MICROBIOTA: A CONSEQUENTIAL THIRD WHEEL IN THE MOSQUITO-PATHOGEN RELATIONSHIP

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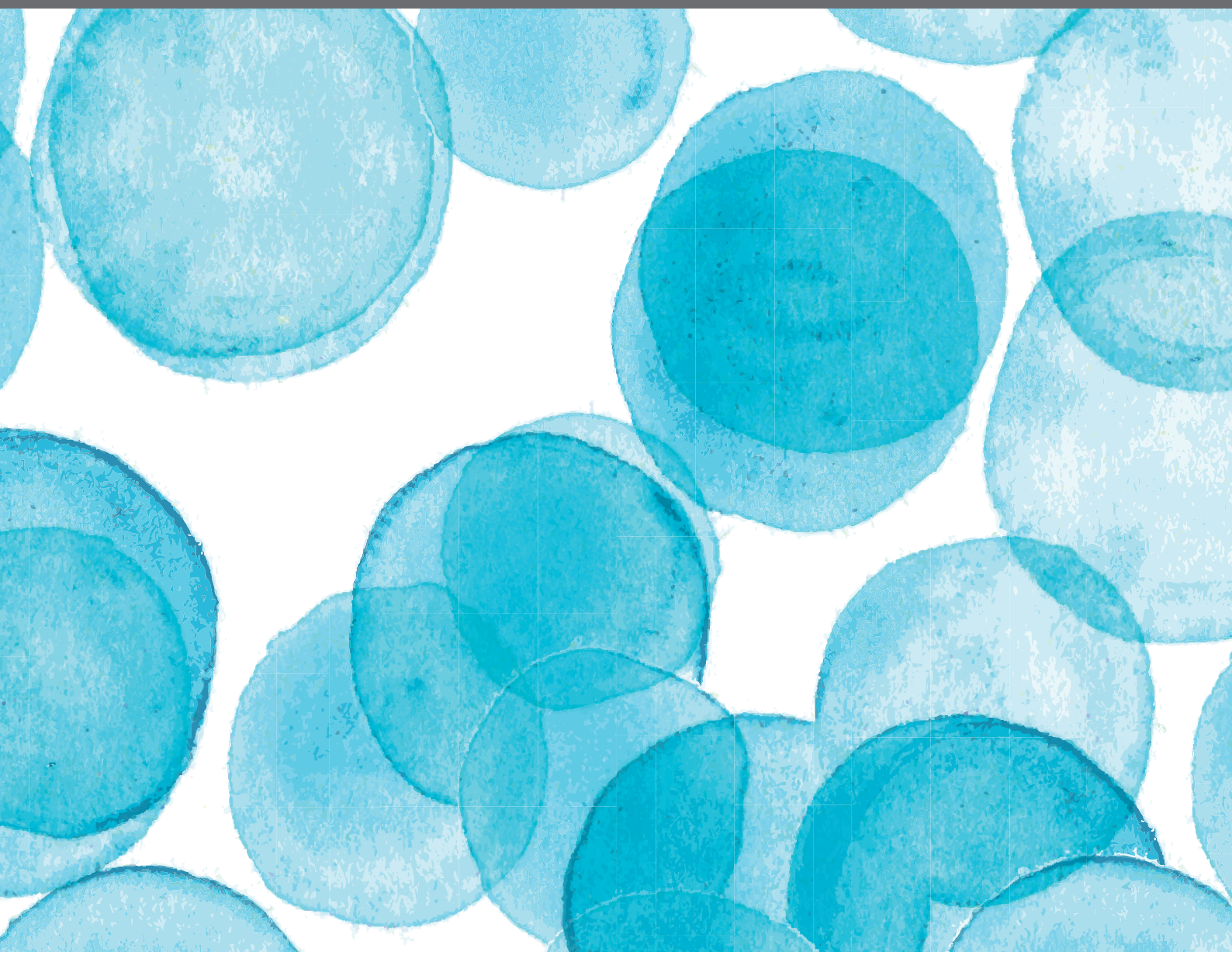

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MICROBIOTA: A CONSEQUENTIAL THIRD WHEEL IN THE MOSQUITO-PATHOGEN RELATIONSHIP

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EDITORIAL
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Editorial: Microbiota: A Consequential Third Wheel in the Mosquito-Pathogen Relationship

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Editorial on the Research Topic

Microbiota: A Consequential Third Wheel in the Mosquito-Pathogen Relationship

Mosquitoes are by far the most important vectors of human disease. There are hundreds of millions of cases of dengue annually, while Chikungunya and Zika have recently caused major outbreaks. Malaria remains a major driver of poverty in sub-Saharan Africa where it is responsible for about 400,000 deaths each year. In addition, about 50 million cases of lymphatic filariasis still occur annually.

The microbial communities harbored by mosquitoes have been the focus of great scientific interest since the discovery of their significant impact on disease transmission, via their influence on mosquito physiology and permissiveness to infection. In the 1990s and 2000s, the gut microbiota was found to limit parasitic infection in *Anopheles* malaria vector mosquitoes in experiments that used antibiotic treatments. It was subsequently demonstrated that *Wolbachia* endosymbionts could protect their insects hosts against viruses. In the 2010s, as high-throughput DNA sequencing became increasingly available to researchers, a more thorough description of the mosquito microbiota composition was generated and correlated to environmental or experimental parameters. Toward the end of the 2010s many more functional studies on mosquito/microbiota interactions were being carried out, and several countries had started to experiment with *Wolbachia*-infected mosquitoes as a public health measure to limit dengue transmission. These advances have culminated in a widespread appreciation that vector-pathogen interactions must be investigated in the context of a consequential third player, the microbiota.

In the article collection "Microbiota: A Consequential Third Wheel in the Mosquito-Pathogen Relationship," we gathered state-of-the-art research on the microbiota of mosquitoes, including bacteria and eukaryotic microbes. This collection examines the interplay between three types of research in the field: functional characterization of host-microbe and microbe-microbe interactions, description of the microbiota system composition, and the design of microbiota-based tools to block disease transmission. These three aims are being advanced in parallel and are indeed interdependent, as functional characterizations identify specific attributes that can be used in the field if the system is better understood, whether functionally or taxonomically, while observations from applied research in the field bring novel questions to the basic research directions and a good description of the microbiota gives essential clues about how host-microbe associations work and how interventions may affect microbiota systems and what the consequences of this may be.

Historically, the first microbes used in the fight against vector borne diseases were entomopathogens, notably *Bacillus thuringiensis* var. *israelensis,* which is still widely used as a

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vector control tool against mosquitoes and other insect pests. Based on a screen for biopesticide activity in soil samples, Barbieri et al. have identified a new spore-producing bacterial isolate, *Brevibacillus laterosporus* SAM19, which is 10 times more efficient at killing *Aedes albopictus* than the reference *B. laterosporus* strain LMG15441 (Barbieri et al.). This biopesticide candidate may be used in a combination with other bacteria, such as *B. thuringiensis* var. *israelensis* and *Lysinibacillus sphaericus*, to avoid the evolution of resistance. Vector control may also be achieved using secondary metabolites produced by bacteria. For instance, prodigiosin produced by *Serratia marcescens* is known to have some larvicidal activity against several mosquito species as well as some antibacterial activity. Using prodigiosin-deficient bacteria, Heu et al. found that secondary metabolites, notably prodigiosin and/or serratamolide, participate in the virulence of *S. marcescens* in *Aedes aegypti* in adults and larvae, and in its antibacterial effect on several members of the mosquito microbiota. Their *in vitro* assays indicate that secondary metabolites are also essential in proteolytic and haemolytic activities (Heu et al.). Kulkarni et al. studied the impact of priming on infections by two strains of *Serratia* or *Enterobacter* in mosquitoes (Kulkarni et al.). They found that a preliminary oral infection by either strain had a protective effect on *Anopheles* mosquitoes after a subsequent septic challenge with the same bacterium. Their transcriptomic analysis discriminates between *Serratia*-infected mosquitoes that have been subjected to different priming conditions.

