ in their behaviour and ability to transmit the malaria parasite to human hosts[1]. Three members of the *An. gambiae* complex are found in South Africa. The major malaria vector *An. arabiensis*, a minor malaria vector *An. merus* and a non-malaria vector *An. quadriannulatus* are vectors that are all found in Africa within the malaria endemic provinces of South Africa[2]. Currently, the only species implicated in malaria transmission in South Africa is *An. arabiensis*[3].

Anopheles arabiensis is one of the dominant malaria vector species in Sub-Saharan Africa[1,4]. *Anopheles arabiensis* exhibits anthropophilic (human biting), zoophilic (animal biting), endophagic (indoor feeding), exophagic (outdoor feeding), endophilic (indoor resting) and exophilic (outdoor resting) behavioural patterns[1,5,6].

The members of the *An. gambiae* complex found in South Africa differ from *An. gambiae ss* and *An. coluzzi* which commonly display exophily and exophagy. Yet, they differ markedly in their vector competence[1]. As such, behavioural differences alone cannot account for these differences. There would also be a molecular basis for these differences, particularly in terms of immunological underpinnings.

To highlight an example of the role of immunity, *An. quadriannulatus* can be artificially infected with *Plasmodium falciparum* at low efficiency[7], even though this is not usually observed in the wild. The prophenoloxidase system of *An. quadriannulatus* is initiated at lower levels than the major vector *An. gambiae*[8]. This highlights the

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importance of the immunological response to the parasite. An increased understanding of the immunological differences between vectors and non-vectors would inform on future transmission blocking efforts.

A key factor in determining vector competence is the gut microbiota of the mosquito. The microbiota of the midgut in a mosquito plays crucial roles in development, reproduction, immunity, and vector competence[9]. The composition of the midgut varies with the life cycle stage and diet[10]. The larval gut microbiome is crucial for development as gut-sterilised mosquitoes cannot pupate[10–12]. Larvae feed on the contents of the surrounding water as well as drink the surrounding water. This suggests that the diet obtained from the environment is important in the survival and replacement of new bacteria in the midgut[13,14].

For malaria to be transmitted by an *Anopheles* mosquito, an infectious blood meal must be ingested and make it through the developmental bottlenecks in the midgut and salivary glands. The midgut is the first and main bottleneck of parasite development, as this is where the parasite undergoes the sexual stages of reproduction. The success of the escape of the motile ookinetes from the midgut is crucial for successful parasite proliferation[15]. Therefore, the study of the dynamics of the midgut of the mosquito is a key field of study to develop alternative vector control strategies.

The midgut microbiota of the *Anopheles* mosquito contributes to the ability of the mosquito to contract and transmit the *Plasmodium* parasite as it is involved with the immunity of the mosquito[16,17]. The midgut microbiota protects the mosquito against *Plasmodium* infection through various pathways. The bacteria in the midgut can act as a physical barrier after a blood meal has been ingested, preventing penetration of the parasite into the midgut wall[18]. The bacteria in the midgut can directly affect the parasite by producing antimicrobial compounds or by inducing oxidative stress. In addition to these other defence mechanisms, the bacteria can activate the NF-κB dependent Immune-deficiency (imd) pathway to fight *Plasmodium* infection[15].

A search of the term "*Anopheles* microbiome" on Pubmed has increased from 3 records in 2010 to 23 in 2020. This indicates a slow but steady increase of the information in this field. Although the field of mosquito microbiome studies are advancing, the studies tend to be limited in the species studies, typically focussing on *An. stephensi*, *An. coluzzi* and *An. gambiae ss*, examples of studies includes Sharma et al.; Galeano-Castañeda et al.; Saab et al.[17,19,20]. Studies of the microbiome of *An. arabiensis* and other members of the *An. gambiae* complex are notably lacking. Characterising the gut microbiota of *Plasmodium* refractory species could provide information on the role of gut microbiota in this phenotype. This study aimed to characterise the dynamic gut microbiota of the minor vector *An. merus* and the non-vector *An. quadriannulatus*. Furthermore, this will be compared to the dynamic gut microbiome of insecticide resistant and susceptible *An. arabiensis*.

Results

Culture-dependent characterisation of the dynamic gut microbiota by MALDI-TOF MS. The species identified by MALDI-TOF MS were dominated by Gram-negative bacteria, specifically γ-proteobacteria of the family *Enterobacteriaceae* (0.41). The remaining γ-proteobacteria were from the families of *Yersiniaceae* (0.09), *Aeromonadaceae*, (0.08). *Pseudomonadaceae* (0.07), *Moraxellaceae* (0.05), and *Morganellaceae* (0.02). The next most abundant classes were the *Bacilli* (0.02) and *Actinobacteria* (0.01), which constituted the majority of the Gram-positive bacteria identified. The final significant Gram-negative class was the *Weeksellaceae* (0.08) family. The results of the MALDI-TOF study are summarised in Supplementary Table S1.

Culture-independent characterisation of the dynamic gut microbiota by 16S rRNA gene sequencing. *Alpha diversity determined by 16S sequencing.* When comparing the alpha diversity of the complete groups, the species richness indices (a measure of the total number of species in the relevant strain and treatment) Chao1 and ACE (summarised in Table 2) indicate a significant difference between the strains. Species evenness indicators (a measure of relative abundance of species in the relevant strain and treatment), Shannon and Simpson (summarised in Table 3), do not indicate a significant difference. Chao 1 indices indicated a significant difference between strains (Kruskal–Wallis One-way ANOVA: $p < 0.01$). SENN (*An. arabiensis*-insecticide susceptible) had a significantly higher index than SENN DDT (*An. arabiensis*-insecticide resistant) ($p = 0.01$), as well as SANGWE (*An. quadriannulatus*) ($p = 0.01$) and MAFUS (*An. merus*) ($p < 0.01$) (Fig. 1A). Similarly, with ACE indicators, there was a significant difference between the strains (Kruskal–Wallis One-way ANOVA: $p = 0.03$). SENN had a significantly higher ACE index than SENN-DDT ($p = 0.02$). Although SENN did not have a significantly different ACE index than MAFUS ($p = 0.05$), it did have a significantly higher ACE index than SANGWE ($p = 0.02$). MAFUS and SANGWE did not differ in ACE indices ($p = 0.13$) (Fig. 1B).

Overall, the Shannon diversity indices of the strains did not differ (Kruskal–Wallis One-way ANOVA: $p = 0.14$). SANGWE had a significantly higher Shannon diversity index than SENN ($p = 0.03$) as did MAFUS ($p = 0.02$). SENN DDT did not differ significantly from SENN ($p = 0.44$) (Fig. 1C). The Simpson's diversity index was also not significantly different (Kruskal–Wallis One-way ANOVA: $p = 0.14$). The only significant difference in Simpson's index was significantly higher indices in MAFUS than SENN DDT ($p < 0.01$) (Fig. 1D).

When comparing alpha diversity between the life stages of the strains, the Chao 1 index of the fourth instar larvae was significantly higher than that of 15-day old sugar fed adults ($p < 0.01$) and 15-day old blood fed adults ($p = 0.02$). The ACE index of the fourth instar larvae was significantly higher than that of 3-day old adults ($p = 0.02$), 15-day old non-blood fed adults ($p < 0.01$), and 15-day old blood fed adults ($p < 0.01$). The Shannon index of the larvae was significantly higher than that of 3-day old adults ($p < 0.01$), 15-day old non-blood fed adults ($p < 0.01$) and 15-day old blood fed adults ($p < 0.01$). The Simpson diversity index of the larvae was significantly higher than that of 3-day old adults ($p < 0.01$), 15-day old non-blood fed adults ($p < 0.01$) and 15-day old blood fed adults ($p < 0.01$). There was no difference in species richness between older blood fed and non-blood fed adults as Chao 1 indices did not differ significant ($p = 0.71$) neither did ACE indices differ ($p = 0.63$). This was