Moving beyond entomopathogens, members of the microbiota can also protect their host against infection by human pathogens, and therefore can decrease the vector competence of host mosquitoes. The most well-known example is that of *Wolbachia* endosymbionts, which naturally limit their host's susceptibility to infection by several viruses including Dengue and Zika in mosquitoes. These symbionts have been shown to manipulate mosquito reproduction to facilitate their spread through host populations. Indeed, among other phenomena, they can induce cytoplasmic incompatibility. This is essentially a male sterility phenotype that can only be rescued if the male mates with a female that carries the same *Wolbachia* strain. *Wolbachia*-colonized females, which can reproduce with any male, tend to have a higher fitness compared to non-infected females that are only compatible with non-infected males. Due to their capacity to invade mosquito populations and to reduce vector competence, these endosymbionts have been released at scale in last decade in several countries and are now considered a potentially transformative tool to fight arbovirus outbreaks. These *Wolbachia*-based control strategies are dependent on two of *Wolbachia's* phenotypes, protection against pathogens and cytoplasmic incompatibility. Notably, there are still many aspects of these two phenotypes that are not well-understood, including the nature of interactions between different strains in the same mosquito. Liang et al. investigated cytoplasmic incompatibility in the context of *Wolbachia* inter-strain competition (Liang et al.). They found that an infection with three strains of *Wolbachia* (wAlbA, wAlbB, and wMel) in *Ae. albopictus* led to a suppression of cytoplasmic incompatibility that is dependent on wAlbA. They also found differences between mosquito strains in the fitness cost of harbouring triple *Wolbachia* infections. Lu et al. investigated cellular aspects of DENV and ZIKV infection inhibition and showed that *Wolbachia* inhibits virion binding to mosquito Aag2 cells in a *Wolbachia*-density dependent manner, notably via the downregulation of dystroglycan and tubulin expression (Lu et al.).

Similar to *Wolbachia* in *Aedes* mosquitoes, *Microsporidia MB* is a fungi-related member of the *Anopheles* microbiota which has recently been proposed as a candidate tool to reduce malaria transmission, as it has been found to inhibit *Plasmodium falciparum* development in the mosquito gut. Focusing on how this symbiont is transmitted between mosquitoes, Nattoh et al. showed via co-housing experiments that horizontal transmission in *Anopheles arabiensis* occurs via mating (Nattoh et al.). *Microsporidia MB* can be detected in the seminal fluid of males, and can be vertically transmitted after a female is infected via mating. After screening several species sharing the habitats of *An. arabiensis*, the authors suggest that *Microsporidia MB* may be an *Anopheles*-specific symbiont. Another fungal member of the mosquito microbiota is the yeast *Wickerhamomyces anomalus*. The *W. anomalus*-host interaction was reviewed by Cappelli et al. Strains of this yeast produce a killer-toxin that is antimicrobial, since their approval by the European Food and Safety Authority these strains are now used in the agro-food sector to control mold and bacteria. Killer-toxin-producing *W. anomalus* is found in mosquitoes and has antiplasmodial activity, supporting its potential for use as a symbiotic-based control tool against malaria transmission. Other eukaryotes may also affect malaria transmission, but despite this the eukaryotic microbiota composition is still poorly described. While analysing the composition of the eukaryotic microbiota in *Anopheles* collected in Kenya, Burkina Faso, and Republic of Guinea, Cuesta et al. found that region of collection was the primary driver of microbiota differences and identified a new taxon in the *Ophryocystis* genus, which is highly prevalent in Kenyan mosquito samples (Cuesta et al.). As it belongs to Apicomplexa (the same phylum as *Plasmodium*), they suspect that interactions between both parasites may occur in *Anopheles* mosquitoes.

The bacterial microbiota has long been known to naturally limit infection of *Anopheles* mosquitoes by *Plasmodium*, yet this had not been tested in *Culex pipiens*, a vector for avian malaria. Martínez-de la Puente et al. observed that prevalence of *Plasmodium relictum* in saliva was higher in antibiotic-treated mosquitoes (Martínez-de-la-Puente et al.). They also detected a negative impact of the microbiota on mosquito life span in mosquitoes infected with *P. relictum*.

Another alternative route to using microbes for transmission blocking involved genetically-engineering them to inhibit infection of their mosquito host by parasites or viruses. This approach, called paratransgenesis, has been applied to several bacterial species including *Asaia* sp. and has been shown to efficiently limit *Plasmodium* infection in laboratory-reared *Anopheles*. Grogan et al. studied whether they could engineer an improved excretion of antiplasmodial effectors by *Asaia* sp. (Grogan et al.). They identified several novel secretion signals, including two which more efficiently excrete proteins and induce a

higher level of antiplasmodial inhibition than their initial paratransgenetic strain.

Interactions between microbes will impact the success of any microbe-based approach applied to mosquitoes. For example, competitive interactions have been observed between *Wolbachia* and other members of the mosquito microbiota. Scolari et al. investigated the composition of the bacterial microbiota in *Ae. albopictus* and report correlations between *Wolbachia* and within-sample diversity (Scolari et al.). They also observed that developing larvae affect the microbial communities in their breeding water, corroborated with changes in pH and solutes. Conversely, environmental conditions during larval development, including microbiota composition and diet or larval density, have been found to impact larvae and even to have long-lasting effects during mosquito adulthood. MacLeod et al. more specifically examined how the amount of larval food affects mosquitoes and their microbiota (MacLeod et al.). They found that food abundance during larval development not only positively affects adult size, but also microbiota abundance and, though to a lesser extent, microbiota composition. Martinson and Strand used larvae colonised with a controlled microbiota to further investigate the impact of diet and microbiota composition on larval development (Martinson and Strand). They found that diet and microbiota both affect development success. Notably, microbiota composed of seven taxonomically-diverse bacteria better supports larval development than microbiota composed of each single bacterium even when larvae are provided a rich fish-food diet.

Last but not least, Gabrieli et al. reviewed the trilogy between the mosquito immune system, the microbiota and transmitted pathogens (Gabrieli et al.). They focus on the basic understanding of the interactions between the microbiota and the mosquito immune system before describing how the microbiota can be used to limit disease transmission via paratrangenesis or via the use of *Wolbachia*. They also contextualize this review with a section devoted to other insect vectors, including tsetse and sandflies.

Together, this article collection gathered studies focused on a diversity of mosquito microbe interactions, which reflects how the mosquito microbiome field is moving forward on many fronts. Through these studies and others, we have begun to understand the complexity of microbial communities and have even started to attribute certain functions to specific microbial members. In addition, these findings are advancing the prospect of symbiont-based control strategies (and paratransgenic control strategies). In the longer term, researchers in this field will hope to build on these studies to understand the precise causes and consequences of microbiota shifts and find ways to use the mosquito microbiota community or specific members to efficiently control vector borne diseases.

AUTHOR CONTRIBUTIONS

MG: writing—original draft. MG, GF, and JH: writing—review and editing. All authors contributed to the article and approved the submitted version.

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Wolbachia Inter-Strain Competition and Inhibition of Expression of Cytoplasmic Incompatibility in Mosquito

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Successful field trials have been reported as part of the effort to develop the maternally transmitted endosymbiontic bacteria *Wolbachia* as an intervention agent for controlling mosquito vectors and their transmitted diseases. In order to further improve this novel intervention, artificially transinfected mosquitoes must be optimized to display maximum pathogen blocking, the desired cytoplasmic incompatibility (CI) pattern, and the lowest possible fitness cost. Achieving such optimization, however, requires a better understanding of the interactions between the host and various *Wolbabachia* strains and their combinations. Here, we transferred the *Wolbachia w*Mel strain by embryonic microinjection into *Aedes albopictus*, resulting in the successful establishment of a transinfected line, HM (*w*AlbA*w*AlbB*w*Mel), with a triple-strain infection comprising *w*Mel, *w*AlbA, and *w*AlbB. Surprisingly, no CI was induced when the triply infected males were crossed with the wild-type GUA females or with another triply infected HC females carrying *w*Pip, *w*AlbA, and *w*AlbB, but specific removal of *w*AlbA from the HM (*w*AlbA*w*AlbB*w*Mel) line resulted in the expression of CI after crosses with lines infected by either one, two, or three strains of *Wolbachia*. The transinfected line showed perfect maternal transmission of the triple infection, with fluctuating egg hatch rates that improved to normal levels after repeated outcrosses with GUA line. Strain-specific qPCR assays showed that *w*Mel and *w*AlbB were present at the highest densities in the ovaries and midguts, respectively, of the HM (*w*AlbA*w*AlbB*w*Mel) mosquitoes. These finding suggest that introducing a novel strain of *Wolbachia* into a *Wolbachia*-infected host may result in complicated interactions between *Wolbachia* and the host and between the various *Wolbachia* strains, with competition likely to occur between strains in the same supergroup.