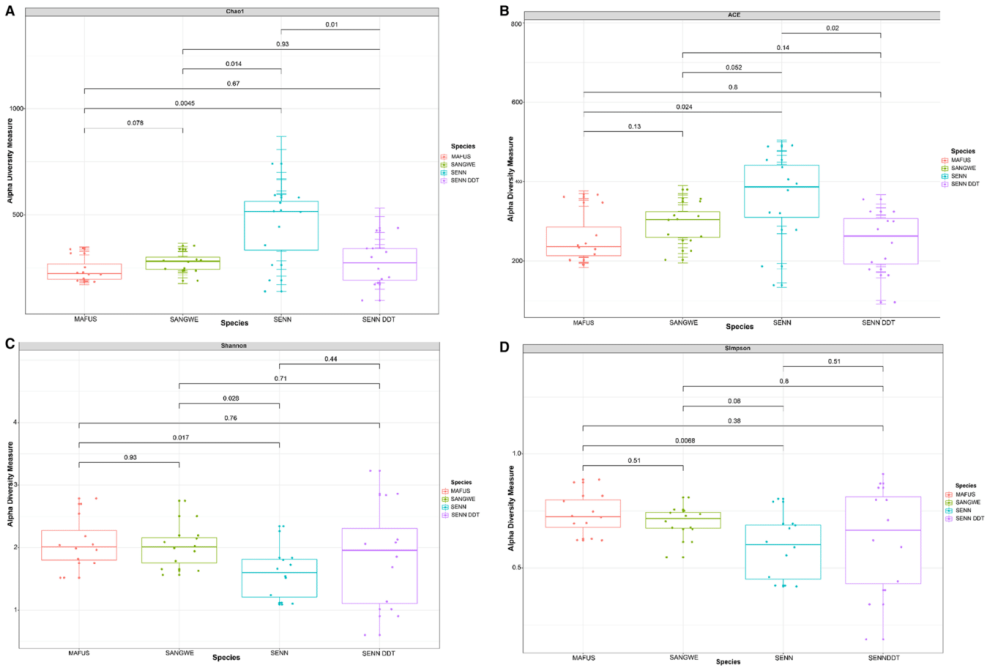

Figure 1. Comparison of alpha diversity between the zoophilic members of the *An. gambiae* complex. (**A**) Chao1 index. (**B**) Abundance-based Coverage Estimator (ACE) index. (**C**) Shannon diversity index. (**D**) Simpson diversity index.

also true for species evenness, as there were no significant differences in Shannon diversity indices ($p = 0.41$) or Simpson diversity indices ($p = 0.93$) (Supplementary Table S2).

Within the strains, only MAFUS had a significant difference within Chao1 index between the life stages (Kruskal–Wallis One-way ANOVA: $p = 0.03$). There was no difference in ACE indices between the life stages of SENN ($p = 0.31$), SENN DDT ($p = 0.44$), MAFUS ($p = 0.05$) and SANGWE ($p = 0.07$). When examining indices examining evenness rather than the richness, there was a significant difference in Shannon diversity indices between life stages in SANGWE ($p = 0.03$) as well as MAFUS ($p = 0.03$), but not in SENN ($p = 0.09$) or SENN DDT ($p = 0.08$). When examining Simpson diversity indices, only SENN had a significant difference in indices between life stages ($p = 0.04$) (Supplementary Table S2). The variation in the findings is confirmed by rarefaction analysis, with larval diversity being the greatest in all samples. This analysis also confirms the depth of sequencing (Fig. 2A). Alpha diversities indices are summarised in Tables 1 and 2.

Beta diversity analysis determined by 16S sequencing. Two β-diversity analyses were performed, and both confirmed the same patterns. NMDS analysis demonstrated that MAFUS (*An. merus*) and SANGWE (*An. quadriannulatus*) clustered together and were significantly different from SENN (*An. arabiensis*-insecticide susceptible) and SENN DDT (*An. arabiensis*-insecticide resistant) (PERMANOVA: $p = 0.01$, $F = 6.59$) (Fig. 2B). Principle Co-ordinates analysis (PCoA) also confirmed this finding (PERMANOVA: $p = 0.01$, $F = 6.59$). There was also a significant difference in β-diversity of larvae and 15-day old adults, with 3-day old females overlapping the different age groups (PERMANOVA: $p = 0.01$, $F = 4.89$) (Fig. 2C).

Comparison of bacterial species overlaps and differential abundance in mosquito strains. When examining the overlap in bacterial specimens identified, all four strains had 17 families in common. SENN (*An. arabiensis*-insecticide susceptible) and SENN DDT (*An. arabiensis*-insecticide resistant) shared 6 families, while SANGWE (*An. quadriannulatus*) and MAFUS (*An. merus*) shared only one family (Fig. 3A). When examining overlapping genera, all four strains had 33 genera in common. MAFUS and SENN DDT had the most unique genera with four each. There were 14 shared genera between SENN and SENN DDT, but only 4 shared between MAFUS and SANGWE (Fig. 3B). When examining species composition, MAFUS had 10 unique species, the most of any of the strains. SANGWE had 4 unique species, and SENN DDT had 3 unique species. SENN had no unique species present (Fig. 3C).

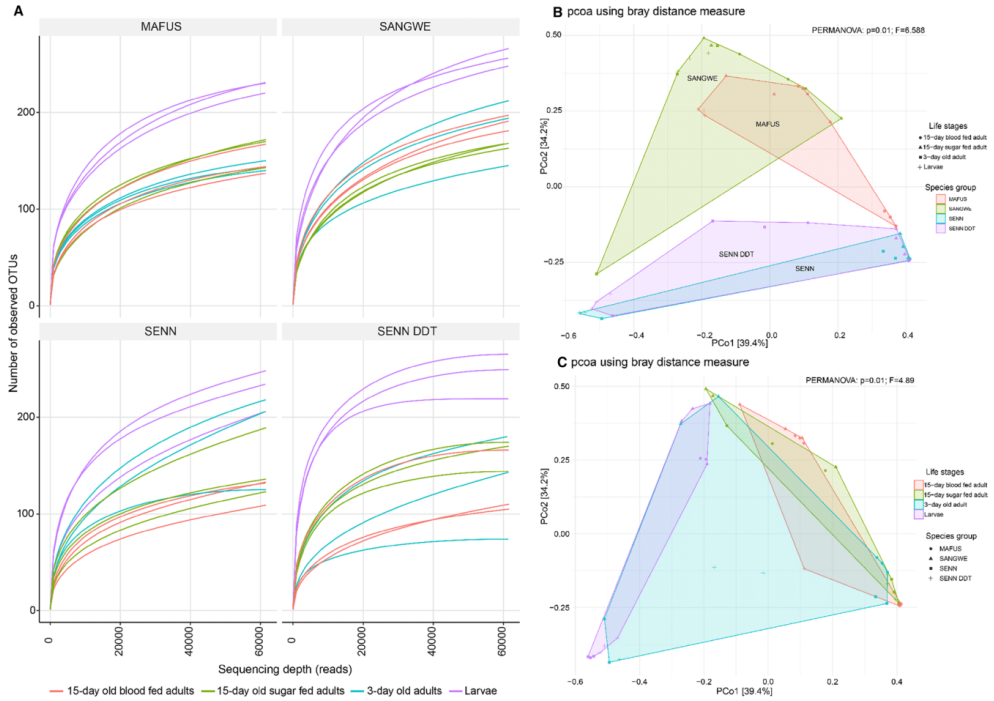

Figure 2. Rarefaction curves and beta diversity plots of the *An. gambiae* complex. (**A**) rarefaction curves of MAFUS (*An. merus*), SANGWE (*An. quadriannulatus*), SENN (*An. arabiensis*-insecticide susceptible) and SENN-DDT (*An. arabiensis*-insecticide resistant). (**B**) Principle Coordinates Analysis plot of beta-diversity highlighting the differences between strains. (**C**) Principal Component Analysis plot of beta-diversity highlighting differences between life stages.

	SENN *An. arabiensis*	SENN DDT *An. arabiensis*	*An. merus*	*An. quadriannulatus*	SENN *An. arabiensis*	SENN DDT *An. arabiensis*	*An. merus*	*An. quadriannulatus*
	Chao1 diversity indices				**ACE diversity indices**			
Fourth instar larvae	151	115	114	179	160	115	111	127
3-day adults	65.1	84.6	75.4	91.4	67.7	85.9	74.1	93.7
15-day old non-blood fed adults	105	62.2	74.4	97.7	104	63.3	70.5	94.7
15-day old blood fed adults	83.1	82.9	71.6	103	71.9	69.6	71.3	130

Table 1. Chao-1 and ACE diversity indexes of the bacteria located in the midgut at the different life stages of female members of the *Anopheles gambiae* complex for 16S rRNA sequencing.

Differential abundance was assessed between strains. For each pairwise comparison, genera fold changes with adjusted p-values (q-value) < 0.01 were considered differentially abundant. When comparing SENN to SANGWE *Aeromonas* and *Aquitalea* had the greatest fold increased abundance in SENN, and *Mesorhizobium* and *Sphingopyxis* having the smallest fold change. Notable abundance differences were increased *Elizabethkingia* and *Rhanella* in SENN and increased *Pseudomonas*, *Klebsiella* and *Serratia* in SANGWE. Similarly, *Aquitalea* and *Aeromonas* had the greatest fold increased abundance in SENN DDT compared to SANGWE, and *Mesorhizobium* and *Microbacterium* having the smallest fold change. Notable abundance differences were higher abundances of *Rhanella* and *Elizabethkingia* in SENN-DDT, and increased abundances of *Pseudomonas* and *Klebsiella* in SANGWE.

	SENN *An. arabiensis*	SENN DDT *An. arabiensis*	*An. merus*	*An. quadriannulatus*	SENN *An. arabiensis*	SENN DDT *An. arabiensis*	*An. merus*	*An. quadriannulatus*
	Shannon diversity indices				Simpson diversity indices			
Fourth instar larvae	2.05	2.90	2.62	2.37	0.896	0.754	0.859	0.738
3-day adults	1.65	1.16	1.93	1.91	0.438	0.650	0.681	0.702
15-day old non-blood fed adults	1.44	1.83	1.92	1.61	0.660	0.518	0.743	0.615
15-day old blood fed adults	1.15	1.41	1.65	2.00	0.5482	0.451	0.660	0.741

Table 2. Shannon and Simpson diversity indexes of the bacteria located in the midgut at the different life stages of female members of the *Anopheles gambiae* complex for 16S rRNA sequencing.

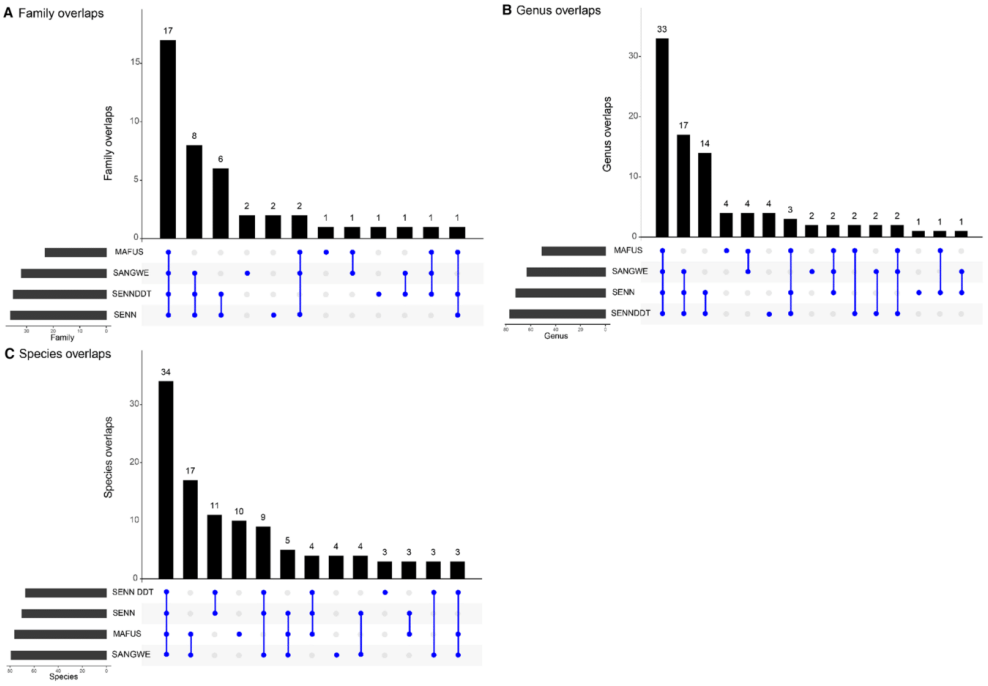

Figure 3. Overlapping midgut bacteria identified between the different mosquito strains. The blue dots represent the number of overlaps per strain, with single dots indicating the number of unique families/genera/species per strain. (**A**) Comparison of common families. There are 17 common families between all four strains. (**B**) Comparison of common genera. There are 33 common genera between all four strains. (**C**) Comparison of common species. There are 34 common species between all strains.

When examining differential abundance between MAFUS and the two *An. arabiensis* strains, SENN and SENN DDT, *Serratia* was the genus with the greatest differential abundance in both SENN and SENN DDT compared to MAFUS. The smallest differential abundance in both SENN and SENN DDT compared to MAFUS are *Sphingopyxis* and *Mesorhizobium*. *Pseudomonas* and *Enterobacter* were more differentially abundant in MAFUS than either SENN or SENN DDT. When comparing the differential abundance of MAFUS and SANGWE, *Acromobacter*, *Sphingobacterium* and *Elizabethkingia* had the greatest differential abundance in MAFUS. *Mycobacterium*, *Leucobacterium* and *Polynucleobacter* had the least differential abundance in MAFUS. There were no genera differentially abundant between SENN and SENN DDT at q-value < 0.01 significance level. When using a less stringent significance level of q-value < 0.05, differentially abundant species were largely observed when comparing blood-fed females. *Klebsiella* were more abundant in SENN, while *Rhanella*, *Aeromonas* and *Serratia* were more abundant in SENN DDT (Fig. 4). With the same adjustment, *Cedecea*, *Klebsiella*, *Enterobacter* and

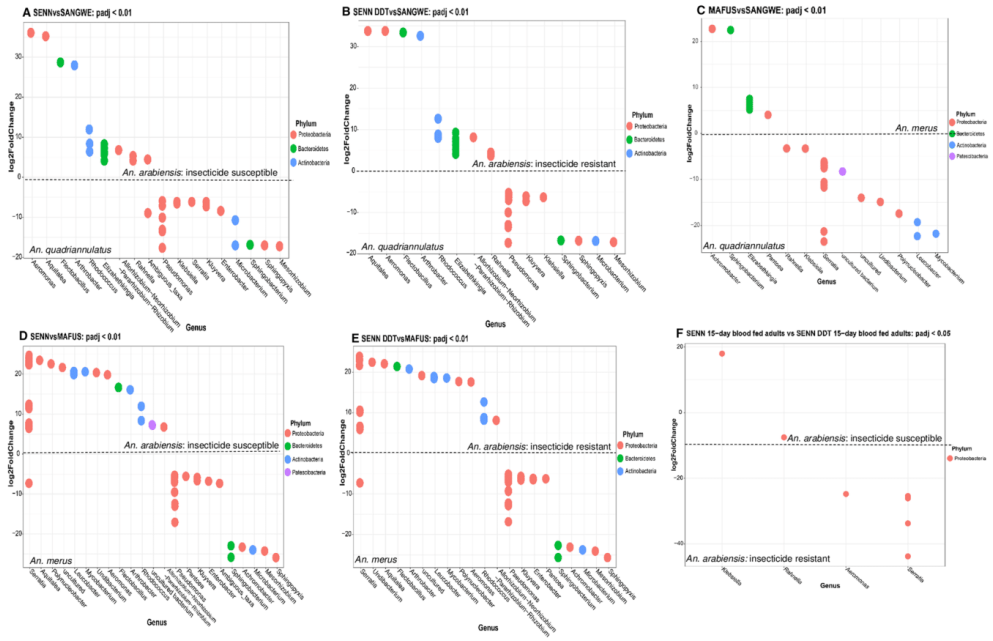

Figure 4. Comparison of differential abundance of genera between strains. (**A**) Differential abundance of genera in SENN vs SANGWE at an 99% Confidence interval. (**B**) Differential abundance of genera in SENN-DDT vs SANGWE at a 99% Confidence interval. (**C**) Differential abundance of genera in MAFUS vs SANGWE at a 99% Confidence interval. (**D**) Differential abundance of genera in SENN vs MAFUS at a 99% Confidence interval. (**E**) Differential abundance of genera in SENN vs SANGWE at an 99% Confidence interval. (**F**) Differential abundance of genera in 15-day bloodfed SENN vs 15 -day bloodfed SENN-DDT at a 95% confidence interval. No genus was found to be significantly abundantly expressed between these two strains overall at the 99% confidence interval. The dotted line at 0 represents the change from over representation to underrepresentation of bacterial genera. The species name on either side of the dotted line where the relevant bacterial species are abundant.. Each dot represents a single OTU.