Keywords: *Wolbachia*, dengue, cytoplasmic incompatibility, *Aedes albopictus*, transinfection, inter-strain competition

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INTRODUCTION

A rapid increase in the number of arbovirus diseases transmitted by mosquitoes, such as dengue and Zika, in recent decades has underscored the urgency in developing effective intervention strategies (Velayudhan, 2012; Katzelnick et al., 2017). The insufficiency of traditional control approaches, including vaccines, drugs, and chemical insecticides, has led to significant efforts to develop novel

vector control methods to combat disease transmission. Rather than using chemical insecticides to directly kill the vector, an approach that is being challenged by the rapid development of insecticide resistance and the negative impacts on both the environment and non-target insect species, these new tools have focused on modifying the mosquito population in a species-specific manner, with the goal of either reducing the mosquito's ability to host a pathogen or suppressing (or even eliminating) the mosquito population to break the viral transmission between vector and host (Achee et al., 2015). Among these strategies, *Wolbachia*-based interventions have recently shown encouraging results in field trials, successfully demonstrating either reduced dengue transmission through *Wobachia*-induced viral inhibition in the mosquitoes or the elimination of the *Aedes* vector populations through *Wobachia*-induced incompatible mating (Hoffmann et al., 2011; Mains et al., 2016; Nazni et al., 2019; Zheng et al., 2019; Crawford et al., 2020).

Estimated to infect >65% of all insect species, *Wolbachia* are maternally transmitted endosymbiotic bacteria belong to the order Rickettsiales and family Anaplasmataceae (Werren et al., 2008). Designated based on their naturally associated host species and divided into eight supergroups, different *Wolbachia* strains can interact with their hosts in their own manner, with phenotypes determined by the genetic background of both *Wolbachia* and the host, as well as the environment (McGraw et al., 2002; Werren et al., 2008; Ross et al., 2017). In the mosquito and many other insects, *Wolbachia* causes a reproduction alteration known as cytoplasmic incompatibility (CI), in which early embryonic death occurs when the *Wolbachia*-infected male mates with an uninfected female or a female carrying a different strain of *Wolbachia*. The CI can be rescued, resulting in compatible mating, if the *Wolbachia* strain carried by the male is also present in the female. Recent studies have shown that two CI determination genes, *cifA* and *cifB*, in *Wolbachia* modify the sperm development to induce CI, but only *cifA* mediates CI rescue in females (or eggs; LePage et al., 2017; Shropshire et al., 2018; Beckmann et al., 2019; Chen et al., 2019). However, it is still unknown how these CI factors interact with their host targets and how the CI determination factors of different *Wolbachia* strains interact with each other to induce CI expression in a host with a *Wolbachia* superinfection.

Since the ability to generate novel *Wolbachia* symbiosis (transinfection) in mosquitoes was first developed through embryonic microinjection (Xi et al., 2005a,b, 2006), a number of transinfected mosquito lines carrying different *Wolbachia* strains have been established and characterized, with the goal of using them for disease control (Xi et al., 2005b, 2006; McMeniman et al., 2009; Walker et al., 2011; Blagrove et al., 2012; Joubert et al., 2016; Ant et al., 2018; Ross et al., 2019). Many of these transinfected mosquito lines show different levels of resistance to dengue, Zika, and Chikungunya viruses, with the strength of the viral inhibition being associated with the density of *Wolbachia* in somatic tissues such as the midgut and salivary glands, where the viruses reside, migrate, and replicate. Whereas transinfected lines with each of three *Wolbachia* strains – *w*Mel, *w*AlbB, and *w*Pip – have been well characterized and successfully tested in field trials (Hoffmann et al., 2011; Nazni et al., 2019; Zheng et al., 2019), significant interest remains in developing improved transinfected lines with maximal viral blocking and optimal fitness under field conditions in order to reach the highest efficiency in disease control or to be able to replace the released lines if viruses develop resistance to the released strains in the future (Ross et al., 2019).

Naturally carrying two *Wolbachia* strains, *w*AlbA and *w*AlbB, *Aedes albopictus* is the world's most invasive mosquito vector and an epidemiologically important vector for many arboviruses. As the density of these two native *Wolbachia* is too low to induce viral inhibiton in *Ae. albopictus* (Lu et al., 2012), efforts have been made to introduce novel strains into this mosquito species to develop transinfected lines that are both incompatible with the wild-type line and resistant to viruses (Blagrove et al., 2013; Zheng et al., 2019). Experiments are often designed by either directly adding a novel strain to *Ae. albopictus* to generate a superinfection (Fu et al., 2010; Suh et al., 2016; Ant and Sinkins, 2018; Zheng et al., 2019) or replacing the native *Wolbachia* with a novel strain, by removing the native *Wolbachia* with an antibiotic and then introducing the novel strain (Xi et al., 2006; Blagrove et al., 2012). The first approach results in a triple infection to induce an unidirectional CI with wild-type mosquitoes (Fu et al., 2010; Zheng et al., 2019), with the advantage that *Wolbachia* invade and spread into the population more effectively than does the second (replacement) approach, which often induces a bi-directional CI (Xi et al., 2006; Blagrove et al., 2012). However, for a host with a triple-strain infection, the outcome of the transinfection is difficult to predict, given the complicated interactions between the various *Wolbachia* strains and between *Wolbachia* and the host (Suh et al., 2016; Ant and Sinkins, 2018). When a tripe-strain infection comprising *w*Mel, *w*AlbA, and *w*AlbB was previously established in *Ae. albopictus*, very low egg hatch rates were observed in both the self-cross of the transinfected line and the compatible cross of the transinfected females with wild-type males (Ant and Sinkins, 2018), suggesting that the ability of this *Wolbachia* triple-strain infection to recue CI modification was compromised due to unknown inter-strain interactions.

We previously developed the transinfected *Ae. albopictus* line HC, featuring another triple infection with *w*Pip, *w*AlbA, and *w*AlbB (Zheng et al., 2019). The HC line induces complete unidirectional CI in crosses with the wild-type line, with intact ability of HC females to rescue CI when mated with either wild-type or HC males (Zheng et al., 2019). In the present study, we have introduced *w*Mel into *Ae. albopictus* and generated the transinfected line, HM (*w*AlbA*w*AlbB*w*Mel), infected with *w*Mel, *w*AlbA, and *w*AlbB. The transinfected line show complete efficiency in maternal transmission of the triple infection, with *w*Mel showing the highest density in ovaries. Multiple crosses showed that the ability of *w*Mel to induce CI was blocked by *w*AlbA in the HM (*w*AlbA*w*AlbB*w*Mel) line and that double infection with *w*Mel and *w*AlbB induced a high level of CI in crosses with the lines infected with either a single, double, or triple infection.

MATERIALS AND METHODS

Mosquito Lines and Maintenance

Two wild-type *Ae. albopictus* lines, HOU (Xi et al., 2005a) and GUA (Zheng et al., 2019), carrying a native superinfection with *w*AlbA and *w*AlbB were used in this study. Two transinfected *Ae. albopictus* lines, HB and HC, carrying a single *w*AlbB infection and a triple infection with *w*Mel, *w*AlbA, and *w*AlbB, respectively, had been generated previously (Xi et al., 2005a; Zheng et al., 2019) and were used in the CI crosses. The transinfected *Aedes aegypti* MGYP2 line (Walker et al., 2011), carrying *w*Mel, was used as a donor to generate the HM (*w*AlbA*w*AlbB*w*Mel) line.

All the mosquito lines were maintained on a 10% sugar solution at 27 ± 1°C and 80 ± 10% relative humidity, with a 12:12 h light:dark photoperiod, according to standard rearing procedures. For routine colony maintenance and experimental studies, female mosquitoes were provided with either human (for the MGYP2 line) or sheep (for the other lines) blood at day-7 post-eclosion, and eggs were collected 2 days post-blood meal.

Transinfection to Generate the HM (*w*AlbA*w*AlbB*w*Mel) Line

The HM (*w*AlbA*w*AlbB*w*Mel) line was generated by transfer of *w*Mel from *Ae. aegypti* MGYP2 to *Ae. albopictus* HOU using embryonic microinjection according to the approach described previously (Xi et al., 2005a,b). In brief, cytoplasm from donor embryos was transferred into the posterior of 60–90-min-old recipient embryos using an IM300 microinjector (Narishige Scientific). After injection, the embryos were incubated at 85% relative humidity and 27°C for 1 h, then transferred to wet filter paper. Embryos were allowed to mature for 5–7 days before hatching. Females (G0) developing from the surviving embryos were isolated and mated with HOU males. After blood-feeding and oviposition, G0 females were tested for *w*Mel infection by PCR using strain-specific primers as described below. G1 females were again crossed with HOU males, blood-fed, isolated, and allowed to oviposit. The offspring from *w*Mel-positive G1 were selected for the next screen, and this process was repeated until the *w*Mel maternal transmission rate reached 100%. Diagnosis of *Wolbachia* *w*AlbA and *w*AlbB was also performed to ensure that the transinfected line carried the triple infection.

PCR Assays of *Wolbachia* Infection

Primers were designed for strain-specific diagnosis of four different strains on the basis of the sequence of the gene encoding the *Wolbachia* surface protein *wsp*. The primers for *w*AlbA were: forward 5′-GTGTTGGTGCAGCGTATGTC-3′; reverse 5′-GCACCAGTAGTTTCGCTATC-3′. The primers for *w*AlbB were: forward 5′-ACGTTGGTGGTGCAACATTTG-3′; reverse 5′-TAACGAGCACCAGCATAAAGC-3′. The primers for *w*Mel were: forward 5′-CCTTTGGAACCCGCTGTGAATG-3′; reverse 5′-GCCTGCATCAGCAGCCTGTC-3′. The primers for *w*Pip were: forward 5′-TATTTCCCACTATATCCCTTC-3′; reverse 5′-GGATTTGACCTTTCCGGC-3′. The primers given below for mosquito rps6 have been reported previously (Molina-Cruz et al., 2005): forward 5′-CGTCGTCAGGAACGTATTCG-3′; and reverse 5′-TCTTGGCAGCCTTGACAGC-3′. Standard curves were generated for each of the genes listed above to convert the Ct value from quantitative PCR (qPCR) to the copy number of target sequences.

Genomic DNA was extracted from the samples using a Thermo Scientific Phire Animal Tissue Direct PCR Kit (F-140WH). Samples were pre-treated in 20 μl of dilution buffer with 0.5 μl DNARelease Additive. The reaction mixture contained 10 μl 2X Phire Animal Tissue PCR Buffer, 0.4 μl Phire Hot Start II DNA Polymerase, 0.2 μl of both the forward and reverses primer, and 7.2 μl dsH_2O. The regular PCR conditions were: initial denaturation at 98°C for 6 min, followed by 40 cycles of 5 s at 98°C, 5 s at 56°C, and 45 s at 72°C. qPCR was performed using a QuantiTect SYBR Green PCR Kit (Qiagen) and ABI Detection System ABI Prism 7000 (Applied Biosystems, Foster City, CA, United States). Samples were homogenized in 100 μl 1× STE buffer and incubated with 4 μl of roteinase K at 55°C for 1 h, followed by 97°C for 5 min.

Tetracycline Treatment of the HM (*w*AlbA*w*AlbB*w*Mel) Line to Generate the HM2 (*w*AlbB*w*Mel) Line With a Double Infection of *w*Mel and *w*AlbB

Once the HM (*w*AlbA*w*AlbB*w*Mel) mosquitoes had emerged as adults (day 0), they were provided with 0.5 mg/ml tetracycline HCl in a 10% sugar solution. This solution was replaced with a 10% sugar solution from day 3 or 4, and a blood meal was provided on day 7. Two days after the blood feeding, the mosquitoes were provided with oviposition cups containing wet filter paper. These treatments were repeated for four generations. At G3, after blood-feeding, the females were isolated for oviposition. After their eggs collected, individual isofemales were sacrificed to extract genomic DNA, and a PCR assay was used to identify each of the three *Wolbachia* strains. Only the eggs from females showing a double infection with *w*Mel and *w*AlbB were allowed to hatch to establish the line. The isofemale selection described above was repeated at G5 to ensure the removal of *w*AlbA, and the resulting HM2 (*w*AlbB*w*Mel) line carried only the double infection with *w*Mel and *w*AlbB.

Experimental Crosses to Determine CI

Cytoplasmic incompatibility assays were conducted as previously described (Xi et al., 2005a,b). A total of 10 virgin males were mated with 10 virgin females in five replicate cages for each cross. A blood meal was provided to the females at day 7 post-eclosion. Two days after the blood meal, eggs were collected using oviposition cups containing wet filter paper, which was subsequently desiccated for 7 days at 27°C and 80% relative humidity. The eggs were counted and then hatched in water containing 6% (m/v) bovine liver powder. Larvae were counted at the L2-L3 stage to record the hatch rate.

Statistical Analysis

All data were statistically analyzed by GraphPad Prism 5.0 software. ANOVA and Tukey's multiple comparisons test were used to compare egg hatching in CI cross experiment and density of each *Wolbachia* strain in mosquito salivary glands, midguts, and ovaries.

RESULTS

Generation of the *Ae. albopictus* Transinfected Line With a Triple *Wolbachia* Infection: *w*Mel, *w*AlbA, and *w*AlbB

The ability of a single *w*Mel infection to inhibit arbovirus transmission in both *Ae. aegypti* and *Ae. albopictus* (Walker et al., 2011; Blagrove et al., 2012) motivated us to test whether a triple infection with *w*Mel, *w*AlbA, and *w*AlbB could be established in *Ae. albopictus* to produce enhanced viral blocking effects for disease control, and whether there was competition among the various *Wolbachia* strains that might affect the nature of the symbiosis between *Wolbachia* and its mosquito host. The cytoplasm of *w*Mel-infected *Ae. aegypti* (MGYP2) embryos (Walker et al., 2011) was transferred by microinjection into embryos of the *Ae. albopictus* HOU line with a native superinfection of *w*AlbA and *w*AlbB (**Figure 1A**). The virgin females (G0) developed from embryos surviving the microinjection were outcrossed with HOU males to produce offspring (G1). A total of 18 G1 isofemales were outcrossed with HOU males. After their eggs (G2) were collected, PCR assay was used to diagnose the *Wolbachia* strain profile in these females, with 15 of 18 isofemales (83%) being seen to carry the triple *Wolbachia* infection (**Figures 1B,C**); the offspring of the females without a triple infection were discarded. Among the G2 offspring of these triply infected mothers, 18 of 20 (90%) males and 15 of 20 (75%) females maintained a triple *Wolbachia* infection. Without further screening, the offspring from the triply infected G2 females were then pooled together to establish a new transinfected line, hereafter referred to as HM (*w*AlbA*w*AlbB*w*Mel). At G3 and G4, we randomly selected 20 and 10 individuals, respectively, for PCR assay. All of the tested mosquitoes carried a triple infection, indicating a 100% maternal transmission efficiency. Subsequently, the infection status of the HM (*w*AlbA*w*AlbB*w*Mel) line was monitored every other generation from G8 to G24, and all the tested samples (n = 126) were positve, confirming the stability of the triple infection in the HM (*w*AlbA*w*AlbB*w*Mel) line (**Figure 1C**). These results suggest that *w*Mel can coexist with *w*AlbA and *w*AlbB to exhibit symbiosis within *Ae. albopictus*.

Failure of the HM (*w*AlbA*w*AlbB*w*Mel) Line to Induce CI When Crossed With Wild-Type or Transinfected Lines

The ability to induce CI is a key feature that is required in order to develop *Wolbachia*-based strategies for mosquito-borne disease control. We therefore set up a series of reciprocal crosses among the HM (*w*AlbA*w*AlbB*w*Mel), GUA, and HC lines to measure the relative strength of the *w*Mel-mediated CI expression. All compatible crosses yielded egg hatch rates ranging from 51 to 56% (**Table 1**). Unexpectedly, two presumably incompatible crosses, matings between HM (*w*AlbA*w*AlbB*w*Mel) males and either GUA or HC females, resulted in high egg hatch rates (46.7 and 43.2%, respectively), indicating compatible mating between them. In contrast, consistent with the ability of HC males to induce a strong CI when crossed with GUA females (Zheng et al., 2019), near-complete CI was observed in the crosses between HC males and HM (*w*AlbA*w*AlbB*w*Mel) females (**Table 1**). These results indicate that the ability of *w*Mel to induce CI, as observed previously (Walker et al., 2011; Blagrove et al., 2012), is blocked in the HM (*w*AlbA*w*AlbB*w*Mel) line when it co-exists with *w*AlbA and *w*AlbB.

CI Induction by *w*Mel After Removal of *w*AlbA From the HM (*w*AlbA*w*AlbB*w*Mel) Line in *Ae. albopictus*

In order to understand whether the ability of *w*Mel to induce CI in the HM (*w*AlbA*w*AlbB*w*Mel) line is being blocked by the other two native *Wolbachia* strains, we treated the HM (*w*AlbA*w*AlbB*w*Mel) line with a subdose of tetracycline for four generations and monitored the infection profile by strain-specific PCR from G3 to G5 after tetracycline treatment (**Figures 2A–C**). This treatment resulted in the specific removal of *w*AlbA from the HM (*w*AlbA*w*AlbB*w*Mel) line and establishment of the HM2 (*w*AlbB*w*Mel) line, with a double infection of *w*Mel and *w*AlbB (**Figure 2C**). CI crosses were then performed using HM2 (*w*AlbB*w*Mel), GUA, HC, and an *Ae. albopictus* HB line with a single *w*AlbB infection. Strikingly, we observed a strong, although not complete, CI when HM2 (*w*AlbB*w*Mel) males were crossed with GUA, HC, or HB females (**Table 2**). As expected, HM2 (*w*AlbB*w*Mel) induced bi-directional CI when crossed with the GUA and HC lines, but uni-directional CI when crossed with the HB line. Among all of these incompatible crosses, HC males induced the highest level of CI, with 100% embryonic death. These results indicate that *w*AlbA may block the expression of CI by *w*Mel in the HM (*w*AlbA*w*AlbB*w*Mel) line.