Rhanella had a greater differential abundance in 3-day old SENN and *Zooglea* had the lowest differential abundance. The fold changes associated with the differential abundance are summarised in Tables 3 and 4.

Discussion

The amount of mosquito species where the gut microbiota is being characterised is increasing[12,21–23] and as such the catalogue of microbiome information available for comparison is increasing. The use of MALDI-TOF MS resulted in a markedly less species identified than NGS. These findings were congruent with a recent finding that analysed the microbiota of laboratory and wild *An. arabiensis* by MALDI-TOF MS where similar numbers and species were identified[21]. For the MS technique to be used for larger scale microbiome studies, culture protocols will have to be optimised. This will include optimising the choice of culture media as well as growing plates under anaerobic conditions to maximise the diversity of samples cultured. The use of an alternate database could also potentially improve the quality of analysis. Therefore, although less data was obtained by MALDI-TOF analysis, it is still a viable technique for identifying mosquito gut microbiota.

NGS was the more sensitive technique. This technique, however, are more prone to contamination which can result in misidentification. A common source of contamination is often the DNA extraction kit. In support of this, a study by Mancini et al.[24] demonstrated that some of the most common species associated with kit-borne contamination found in mosquito microbiome study included *Mesorhizobium*, *Phyllobacterium*, *Rhizobium*, *Comamonas*, *Delftia*, *Variovorax* and *Escherichia-Shigella*[24]. In the current study *Phyllobacterium* and *Rhizobium* were not identified, while *Mesorhizobium*, *Comamonas*, *Delftia*, *Variovorax* and *Escherichia-Shigella* were detected at low abundance (< 0.01% prevalence). The human skin contaminants *Corynebacterium* (12 OTUs) and *Streptococcus* (119 OTUs) were also identified at low abundance (< 0.01% prevalence), while *Propionibacterium*, another skin-associated contaminant was not identified at all.

There have been several studies associating gut microbiota with insecticide resistance[22,23,25]. These studies compared the gut microbiota of fenitrothion resistant and susceptible *An. albimanus* populations[23] as well as observing distinct gut microbiota in pyrethroid resistance in *An. gambiae*[22]. The insecticide susceptible SENN

	SENN (*An. Arabiensis*—insecticide susceptible)	SENN-DDT (*An. Arabiensis*—insecticide resistant)	MAFUS (*An. merus*)	SANGWE (*An. quadriannulatus*)
SENN (*An. arabiensis*—insecticide susceptible)		None	*Sphingopyxis* (Log2 fold change: − 25.90) *Mesorhizobium* (Log2 fold change: − 24.23) *Pseudomonas* (Log2 fold change: − 17.04) *Enterobacter* (Log2 fold change: − 6.79)	*Mesorhizobium* (Log2 fold change: − 17.29) *Sphingopyxis* (log fold change: − 16.96) *Pseudomonas* (Log2 fold change: − 13.61) *Klebsiella* (Log2 fold change: − 6.52) *Serratia* (Log2 fold change: − 6.12)
SENN-DDT (*An. arabiensis*—insecticide resistant)	None		*Sphingopyxis* (Log2 fold change: − 25.69) *Mesorhizobium* (Log2 fold change: − 24.03) *Pseudomonas* (Log2 fold change: − 16.91) *Enterobacter* (Log2 fold change: − 6.52)	*Mesorhizobium* (Log2 fold change: − 17.09) *Microbacterium* (Log2 fold change: − 16.87) *Pseudomonas* (Log2 fold change: − 17.04) *Elizabethkingia* (Log2 fold change: − 9.10) *Klebsiella* (Log2 fold change: − 5.03)
MAFUS (*An. merus*)	*Serratia* (Log2 fold change: 24.48)	*Serratia* (Log2 fold change: 23.94)		*Mycobacterium* (Log2 fold change: − 21.91 *Leucobacterium* (Log2 fold change: − 19.27) *Polynucleobacter* (Log2 fold change: − 17.46)
SANGWE (*An. quadriannulatus*)	*Aeromonas* (Log2 fold change: 38.02) *Aquitalea* (Log2 fold change: 35.20) *Elizabethkingia* (Log2 fold change: 8.25) *Rhanella* (Log2 fold change: 5.31)	*Aquitalea* (Log2 fold change: 33.79) *Aeromonas* (Log2 fold change: 33.76) *Rhanella* (Log2 fold change: 4.30)	*Acromobacter* (Log2 fold change: 22.74) *Sphingobacterium* (Log2 fold change: 22.47) *Elizabethkingia* (Log2 fold change: 7.35)	

Table 3. Statistical indicators for differential abundance of OTUs at a 99% confidence interval. The differential abundance is given for the overall life stages. The strain at the top is compared to the strain on the right, with the fold change referring to the change relevant to the strain at the top.

	SENN (*An. arabiensis*—insecticide susceptible) 15-day-blood	SENN-DDT (*An. arabiensis*—insecticide resistant) 15-day-blood
SENN (*An. arabiensis*-insecticide susceptible) 15 day blood		*Rhanella* (Log2 fold change: − 7.54) *Aeromonas* (Log2 fold change: − 24.79) *Serratia* (Log2 fold change: − 43.82)
SENN-DDT (*An. arabiensis*-insecticide resistant) 15 day blood	*Klebsiella* (Log2 fold change: 17.98)	
	SENN (*An. arabiensis*—insecticide susceptible) 3-day-old	**SENN-DDT (*An. arabiensis*—insecticide resistant) 3-day-old**
SENN (*An. arabiensis*-insecticide susceptible)		*Zooglea* (Log2 fold change: -24.86)
SENN-DDT (*An. arabiensis*-insecticide resistant)	*Cedecea* (Log2 fold change: 8.95)	
	Klebsiella (Log2 fold change: 5.01)	
	Enterobacter (Log2 fold change: − 17.46)	
	Rhanella (Log2 fold change: 4.48)	

Table 4. Statistical indicators for differential abundance between SENN and SENN-DDT at a 95% confidence interval. The differential abundances specifically compare relative abundance of OTUs at specific life stages as indicated.

strain had a greater species richness than the insecticide resistant SENN-DDT strain, but this did not translate into a difference in species diversity. Both strains also had overlapping β-diversity. There were also no bacterial species differentially abundant in the strains at the 99% confidence, unlike when compared to other strains. The increased number of species in SENN suggests a reduction in bacterial species due to selection for resistance[23]. However, the presence of species and genera associated with insecticide degradation in both species suggests that this is not a significant contributor to insecticide resistance in the SENN DDT strain. As the SENN DDT strain is fixed for the L1014F mutation as well as having elevated detoxification enzymes[26], it is likely that the contribution of insecticide degrading bacteria to the resistant phenotype is minimal. It is, however, worth noting that *Sphingobacterium*, an insecticide degrading genus[22], was significantly reduced in the insecticide susceptible SANGWE. A previous study found *Microbacterium* was unique to insecticide susceptible *An. albimanus*[23]. In this study, *Microbacterium* had a greater relative abundance in the insecticide susceptible SANGWE and MAFUS, which has low level insecticide tolerance.

Regardless of strains, larvae had the greatest species richness and diversity of all the life stages. This is congruent with previous findings on the dynamic gut microbiota of *An. gambiae*[12], where species richness decreased with age and blood feeding status. The shift in microbiota between the aquatic and aerial stages has been observed previously[10,27]. Furthermore, in an examination of the dynamic gut microbiota of *An. gambiae*, diversity decreased with age and blood feeding status[12,28]. This was confirmed by comparing the differential abundance of the combined groups, where larvae had nineteen genera with a higher differential abundance compared to six in 15-day old females which had multiple blood meals. *Asaia*, *Elizabethkingia*, *Serratia* and *Rhanella* were more abundant in blood fed females, while *Aquitalea*, *Sphingobacterium* and *Sphiyngopyxis* were the most abundantly expressed in larvae. Consistent with previous findings, this represents a shift from aerobic to facultatively anaerobic[29]. Unlike other studies, this study did not examine the direct effect of blood on gut microbiota. Rather, it examined the longer-term effects of multiple blood meals. This is because multiple blood meals are a common feeding strategy (e.g., Norris et al.)[30] which is known to increase the *Plasmodium* infectivity[31]. The lack of species richness and diversity in the adult stages examined in this study suggests that specific bacterial species, rather than population changes contribute to the microbial effect on life history.

A range of factors affect the composition of the gut microbiota. This includes the rearing water, food and sugar source[20]. Like with previous studies, several common species constituted the majority of gut microflora. These include *Enterobacter*, *Rhanella*, *Klebsiella*, *Serratia*, *Pseudomonas*, *Elizabethkingia*, *Asaia* and *Raoultella*. This supports the suggestion of a core gut microbiota. A core microbiome has been suggested to exist for *An. gambiae* and *An. coluzzi*[32]. As the gut microbiome is markedly less diverse than that of the salivary glands and reproductive organs[24,33], it is plausible that a set of bacteria could constitute the core gut microbiota of anophelines. It has also been suggested that the core microbiota persists over time[34], which is also supported by this study.

There have not been many studies that have characterised members of the *An. gambiae* complex. The majority of studies have focused on the major vector *An. gambiae* (e.g., Gimonneau et al.; Boissière et al.)[33,35]. There are fewer examples of *An. arabiensis* to compare to (E'Silva et al.; Barnard et al.)[21,36] and even less for *An. quadriannulatus* and *An. merus*[24]. As such, there are not many databases for to compare the data generated in this study. A notable conservation is that in *An. arabiensis* from the E'Silva study, which constitutes young, unfed females are dominated by *Serratia* as are both *An. arabiensis* strains in this study. Interestingly, this congruency does not hold true for the dominance of *Pseudomonas* in *An. merus* and *An. quadriannulatus* when compared to Mancini et al.[24].

The amount of microbiome data available for members of the An. gambiae complex outside that of the nominal member[33] is relatively limited. A single previous study examined the microbiota of several tissues in nine mosquito species, including *An. gambiae*, *An. arabiensis*, *An. merus* and *An. quadriannulatus*[24]. The present study is more similar to Wang et al.[12] which examined the dynamic gut microbiota of *An. arabiensis*. Although this took place in laboratory strains, the uniformity in larval feeding, sugar feeding and provision of blood meals should limit the rate of false discovery, this cannot be fully guaranteed[20].

Indices that estimate richness (Chao1 and ACE) indicate a significant difference between the two *An. arabiensis* strains with the insecticide susceptible strain having a significantly higher species richness (number of bacteria). This difference did not extend to species diversity, as determined by Shannon and Simpson indices. *Anopheles merus* and *An. quadriannulatus* did not differ in α-diversity but had higher Shannon indices than the insecticide susceptible *An. arabiensis*. Despite the lack of difference in alpha diversity, there is a marked difference in beta diversity between the strains, with the major vector species SENN and SENN DDT clustering separately from the minor vector MAFUS and the non-vector species SANGWE. This is markedly different to the lack of beta diversity between wild *An. gambiae* and *An. coluzzi*[33]. Laboratory reared mosquitoes have been described as having lower gut microbial diversity than their wild counterparts (as reviewed in Dada et al.)[37]. Despite this, a significant difference in beta diversity was observed in the present study.

Anopheles gambiae and *An. coluzzi* are both efficient major vectors of malaria that do not differ in microbial beta diversity. By contrast, *An. arabiensis*, *An. merus* and *An. quadriannulatus* differ in vector competence. This difference in vector competence is unlikely to be explained fully by behaviour. *Anopheles arabiensis*, *An. merus* and *An. quadriannulatus* are all primarily zoophilic and outdoor biting. Therefore, the differences in vector competence may have further molecular underpinnings, such as variable immune responses (e.g., Habtewold et al.)[38]. The differences in gut microbiota between the major, minor, and non-vectors may therefore contribute vector competence, although this needs to be confirmed by *Plasmodium*-infection studies.

Due to their anti-*Plasmodium* activities, *Asaia*, *Pantoea*, *Pseudomonas*, *Enterobacter*, *Serratia* are gaining considerable attention as promising candidates for paratransgenic modifications for vector control strategies[24]. *Pseudomonas putida*, *Pantoea sp* and *S. marcescens* were capable of blocking *Plasmodium* development in vivo when introduced as a sugar meal. *Comamonas* sp., *Acinetobacter sp*., *P. putida* and *P. rhodesiae*, *Pantoea sp*, *S. marcescens* and *E. anophelis* demonstrated *Plasmodium* blocking activity in vitro[39]. The *Enterobacter* isolate Esp_Z inhibits *Plasmodium* development from gametocyte to ookinete by in vivo and in vitro production of reactive oxygen species[40]. When comparing differential abundance of *An. quadriannulatus* to both *An. arabiensis* strains, *Pseudomonas* was more abundant in the former. Additionally, *Serratia* and *Enterobacter* was more abundant in *An. quadriannulatus* compared to the insecticide susceptible *An. arabiensis*. *Pseudomonas*, *Pantoea*, and *Enterobacter* had a greater differential abundance in *An. meus*. This further suggests that differential gut microbiota may contribute to differences in vector competence between the strains *Serratia* had a higher differential abundance in *An. quadriannulatus*. *Serratia*, however, had a greater differential abundance in the *An. arabiensis* strains. It is worth noting, however, that despite the association of *Serratia* with anti-*Plasmodium* defense, this is strain dependent[41]. *Serratia marcescens*, however, is known to degrade several insecticides[23]. The higher expression in the *An. arabiensis* strains may be related more to insecticide resistance than anti-parasite defense.

The microbiota of *An. merus* and *An. quadriannulatus* is poorly examined. Although this study does contribute information to this small pool, there are several limitations to this study. The first limitation is that this study

requires replication on field specimens (as described in Romoli and Gendrin)[15] Secondly, experiments need to be performed examining the effect of *Plasmodium* infection on gut microbial diversity to confirm the species-specific role of microbiota in vector competence. It would also be worth examining changes in the microbiota of the salivary glands after infection. Furthermore, it is worth considering the effect of saltwater breeding on the microbiota of *An. merus*. As an example, *Rhanella* is a freshwater bacterial species[42], potentially explaining the low expression of this species in *An. merus*.

The *An. merus* strain was reared in salt water, but as an osmotolerant species, considerations must be made of the effect of salt concentration on the gut microbiota of this species. The variation in beta diversity between the two *An. arabiensis* strains and the *An. merus* and *An. quadriannulatus* may be due to the evolutionary distance between the species, but the lack of beta diversity between *An. quadriannulatus* and *An. merus* should be taken into account as motivation for the validity of these findings. Although the findings could suggest a role for gut microbiota in differences in *Plasmodium* transmission, these findings must be replicated with wild mosquitoes, and preferably with experiments that examine the changes in gut microbiota before and after *Plasmodium* infection. These experiments are, however, beyond the scope of this study.

In conclusion, this study characterised the dynamic gut microbiota of *An. merus*, *An. quadriannulatus* and *An. arabiensis* by culture-dependent and culture independent techniques. Although α-diversity did not differ greatly, there was a significant difference in β-diversity of the major vector *An. arabiensis* and minor vector *An. merus* and the non-vector *An. quadriannulatus*. This diversity, in conjunction with the decreased relative abundance of genera associated with anti-*Plasmodium* effects in *An. arabiensis* suggests that this difference may be associated with vector competence. Although both the insecticide susceptible and resistant *An. arabiensis* harboured insecticide degrading bacteria, genera associated with insecticide susceptibility was associated with the insecticide susceptible *An. quadriannulatus* strain. Therefore, this study suggests a role for differential microbial diversity in the life history of zoophilic members of the *An. gambiae* complex.

Materials and methods

Mosquito collection and preparation for characterisation. Laboratory reared *Anopheles* mosquitoes used were housed and collected from the Botha de Meillon insectary of the National Institute of Communicable Disease (NICD), Johannesburg, South Africa. Four strains, representing three species were used in this study. The *An. arabiensis* strain SENN is an insecticide susceptible strain originated in Sennar, Sudan. SENN has been in colony since 1980. The *An. arabiensis* strain SENN DDT is an insecticide resistant strain that has continuously been selected for DDT resistance from the SENN strain since 1995. This strain is still being selected for DDT resistance. SENN DDT is fixed for the *kdr* L1014F mutation. It also has elevated cytochrome P450, general esterase as well as Glutathione S-transferase activity. This accounts for the strain's resistance to DDT, permethrin, deltamethrin, λ-cyhalothrin and malathion[26,43,44]. The *An. quadriannulatus* strain (SANGWE) is insecticide susceptible and originated from Zimbabwe. MAFUS is an *An. merus* strain originating from Malahlapanga, South Africa and is tolerant to insecticides. It has been in colony since 2012 and was reared in 50% seawater.