Introduction of a New Host Genetic Background Into the HM (*w*AlbA*w*AlbB*w*Mel) Line to Increase Its Fitness

The newly established HM (*w*AlbA*w*AlbB*w*Mel) line suffered from a strong fitness cost associated with the triple-strain infection, with an extremely low egg hatch rate ranging from 1 to 12% between G2 and G5 (**Figure 3**). Therefore, we outcrossed HM (*w*AlbA*w*AlbB*w*Mel) females with HOU males to remove the potential inbreeding effect, which has been observed to cause a low egg hatch rate in previous transinfected lines (Xi et al., 2005a, 2006). The egg hatch rate increased to 60% at G6, then dropped to 12 and 6% at G10 and G12, respectively (**Figure 3**). From G13 to G27, the egg hatch rate continued

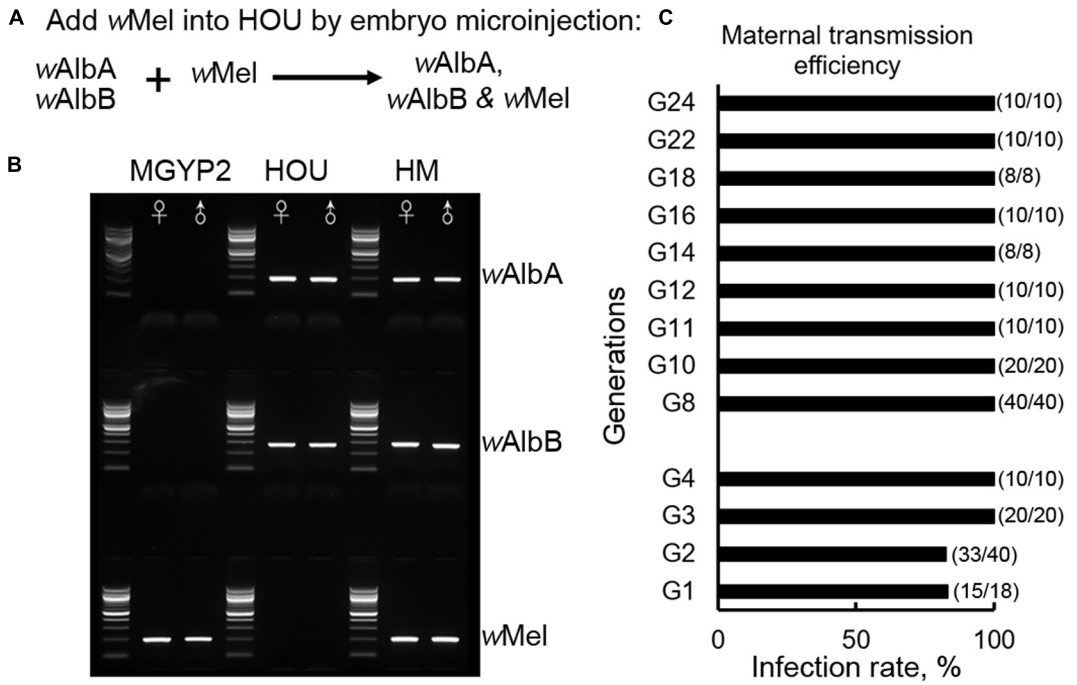

FIGURE 1 | Establishment of the transinfected *Ae. albopictus* HM (*w*AlbA*w*AlbB*w*Mel) line with a triple infection. **(A)** Schematic diagram of the experimental design to establish the HM (*w*AlbA*w*AlbB*w*Mel) line with a *Wolbachia* triple-strain infection. **(B)** Representative results of the strain-specific amplification of the three *Wolbachia* strains, *w*Mel, *w*AlbA, and *w*AlbB, in a PCR assay. MGYP2, the transinfected *Aedes aegypti* line carrying *w*Mel, serving as a donor during the embryonic microinjection experiment in this study. HOU, *Ae. albopictus* HOU line carrying *w*AlbA and *w*AlbB, serving as the recipient. HM, transinfected *Ae. albopictus* line carrying the triple infection *w*Mel, *w*AlbA, and *w*AlbB. **(C)** Maternal transmission efficiency was monitored by randomly selecting individuals from each generation, as indicated, and diagnosis of *Wolbachia* infection by PCR using strain-specific primers. The infection rate was calculated as the percentage of positive individuals in the tested samples at the designated generation.

TABLE 1 | Results of CI crosses among the HM (*w*AlbA*w*AlbB*w*Mel), GUA, and HC lines.

Expected CI type	Cross (♀ × ♂)	Infection type		Percent egg hatch*
		Female	Male	
Incompatible	HC × HM	*w*AlbA,*w*AlbB, *w*Pip	*w*AlbA,*w*AlbB, *w*Mel	43.2 ± 11.6 a
	HM × HC	*w*AlbA,*w*AlbB, *w*Mel	*w*AlbA,*w*AlbB, *w*Pip	0.01 ± 0.04 b
	GUA × HM	*w*AlbA,*w*AlbB	*w*AlbA,*w*AlbB, *w*Mel	46.7 ± 7.8 c
Compatible	HM × GUA	*w*AlbA,*w*AlbB, *w*Mel	*w*AlbA,*w*AlbB	55.5 ± 17.8 c
	HM × HM	*w*AlbA,*w*AlbB, *w*Mel	*w*AlbA,*w*AlbB, *w*Mel	55.1 ± 9.0 c
	HC × HC	*w*AlbA,*w*AlbB, *w*Pip	*w*AlbA,*w*AlbB, *w*Pip	51.3 ± 11.7 c

**Expressed as the mean for 15 replicates/cross type ± standard deviation. Different letters following the data indicate significant differences ($P < 0.001$) by ANOVA-Tukey's multiple comparison test.*

fluctuating and varied from 8 to 65%, indicating that the low egg hatch rate may not be only caused by inbreeding; the maladaptation of the novel triple-strain infection to the HOU genetic background may also have contributed to this fitness cost. Thus, at G16, we started to outcross the HM (*w*AlbA*w*AlbB*w*Mel) females with males of the GUA strain, a wild-type *Ae. albopictus* recently collected from the field in Guangzhou, China (Zheng et al., 2019). A steady increase in the egg hatch rate of the outcrossed HM (*w*AlbA*w*AlbB*w*Mel) line was then observed, from 21% at G19 to 84% at G27 (**Figure 3**), the higher level being similar to that in the GUA strain. Thus, it appears that the GUA genetic background is able to overcome the triple infection-associated decrease in egg hatch rate in the HM (*w*AlbA*w*AlbB*w*Mel) line.

*w*Mel Distribution in Both the Somatic and Germline Tissues in the HM (*w*AlbA*w*AlbB*w*Mel) Line

Wolbachia tissue tropism is an important determining factor underlying its viral blocking effect and maternal transmission. We first compared the densities of the three *Wolbachia* strains, *w*Mel, *w*AlbA, and *w*AlbB, in somatic tissues (salivary glands and midgut) and germline tissues (ovaries) of HM