Larvae were reared in reverse osmosis water at 25 °C (± 2 °C), 80% relative humidity (± 5%) with a 12-h light/dark cycle and a 30-min dusk/dawn cycle[45]. Larvae were fed a mixture of powder Beano™ dog biscuits and yeast. Several life stages of *An. arabiensis* (SENN and SENN DDT), *An. merus* and *An. quadriannulatus* were collected: fourth instar larvae, 3-day old female adults, 15-day old non-blood fed female adults and 15-day old blood fed female adults. Blood feeding took place at the age of 3, 7 and 11 days. Blood was supplied by a single consenting human volunteer, the author Shüné V. Oliver, as per the ethics waiver provided by the University of the Witwatersrand (Ethics waiver number: 09-06-2020-O). Blood was supplied as a live feeding volunteer, and no other individuals besides the author were involved in providing the blood meal. For MALDI-TOF mass spectrometric analysis of the midgut microbiota, 180 female mosquitoes and 60 larvae were used. For 16S rRNA gene sequencing analysis of the midgut microbiota, 180 female mosquitoes and 60 larvae were used.

Matrix assisted laser desorption ionisation-time of flight (MALDI-TOF) mass spectrometric analysis of the midgut microbiota. *Midgut resection and culturing procedure.* Larvae were chilled at 4 °C to minimise their activity and adult female mosquitoes were cold killed at − 70 °C for 5 min prior to midgut resection. Strict sterile conditions were implemented, the forceps, microscope, microscope slides and mosquitoes were sterilised with 70% ethanol. The midguts from the respective samples were dissected using a dissection microscope (Olympus, SZ2-ILST) with a magnification of 40×. Five replicates consisting of three midguts each were suspended in 200 µl of 0.1 M Phosphate Buffered Saline (PBS) pH 7.2.

The extracted guts were aseptically added to 2 ml of Lysogeny Broth (LB broth). The culturing tubes were incubated at 37 °C overnight with constant shaking at approximately 120 rpm. The negative control consisted of PBS in uninoculated LB broth. Each of the samples were plated onto three types of media, namely, Brain Heart Infusion BHI agar (for the culture of fastidious organisms), a 10% blood agar (for the culture of fastidious organisms) and a MacConkey agar (selective for lactose fermenters). All culture plates were obtained from Diagnostic Media Products (South Africa). Single colonies were obtained by streak plating with subsequent incubation at 37 °C for 18 h.

Bacterial identification using MALDI-TOF spectroscopy. The mass spectrometry system used in this study is housed in the Centre for Healthcare-Associated Infections, Antimicrobial Resistance and Mycoses (CHARM) at the National Institute of Communicable Diseases in Johannesburg, South Africa.

To perform the analysis individual bacterial colonies were selected manually from each plate based on the size, colour, growth pattern or shape and transparency. The individual colony was selected and applied to a 96-well MALDI target plate (Bruker Daltonics, Wissembourg, France; cat no. 8280800) in duplicate. Brucker Matrix

HCCA (α-Cyano-4-hydroxycinnamic acid), portioned at 2.5 ± 0.3 mg matrix (Hain Life Sciences) was dissolved in 250 µl Standard solvent (acetonitrile 50%, water 47.5% and trifluoroacetic acid 2.5%) (Sigma-Aldrich). One microlitre of Bruker HCCA Bruker (10 mg Bruker HCCA/mL) was added to each occupied target spot position and left to dry at room temperature.

The target plate was placed into the Brucker Matrix-assisted Laser Desorption Ionising Biotyper System with a benchtop Microflex LT/SH mass spectrometer (Bruker Daltonics, Wissembourg, France). The bacteria were identified on 16 s rRNA spectra as per manufacturer's instructions. For each spectrum obtained a maximum of 100 peaks were used and compared with other peaks in the MBT 7854 MSP Library (Bruker Daltonics, Wissembourg, France; ref no. 182903). The bacteria were accepted as correctly identified if the identification score was ≥ 1.9[46].

For all downstream analysis α-diversity indices were divide into species richness and species evenness indicators. The Chao1 and ACE indices were used to measure species richness, a direct count of the Operational Taxonomic Units (OTUs). The Shannon and Simpson diversity indices were used as measures of species evenness. The Shannon and Simpson diversity index is a measure of diversity which considers both richness as well as their relative abundance or evenness.

16S rRNA gene sequencing analysis of the midgut microbiota. *Midgut dissection and DNA extraction.* The midguts of the female adult mosquitoes and larvae were dissected as previously mentioned however, five replicates consisting of five midguts each were suspended in 200 µl of 0.1 M PBS pH 7.2. To ensure sterile conditions, all equipment and surfaces used were sterilised using 70% ethanol and 3% hydrogen peroxide. An extraction negative control of 0.1 M PBS pH 7.2 only was used during the course of the DNA extraction.

Total genomic DNA from the midgut of the mosquito was extracted using the DNeasy PowerSoil® Kit (QIAGEN: 12888-50). The tubes containing the midguts were centrifuged at 5000×*g* and approximately 150 µl of PBS was discarded, the remaining PBS and midguts were vortexed briefly and added to the PowerBead™ tubes. The protocol was then followed as per manufacturer's instructions.

Amplification of the V3-V4 hypervariable region of the 16S rRNA gene. The V3-V4 hypervariable region of the bacterial 16S rRNA gene was amplified using universal bacterial primers. Forward Primer (5′ GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C 3′)(Integrated DNA Technologies) and Reverse Primer (5′ TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG 3′) (4 nmol Ultramer® DNA Oligo 55 bases : Integrated DNA Technologies)[47]. Library preparation was performed according to the standard instructions of the 16S Metagenomic Sequencing Library Preparation protocol (IlluminaTM, Inc., San Diego, CA, United States). The size of the amplicons were then visualised using the 4200 TapeStation (Agilent Technologies). Two negative controls were used in the amplicon preparation step. The extraction negative control as well as no template control were used in the initial amplification step. No amplification was observed in either of the negative controls.

Libraries were then sequenced on the MiSeq platform using MiSeq Reagent kit v3 (Illumina) and paired-end 2 × 300 bp sequencing was performed at the Sequencing Core Facility, National Institute for Communicable Diseases, South Africa.

Bioinformatic analysis. QIAGEN CLC Genomics Workbench 20 (CLC Bio Qiagen) was used to identify the operational taxonomic units (OTU's) of each of the specimens analysed. Amplicon-based analysis workflows were used for data quality control and OTU clustering.

Sequences were filtered, trimmed and subjected to quality control to ensure clean data was used for clustering. The raw reads were trimmed with a 0.05 quality limit. Clustering of the data was done at a 99% similarity threshold with the SILVA 16S v132 database. The OTU's were assigned to sequences based on 97% identity. The OTU table was then used to obtain a visual representation of the midgut bacteria of each respective sample in the form of a bar plot.

Bacterial diversity analysis was done using raw reads, which were quality controlled and filtered (Q > 20 and length > 50 bp) using fastqc (v0.11.8) and trimGalore (v0.6.4_dev; https://github.com/FelixKrueger/TrimGalore), respectively[48,49]. In addition, trimGalore was used for adapter removal.

All the downstream analyses were performed in R (v3.6.1), including classification, abundance estimations, statistical analysis, and visualisation. Clean reads were pre-processed using dada2 package (v1.12.1)[50], including quality inspection, trimming, dereplication, merging paired-end reads and removal of chimeric sequences.

Taxonomy was assigned to the obtained amplicon sequence variants (ASVs) and the ASV abundance estimates determined using training sequence sets based on the SILVA reference database (v138; https://zenodo.org/record/1172783#.XvCmtkUzY2w).

Ordinations for beta diversity, abundance bar plots, alpha diversity and richness estimates, and heatmaps were generated using the phyloseq package (v1.28.0)[51], ggplot2 (v3.2.1) and AmpVis2 package (v2.6.4)[52]. Data clustering in NMDS were assessed using PERMANOVA (permutation test with pseudo-F ratios) as implemented in the adonis function in the vegan package (https://github.com/vegandevs/vegan).

Kruskal–Wallis rank-sum tests were used to compare alpha diversity between groups. Venn diagrams were generated using Venn Diagram (v1.6.20) and UpsetR (v1.4.0)[53]. Differential abundance analysis between sample groups was performed using DESeq2 (v1.24.0)[54].

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References

1. Sinka, M. E. *et al.* The dominant *Anopheles* vectors of human malaria in Africa, Europe and the Middle East: Occurrence data, distribution maps and bionomic précis. *Parasites Vectors* **2010**(3), 1–34 (2010).
2. Dahan-Moss, Y. *et al.* Member species of the *Anopheles gambiae* complex can be misidentified as *Anopheles leesoni*. *Malar. J.* **2020**(19), 1–9 (2020).
3. Dandalo, L. C. *et al.* Population Dynamics and Plasmodium falciparum (Haemosporida: Plasmodiidae) Infectivity Rates for the Malaria Vector *Anopheles arabiensis* (Diptera: Culicidae) at Mamfene, KwaZulu-Natal, South Africa. *J. Med. Entomol.* **54**, 1758–1766 (2017).
4. Gillies, M. T. & De Meillon, B. The Anophelinae of Africa south of the Sahara (Ethiopian Zoogeographical Region). *Anophelinae Africa south Sahara (Ethiopian Zoogeographical Reg.* (1968).
5. Killeen, G. F., Govella, N. J., Lwetoijera, D. W. & Okumu, F. O. Most outdoor malaria transmission by behaviourally-resistant *Anopheles arabiensis* is mediated by mosquitoes that have previously been inside houses. *Malar. J.* **2016**(15), 1–10 (2016).
6. Mahande, A., Mosha, F., Mahande, J. & Kweka, E. Feeding and resting behaviour of malaria vector, *Anopheles arabiensis* with reference to zooprophylaxis. *Malar. J.* **2007**(6), 1–6 (2007).
7. Takken, W. *et al.* Susceptibility of *Anopheles quadriannulatus* theobald (Diptera: Culicidae) to *Plasmodium falciparum*. *Trans. R. Soc. Trop. Med. Hyg.* **93**, 578–580 (1999).
8. Habtewold, T., Povelones, M., Blagborough, A. M. & Christophides, G. K. Transmission blocking immunity in the malaria non-vector mosquito *Anopheles quadriannulatus* species A. *PLoS Pathog.* **4**, e1000070 (2008).
9. Jayakrishnan, L., Sudhikumar, A. V. & Aneesh, E. M. Role of gut inhabitants on vectorial capacity of mosquitoes. *J. Vector Borne Dis.* **55**, 69 (2018).
10. Coon, K. L., Vogel, K. J., Brown, M. R. & Strand, M. R. Mosquitoes rely on their gut microbiota for development. *Mol. Ecol.* **23**, 2727–2739 (2014).
11. Akorli, J., Namaali, P. A., Ametsi, G. W., Egyirifa, R. K. & Pels, N. A. P. Generational conservation of composition and diversity of field-acquired midgut microbiota in *Anopheles gambiae* (*sensu lato*) during colonization in the laboratory. *Parasites Vectors* **2019**(12), 1–9 (2019).
12. Wang, Y., Gilbreath, T. M., Kukutla, P., Yan, G. & Xu, J. Dynamic gut microbiome across life history of the malaria mosquito *Anopheles gambiae* in Kenya. *PLoS ONE* **6**, 1–9 (2011).
13. Ranasinghe, H. A. K. & Amarasinghe, L. D. Naturally occurring microbiota in dengue vector mosquito breeding habitats and their use as diet organisms by developing larvae in the Kandy district, Sri Lanka. *Biomed Res. Int.* **2020**, 5830604 (2020).
14. Chen, S. *et al.* Multiple factors determine the structure of bacterial communities associated with *Aedes albopictus* under artificial rearing conditions. *Front. Microbiol.* **5**, 605 (2020).
15. Romoli, O. & Gendrin, M. The tripartite interactions between the mosquito, its microbiota and *Plasmodium*. *Parasit. Vectors* **11**, 1–8 (2018).
16. Grogan, C., Bennett, M., Moore, S. & Lampe, D. Novel *Asaia bogorensis* signal sequences for *Plasmodium* inhibition in *Anopheles stephensi*. *Front. Microbiol.* **12**, 1–14 (2021).
17. Sharma, P. *et al.* Altered gut microbiota and immunity defines *Plasmodium vivax* survival in *Anopheles stephensi*. *Front. Immunol.* **11**, 1–13 (2020).
18. Kumar, S., Molina-Cruz, A., Gupta, L., Rodrigues, J. & Barillas-Mury, C. A Peroxidase/dual oxidase system modulates midgut epithelial immunity in *Anopheles gambiae*. *Science* **327**, 1644–1648 (2010).
19. Galeano-Castañeda, Y., Bascuñán, P., Serre, D. & Correa, M. M. Trans-stadial fate of the gut bacterial microbiota in *Anopheles albimanus*. *Acta Trop.* **201**, 105204 (2020).
20. Saab, S. A. *et al.* The environment and species affect gut bacteria composition in laboratory co-cultured *Anopheles gambiae* and *Aedes albopictus* mosquitoes. *Sci. Rep.* **10**, 1–13 (2020).
21. Silva, B. E., Matsena Zingoni, Z., Koekemoer, L. L. & Dahan-Moss, Y. L. Microbiota identified from preserved *Anopheles*. *Malar. J.* **20**, 1–18 (2021).
22. Omoke, D. *et al.* Western Kenyan *Anopheles gambiae* showing intense permethrin resistance harbour distinct microbiota. *Malar. J.* **2021**(20), 1–14 (2021).
23. Dada, N., Sheth, M., Liebman, K., Pinto, J. & Lenhart, A. Whole metagenome sequencing reveals links between mosquito microbiota and insecticide resistance in malaria vectors. *Sci. Rep.* **2018**(8), 1–13 (2018).
24. Mancini, M. V. *et al.* Estimating bacteria diversity in different organs of nine species of mosquito by next generation sequencing. *BMC Microbiol.* **18**, 1–10 (2018).
25. Dada, N. *et al.* Pyrethroid exposure alters internal and cuticle surface bacterial communities in *Anopheles albimanus*. *ISME J.* **2019**(13), 2447–2464 (2019).
26. Oliver, S. V. & Brooke, B. D. The effect of larval nutritional deprivation on the life history and DDT resistance phenotype in laboratory strains of the malaria vector *Anopheles arabiensis*. *Malar. J.* **2013**(12), 1–9 (2013).
27. Minard, G., Mavingui, P. & Moro, C. V. Diversity and function of bacterial microbiota in the mosquito holobiont. *Parasit. Vectors* **6**, 1–12 (2013).
28. Muturi, E. J., Dunlap, C., Ramirez, J. L., Rooney, A. P. & Kim, C. H. Host blood-meal source has a strong impact on gut microbiota of *Aedes aegypti*. *FEMS Microbiol. Ecol.* **95**, fiy213 (2018).
29. Coon, K. L. *et al.* Bacteria-mediated hypoxia functions as a signal for mosquito development. *Proc. Natl. Acad. Sci. USA* **114**, E5362–E5369 (2017).
30. Norris, L. C., Fornadel, C. M., Hung, W. C., Pineda, F. J. & Norris, D. E. Frequency of multiple blood meals taken in a single gonotrophic cycle by *Anopheles arabiensis* mosquitoes in Macha, Zambia. *Am. J. Trop. Med. Hyg.* **83**, 33–37 (2010).
31. Shaw, W. R. *et al.* Multiple blood feeding in mosquitoes shortens the *Plasmodium falciparum* incubation period and increases malaria transmission potential. *PLOS Pathog.* **16**, e1009131 (2020).
32. Segata, N. *et al.* The reproductive tracts of two malaria vectors are populated by a core microbiome and by gender-and swarm-enriched microbial biomarkers OPEN. *Nat. Publ. Gr.* https://doi.org/10.1038/srep24207 (2016).
33. Gimonneau, G. *et al.* Composition of *Anopheles coluzzii* and *Anopheles gambiae* microbiota from larval to adult stages. *Infect. Genet. Evol.* **28**, 715–724 (2014).
34. Tchioffo, M. T. *et al.* Dynamics of Bacterial community composition in the malaria mosquito's epithelia. *Front. Microbiol.* **6**, 1500 (2016).
35. Boissière, A. *et al.* Midgut microbiota of the malaria mosquito vector *Anopheles gambiae* and interactions with *Plasmodium falciparum* infection. *PLoS Pathog.* **8**, 1–12 (2012).
36. Barnard, K., Jeanrenaud, A. C. S. N., Brooke, B. D. & Oliver, S. V. The contribution of gut bacteria to insecticide resistance and the life histories of the major malaria vector *Anopheles arabiensis* (Diptera: Culicidae). *Sci. Rep.* **2019**(9), 1–11 (2019).
37. Dada, N. *et al.* Considerations for mosquito microbiome research from the Mosquito Microbiome Consortium. *Microbiome* **9**, 1–16 (2021).
38. Habtewold, T., Groom, Z. & Christophides, G. K. Immune resistance and tolerance strategies in malaria vector and non-vector mosquitoes. *Parasites Vectors* **2017**(10), 1–12 (2017).
39. Bahia, A. C. *et al.* Exploring *Anopheles* gut bacteria for *Plasmodium* blocking activity. *Environ. Microbiol.* **16**, 2980–2994 (2014).