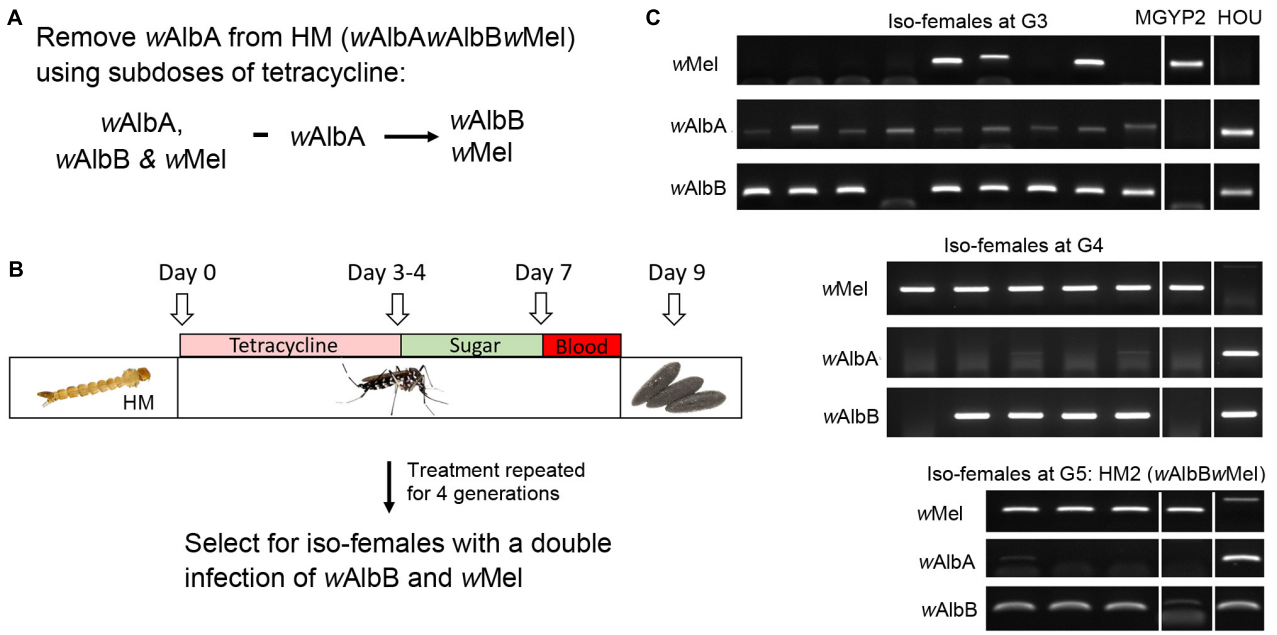

FIGURE 2 | Establishment of the HM2 (*w*AlbB*w*Mel) line carrying the double infection with *w*AlbB and *w*Mel. **(A,B)** Schematic diagram of the experimental approach to remove *w*AlbA from the triply infected HM (*w*AlbA*w*AlbB*w*Mel) line using subdoses of tetracycline. **(C)** Representative results from the PCR screening for isofemales carrying a double infection with *w*AlbB and *w*Mel. *w*AlbA was specifically removed after treatment for four consecutive generations.

TABLE 2 | Results of CI crosses among the HM2 (*w*AlbB*w*Mel), GUA, HB, and HC lines.

Expected CI type	Cross (♀ × ♂)	Infection types		Percent egg hatch*
		Female	Male	
Incompatible	HM2 × HC	*w*AlbB, *w*Mel	*w*AlbA, *w*AlbB, *w*Pip	0 ± 0 a
	HC × HM2	*w*AlbA,*w*AlbB *w*Pip	*w*AlbB, *w*Mel	9.0 ± 7.8 b
	GUA × HM2	*w*AlbA, *w*AlbB	*w*AlbB, *w*Mel	14.6 ± 8.3 b
	HM2 × GUA	*w*AlbB, *w*Mel	*w*AlbA, *w*AlbB	9.3 ± 4.2 b
	HB × HM2	*w*AlbB	*w*AlbB, *w*Mel	14.6 ± 6.1 b
Compatible	HM2 × HB	*w*AlbB, *w*Mel	*w*AlbB	79.6 ± 8.2 c
	HM2 × HM2	*w*AlbB, *w*Mel	*w*AlbB, *w*Mel	64.8 ± 17.7 d
	HB × HB	*w*AlbB	*w*AlbB	83.7 ± 6.9 c
	HC × HC	*w*AlbA, *w*AlbB, *w*Pip	*w*AlbA,*w*AlbB *w*Pip	80.8 ± 7.8 c
	GUA × GUA	*w*AlbA,*w*AlbB	*w*AlbA,*w*AlbB	85.6 ± 4.8 c

**Expressed as the mean for 15 replicates/cross type ± standard deviation. Different letters following the data indicate significant differences ($P < 0.001$) by ANOVA-Tukey's multiple comparison test.*

(*w*AlbA*w*AlbB*w*Mel) mosquitoes by qPCR. In the salivary glands at G6, the density of *w*AlbB was significantly higher than that of *w*AlbA, but there was no significant difference in density between *w*Mel and *w*AlbA or between *w*Mel and *w*AlbB (**Figure 4A**). In the midgut, a higher density of *w*AlbB than either *w*Mel or *w*AlbA was observed, whereas the densities of *w*Mel and *w*AlbA did not differ significantly (**Figure 4B**). These results indicates that *w*AlbB is dominant in the somatic tissue of HM (*w*AlbA*w*AlbB*w*Mel) mosquitoes. In contrast, a higher density of *w*Mel than of *w*AlbA or *w*AlbB is apparent in HM (*w*AlbA*w*AlbB*w*Mel) ovaries (**Figure 4C**). This distribution pattern was consistently maintained at G6 and G18 despite some degree of fluctuation.

To better understand the strain-specifc interactions in transinfected mosquitoes with triple infections, we also compared the densities of *w*Pip, *w*AlbA, and *w*AlbB in HC ovaries and observed a different order of *Wolbachia* density: *w*Pip > *w*AlbA > *w*AlbB (**Figure 4C**). Consistent with previous observations (Lu et al., 2012), *w*AlbB was present at a higher level than was *w*AlbA in the ovaries of HOU mosquitoes, from which both the HC and HM (*w*AlbA*w*AlbB*w*Mel) lines were originally derived (**Figure 4C**). We further compared the density of the same *Wolbachia* strain in ovaries across various mosquito lines to examine the impact of the host's genetic background on infection levels. *w*AlbA showed its highest level of infection in HM (*w*AlbA*w*AlbB*w*Mel) ovaries at G6 but decreased by 7.8-fold

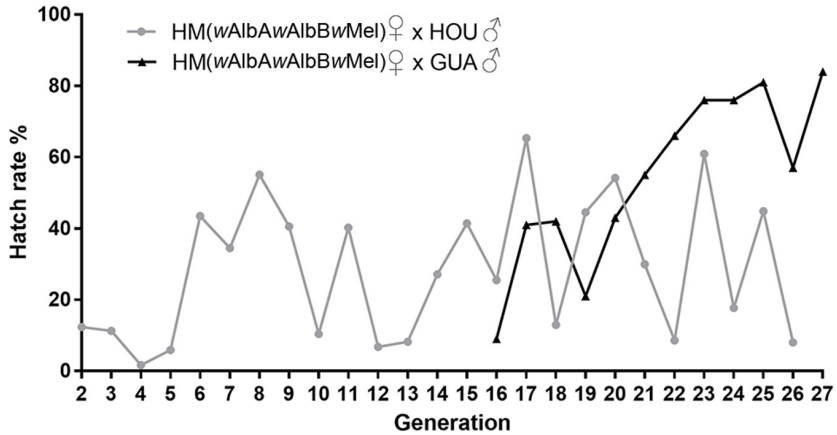

FIGURE 3 | Egg hatch rate of HM (*w*AlbA*w*AlbB*w*Mel) females outcrossed with wild-type males from G2 to G27. Egg hatch was calculated as the percentage of eggs hatched divided by the total number of eggs (see **Supplementary Table 1**). Outcrosses are indicated as female × male. HM (*w*AlbA*w*AlbB*w*Mel), the transinfected *Ae. albopictus* line carrying the triple infection with *w*Mel, *w*AlbA, and *w*AlbB. HOU and GUA, two wild-type *Ae. albopictus* lines carrying *w*AlbA and *w*AlbB.

at G18, when it reached a level closer to that in HOU ovaries. The density of *w*AlbB was stable in HM (*w*AlbA*w*AlbB*w*Mel) ovaries from G6 to G18 and was consistently maintained at a level significantly higher than that in both the HOU and HC lines (**Figure 4D**). The density of *w*Mel decreased by 47% in HM (*w*AlbA*w*AlbB*w*Mel) ovaries from G6 to G18 (**Figure 4D**) but was still much higher than that of the other *Wolbachia* strains. Interestingly, as compared to HOU ovaries, *w*AlbA was 1,022-fold higher in HM (*w*AlbA*w*AlbB*w*Mel) ovaries at G6, and *w*AlbB was 1,411-fold lower in HC ovaries (**Figure 4D**). Taken together, these results indicate that *Wolbachia* density is regulated in triply infected *Ae. albopictus* in a strain-, host-, and temporally specific manner.