40. Cirimotich, C. M. *et al.* Natural microbe-mediated refractoriness to *Plasmodium* infection in *Anopheles gambiae*. *Science* **332**, 855–858 (2011).
41. Bai, L. *et al.* A gut symbiotic bacterium *Serratia marcescens* renders mosquito resistance to *Plasmodium* infection through activation of mosquito immune responses. *Front Microbiol.* **10**, 1580 (2019).
42. Koczura, R., Mokracka, J. & Makowska, N. Environmental isolate of *Rahnella aquatilis* harbors class 1 integron. *Curr. Microbiol.* **72**, 64–67 (2016).
43. Oliver, S. V. & Brooke, B. D. The effect of elevated temperatures on the life history and insecticide resistance phenotype of the major malaria vector *Anopheles arabiensis* (Diptera: Culicidae). *Malar. J.* **2017**(16), 1–13 (2017).
44. Oliver, S. V. & Brooke, B. D. The role of oxidative stress in the longevity and insecticide resistance phenotype of the major malaria vectors *Anopheles arabiensis* and *Anopheles funestus*. *PLoS ONE* **11**, e0151049 (2016).
45. Hunt, R. H., Brooke, B. D., Pillay, C., Koekemoer, L. L. & Coetzee, M. Laboratory selection for and characteristics of pyrethroid resistance in the malaria vector *Anopheles funestus*. *Med. Vet. Entomol.* **19**, 271–275 (2005).
46. Seng, P. *et al.* Ongoing revolution in bacteriology: Routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Clin. Infect. Dis.* **49**, 543–551 (2009).
47. Klindworth, A. *et al.* Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* **41**, e1–e1 (2013).
48. Wood, D. E., Lu, J. & Langmead, B. Improved metagenomic analysis with Kraken 2. *Genome Biol.* **2019**(20), 1–13 (2019).
49. Ondov, B. D., Bergman, N. H. & Phillippy, A. M. Interactive metagenomic visualization in a Web browser. *BMC Bioinform.* **12**, 1–10 (2011).
50. Callahan, B. J. *et al.* DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* **2016**(13), 581–583 (2016).
51. McMurdie, P. J. & Holmes, S. phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE* **8**, e61217 (2013).
52. Andersen, K. S., Kirkegaard, R. H., Karst, S. M. & Albertsen, M. ampvis2: An R package to analyse and visualise 16S rRNA amplicon data. *BioRxiv* https://doi.org/10.1101/299537 (2018).
53. Conway, J. R., Lex, A. & Gehlenborg, N. *UpSetR: An R Package for the Visualization of Intersecting Sets and Their Properties* https://doi.org/10.1093/bioinformatics/btx364.
54. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **2014**(15), 1–21 (2014).

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Author contributions
A.S.: performed experiments, analysed data, produced initial draft of manuscript. Z.T.H.K.: performed NGS sequencing runs, manuscript editing. S.K.: bioinformatics analysis, manuscript editing. M.A.: bioinformatics analysis, project administration, manuscript editing. A.I.: manuscript editing. S.V.O.: conceived project, obtained funding, project administration, produced final draft of the manuscript. All authors reviewed the manuscript.

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Competing interests
The authors declare no competing interests.

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Appendix 3: Nextera® Index Adapter Sequences

Table 1 Index adapter sequences

Index 1 (i7)	**Sequence**	**Index 2 (i5)**	**Sequence**
N701	TAAGGCGA	N502	CTCTCTAT
N702	CGTACTAG	N503	TATCCTCT
N703	AGGCAGAA	N504	AGAGTAGA
N704	TCCTGAGC	N505	GTAAGGAG
N705	GGACTCCT	N506	ACTGCATA
N706	TAGGCATG	N507	AAGGAGTA
N707	CTCTCTAC	N508	CTAAGCCT
N708	CAGAGAGG	N517	GCGTAAGA
N709	GCTACGCT		
N710	CGAGGCTG		
N711	AAGAGGCA		

Appendix 4: Bacteria Involved in Heavy Metal Metabolism

Table 1 Bacteria genera involved in heavy metals metabolism that were identified in the gut of three-day old SENN, SENN DDT, and F_1 population of *An. arabiensis*

All bacterial genera are tolerant to heavy metals. Those that degrade heavy metals are highlighted in bold.

	SENN			**SENN DDT**			**F_1 population**		
	Untreated	Cadmium	Copper	Untreated	Cadmium	Copper	Untreated	Cadmium	Copper
Aeromonas	X		X	X	X	X			
Chryseobacterium		X	X	X	X	X			
Yersinia	X	X	X	X	X	X			
Delftia		X		X		X			
Enterococcus							X	X	X
Bacillus							X	X	X
Klebsiella							X	X	X
Enterobacter							X	X	X
Microbacterium	X	X	X	X	X	X			
Pseudomonas		X		X		X	X	X	X
Sphingomonas		X							
Acinetobacter	X	X	X						X
Corynebacterium							X	X	X
Streptococcus							X	X	X
Rahnella		X					X	X	X
Staphylococcus							X	X	X
Rhodopseudomonas		X	X					X	

Table 2 Bacteria genera involved in heavy metals metabolism that were differentially abundant in the gut of three-day old SENN, SENN DDT, and F_1 population of *An. arabiensis*

All bacterial genera are tolerant to heavy metals. Those that degrade heavy metals are highlighted in bold.

	SENN			**SENN DDT**			**F_1 population**		
	Untreated	Cadmium	Copper	Untreated	Cadmium	Copper	Untreated	Cadmium	Copper
Aeromonas	X		X	X					
Chryseobacterium			X	X	X	X			
Yersinia	X					X			
Delftia				X		X	X		
Enterococcus							X	X	
Bacillus							X		
Klebsiella							X	X	X
Enterobacter							X	X	X
Microbacterium							X	X	X
Pseudomonas				X			X		
Acinetobacter	X	X	X						
Corynebacterium							X	X	X
Streptococcus							X	X	X
Rahnella								X	X
Staphylococcus								X	X
Rhodopseudomonas			X						

Appendix 5: Fungal Primers

Table 3. Primer sequences used for ITS1 sequencing.

Forward primer set		
Name	**Sequence (5')**	**Overhang adapter sequence**
ITS forward primer 1	CTTGGTCATTTAGAGGAAGTAA	5' TCGTCGGCA GCGTCAGATG TGTATAAGAG ACAG–[locus-specific sequence]
ITS forward primer 2	CTCGGTCATTTAGAGGAAGTAA	
ITS forward primer 3	CTTGGTCATTTAGAGGAACTAA	
ITS forward primer 4	CCCGGTCATTTAGAGGAAGTAA	
ITS forward primer 5	CTAGGCTATTTAGAGGAAGTAA	
ITS forward primer 6	CTTAGTTATTTAGAGGAAGTAA	
ITS forward primer 7	CTACGTCATTTAGAGGAAGTAA	
ITS forward primer 8	CTTGGTCATTTAGAGGTCGTAA	
Reverse Primer Set		
ITS reverse primer 1	GCTGCGTTCTTCATCGATGC	5' GTCTCGTGGG CTCGGAGATG TGTATAAGAG ACAG–[locus-specific sequence]
ITS reverse primer 2	GCTGCGTTCTTCATCGATGG	
ITS reverse primer 3	GCTACGTTCTTCATCGATGC	
ITS reverse primer 4	GCTGCGTTCTTCATCGATGT	
ITS reverse primer 5	ACTGTGTTCTTCATCGATGT	
ITS reverse primer 6	GCTGCGTTCTTCATCGTTGC	
ITS reverse primer 7	GCGTTCTTCATCGATGC	

Appendix 6: Additional Sequencing Information

Additional information for the sequencing data, including OTU taxa and abundance tables, processed reads information, and sample metadata is available using the following link:

https://drive.google.com/drive/folders/1HynOispPmJsQhwuqoMTi3_CMPx8HyI5-?usp=sharing