DISCUSSION

We have demonstrated the successful establishment of a novel triple *Wolbachia* infection with *w*Mel, *w*AlbA, and *w*AlbB in the *Ae. albopictus* HM (*w*AlbA*w*AlbB*w*Mel) line, with 100% maternal transmission efficiency. Experimental crosses showed that CI is not induced when HM (*w*AlbA*w*AlbB*w*Mel) males mate with either GUA or HC females, but removal of *w*AlbA from the HM (*w*AlbA*w*AlbB*w*Mel) mosquitoes results in CI when these mosquitoes are crossed with three *Ae. albopictus* lines carrying either a single- (HB), double- (GUA), or triple- (HC) strain infection. Despite a severe reduction in the egg hatch rate associated with the triple infection, the rate was returned to normal levels by outcrossing with the wild-type GUA line, but not the HOU line. Among three different *Wolbachia* strains, *w*Mel and *w*AlbB were highest in density in the ovaries and midguts, respectively, of HM (*w*AlbA*w*AlbB*w*Mel) mosquitoes, whereas *w*Pip and *w*AlbB were present in the highest and lowest levels, respectively, in HC ovaries. The densities of *w*AlbA and *w*Mel, but not *w*AlbB, were reduced from G6 to G18 in HM (*w*AlbA*w*AlbB*w*Mel) ovaries. These results indicate the existence of complicated interactions in term of both tissue tropism and CI expression when various *Wolbachia* strains co-exist in a host, providing important information to guide the design and establishment of transinfections in mosquito with optimal *Wolbachia* strains or their combination for disease control.

Our results indciate that competition for tissue colonization may occur between *Wolbachia* strains in the same supergroup. In the phylogeny of *Wolbachia*, both *w*Mel and *w*AlbA belong to supergroup A, whereas *w*AlbB and *w*Pip belong to supergroup B (Werren et al., 2008). With the introduction of *w*Mel into *Ae. albopictus* HOU mosquitoes carrying *w*AlbA and *w*AlbB, we observed that the density of *w*AlbA decreased by 7.8-fold in HM (*w*AlbA*w*AlbB*w*Mel) ovaries from G5 to G18, but *w*AlbB density remained stable. The level of *w*Mel infection also decreased from G5 to G18, but this decrease could have been caused by either the adaption of *w*Mel to a novel host background or competition from *w*AlbA, or both. Consistent with a previous report (Lu et al., 2012), the *w*AlbB density was higher than that of *w*AlbA in HOU ovaries. In the triply infected HC mosquitoes, generated by transfer of *w*Pip to HOU mosquitoes (Zheng et al., 2019), *w*AlbB was suppressed to a minimal level in the ovaries. Specifically, the density of *w*AlbB (5.6×10^{-3} *wsp*/*rps6*) was 7,934- and 5,226-fold lower than that of *w*Pip (44.6 *wsp*/*rps6*) or *w*AlbA (29.4 *wsp*/*rps6*), respectively. It is worth noting that this low number of *w*AlbB was still sufficient to induce CI, given that unidirectional CI has been observed in crosses of HC and GUA mosquitoes (Zheng et al., 2019). Thus, when *Wolbachia* is being introduced into an infected host, choosing a novel strain belonging to a supergroup different from that of the orginal infection may prove useful for avoiding competition. Caution should be used if the native strain provides an essential benefit to the host, since the novel strain will likely outcompete the native strain in the transfected line, based on our observations from the HM (*w*AlbA*w*AlbB*w*Mel) and HC lines.

Competition for CI induction can also occur among different strains within the same supergroup. Although a single *w*Mel infection is able to induce CI in both *Ae. aegypti* and *Ae.*

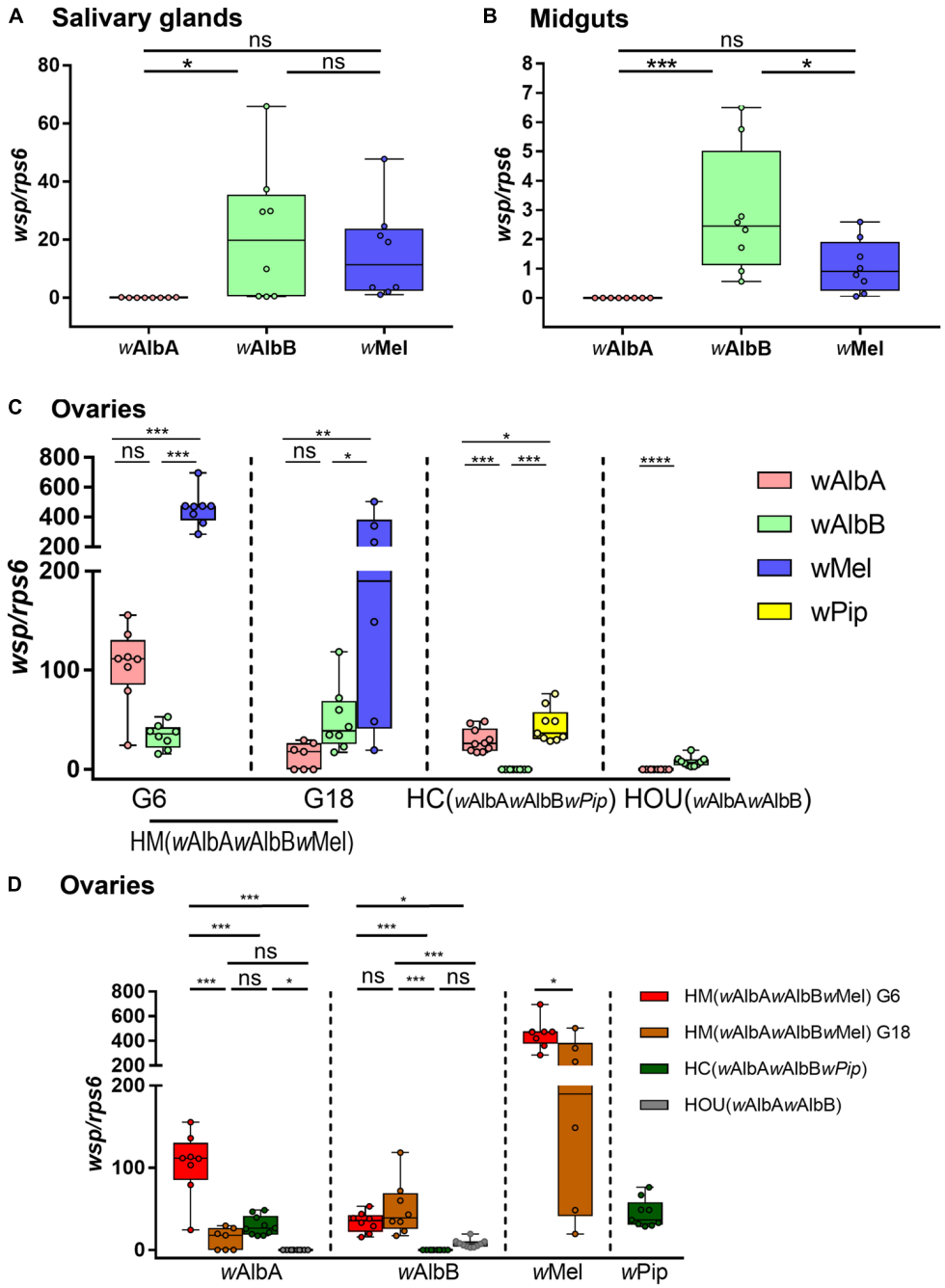

FIGURE 4 | The densities of various *Wolbachia* strains in the salivary glands, midguts, and ovaries of HM (*w*AlbA*w*AlbB*w*Mel) mosquitoes. The densities of *w*Mel, *w*AlbA, and *w*AlbB in salivary glands **(A)** and midguts **(B)** of HM (*w*AlbA*w*AlbB*w*Mel) mosquito were measured by quantitative PCR (qPCR). The densities of the three *Wolbachia* strains in the ovaries of HM (*w*AlbA*w*AlbB*w*Mel) mosquitoes from two generations, G6 and G18, were compared within **(C)** and across **(D)** mosquito lines. HC (*w*AlbA*w*AlbB*w*Pip), the transinfected *Ae. albopictus* line with *w*AlbA, *w*AlbB, and *w*Pip infections. HOU (*w*AlbA*w*AlbB), the wild-type *Ae. albopictus* line with *w*AlbA and *w*AlbB infections. The copy number of the *Wolbachia wsp* gene was normalized by the mosquito *rps6* gene (see **Supplementary Table 2**). The center of a box plot shows the median of 6–10 replicates, edges show upper and lower quartiles, and bars indicate maximum and minimum values. Dots show values from individual biological replicates. $^{****}P < 0.0001$; $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$; ns, not significant; ANOVA and Tukey's multiple comparisons test.

albopictus (Walker et al., 2011; Blagrove et al., 2012), HM (*w*AlbA*w*AlbB*w*Mel) males did not induce CI when crossed with either GUA or HC females. After removal of *w*AlbA from the HM (*w*AlbA*w*AlbB*w*Mel) line, however, we observe a strong CI expression in crosses of HM2 (*w*AlbB*w*Mel) with either GUA, HC or HB. These results indicate that the ability of *w*Mel to modify the HM (*w*AlbA*w*AlbB*w*Mel) sperm may be blocked by the presence of *w*AlbA, instead of *w*Mel-modified

sperm being rescued by *w*AlbA or *w*AlbB in HC or GUA mosquitoes. Consistent with our observations concerning HM2 (*w*AlbB*w*Mel) crosses, double infection of *w*AlbB and *w*Mel in transinfected *Ae. aegypti* was able to induce CI in the crosses with either non-infected, *w*AlbA-, *w*AlbB-, or *w*Mel-infected lines (Joubert et al., 2016). A similar effort to develop a triple infection (*w*AlbA, *w*AlbB, and *w*Mel) in *Ae. Albopictus* has been previously reported, but it resulted in very different outcomes: the triply infected line was self-incompatibility, its female was incompatible with wild-type male, and its male induced CI when crossed with wild-type females (Ant and Sinkins, 2018). It appears that the ability to rescue CI modification is compromised in their triply infected female, wherea the ability to induce CI is inhibited in our triply infected male. One possible explaination for the difference from our study is that the *w*AlbA density in the embryos of their triply infected line was inhibited to such an extent that it was impossible for *w*AlbA to rescue the CI modification in the males; in contrast, in our case the infection level of *w*AlbA was not significantly reduced in the HM (*w*AlbA*w*AlbB*w*Mel) ovaries when compared to wild-type. A similar experiment with different observation indicates complicated *Wolbachia*-host interactions when multiple strains coexsit and stresses the importance of repeating transinfection experiments with different genetic backgrounds of both donor and recipient strains as a way to obtain a useful combination of parameters.

Blocking by *w*AlbA of the *w*Mel-induced modification of sperm in the HM (*w*AlbA*w*AlbB*w*Mel) line suggests a potentional competition for host targets of CI factors between *w*Mel and *w*AlbA. Recent studies have suggested a "two-by-one" model underlying the CI mechanism in which *Wolbachia*-induced sperm modification is determined by two CI factors, *cifA* and *cifB*, whereas CI rescue is determined only by *cifA* (LePage et al., 2017; Shropshire et al., 2018; Beckmann et al., 2019; Chen et al., 2019). Further evidence has suggested that *cifB* targets nuclear protein import and protamine-histone exchange and that *cifA* rescues embryos by restricting the access of *cifB* to its targets (Beckmann et al., 2019). We hypothesize that the *cifB* genes of *w*Mel and *w*AlbA are very similar, so that they bind to the same sites that affect the host's nuclear protein import and then are translocated to the nucleus, where their substrates for sperm modification reside. The affinity of native *w*AlbA for host targets may be higher than that of *w*Mel, thus preventing the *w*Mel from entering the nucleus to induce CI expression.

Very low rates of egg hatching were observed in the HM (*w*AlbA*w*AlbB*w*Mel) line before G6. Suprisingly, the outcross with wild-type HOU only increased egg hatch rates temporarily in some generations (e.g., G6, G17, and G23); in these cases, there was an immediate decline afterward, resulting in a fluctuation wave across 26 generations. When the HC line was initially established, low hatch rates were also observed for almost a year. The situation was different for the other transfected lines that we established, in that egg hatching quickly returned to a normal level after the outcrosses with wild-type for several consecutive generations (Xi et al., 2005a,b, 2006). It would presumably be more challenging for the host to establish a symbiotic relationship with a *Wolbachia* triple strain than with a single or double strain because of the overload of symbionts and the complicated interactions between various strains and the host. Interestingly, outcrosses of HM (*w*AlbA*w*AlbB*w*Mel) with another wild-type line, GUA, effectively recovered normal egg hatch rates, indicating that the GUA genetic background can facilitate the host's adaptation to the novel triple infection. Because HM (*w*AlbA*w*AlbB*w*Mel) was derived from HOU, which had been maintained for a long time in the laboratory, outcrosses with HOU may not be able to introduce as much genetic heterogeneity to foster a novel symbiosis as can outcrosses with GUA, which was recently established from field samples (Zheng et al., 2019).

Here, we have demonstrated the successful establishment of a transinfected *Ae. albopictus* HM (*w*AlbA*w*AlbB*w*Mel) line carrying a *Wolbachia* triple-strain infection. Unfortunately, the newly introduced *w*Mel strain failed to induce CI in this triply infected line, and our experimental evidence indicates that its ability to modify the sperm was blocked by the native strain, *w*AlbA. Further studies are needed to compare the CI determination factors associated with *w*Mel and *w*AlbA and to understand the molecular mechanism undergirding their potential competition in utilizing host targets for CI expression. The tissue tropism of the three *Wolbachia* strains in the HM (*w*AlbA*w*AlbB*w*Mel) line indicates their complicated interactions, with competition likely to happen between *Wolbachia* strains in the same supergroup. The differences in both CI expression and *Wolbachia* tissue tropism between the two triply transinfected lines HM (*w*AlbA*w*AlbB*w*Mel) and HC also indicate that caution is necessary when predicting the outcome of transinfected lines with multiple infections. These results provide important information to guide the future selection of *Wolbachia* strains for the development of transinfected lines in order to obtain the maximum pathogen-blocking efficiency, the lowest fitness cost, and ideal CI patterns.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

XL and ZX contributed to conceptualization, methodology, validation, formal analysis, investigation, data curation, and original draft preparation. JL was responsible for CI crosses. GB performed embryo microinjection. ZX supervised the study. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01638/full#supplementary-material

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Wolbachia Inhibits Binding of Dengue and Zika Viruses to Mosquito Cells

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As traditional approaches to the control of dengue and Zika are insufficient, significant efforts have been made to develop utilization of the endosymbiotic bacterium *Wolbachia* to reduce the ability of mosquitoes to transmit pathogens. Although *Wolbachia* is known to inhibit flaviviruses in mosquitoes, including dengue virus (DENV) and Zika virus (ZIKV), it remains unclear how the endosymbiont interferes with viral replication cycle. In this study, we have carried out viral binding assays to investigate the impact of the *Wolbachia* strain *w*AlbB on the attachment of DENV serotype 2 (DENV-2) and ZIKV to *Aedes aegypti* Aag-2 cells. RNA interference (RNAi) was used to silence a variety of putative mosquito receptors of DENV that were differentially regulated by *w*AlbB in Aag-2 cells, in order to identify host factors involved in the inhibition of viral binding. Our results showed that, in addition to suppression of viral replication, *Wolbachia* strongly inhibited binding of both DENV-2 and ZIKV to Aag-2 cells. Moreover, the expression of two putative mosquito DENV receptors – dystroglycan and tubulin – was downregulated by *w*AlbB, and their knock-down resulted in the inhibition of DENV-2 binding to Aag-2 cells. These results will aid in understanding the *Wolbachia*-DENV interactions in mosquito and the development of novel control strategies for mosquito-borne diseases.

Keywords: *Wolbachia*, dengue, Zika, viral entry, mosquito

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INTRODUCTION

Dengue virus (DENV), a member of the family *Flaviviridae*, is the causative agent of dengue fever, dengue hemorrhagic fever, and dengue shock syndrome. As a major public health problem, with approximately 2.5 billion people at risk of pathogen transmission, DENV causes up to 50 million infections annually, in over 100 endemic countries, with 22,000 deaths mainly among children (Bhatt et al., 2013). Zika virus (ZIKV) is another flavivirus, which, in 2016, the World Health Organization (WHO) declared a Public Health Emergency of International Concern, because of its outbreak in the Americas and the widespread microcephaly and other neurological disorders it caused. Both DENV and ZIKV are transmitted to humans by *Aedes* mosquitoes, including the two species *Aedes aegypti* and *Aedes albopictus*. Lack of effective vaccines and antiviral therapies means that vector control is the primary intervention tool, which has been insufficient to prevent the global spread of dengue (Guzman et al., 2010). In order to meet the challenge of controlling DENV and ZIKV, innovative approaches – including *Wolbachia*-based replacement and suppression of mosquito vector populations – are currently under development for disease control (Hoffmann et al., 2011; Zheng et al., 2019).

Flaviviruses, including DENV and ZIKV, are enveloped positive-strand RNA viruses with a genome of approximately 11 kilobases, which have a single open reading frame encoding three structural proteins – capsid (C), membrane (M), and envelope (E) protein – and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5; Mukhopadhyay et al., 2005; Harris et al., 2006). The viral life cycle begins with the binding of virions to their cellular receptors on the surface of susceptible host cells (Yazi Mendoza et al., 2002; Thepparit and Smith, 2004; Reyes-Del Valle et al., 2005; Mercado-Curiel et al., 2006), followed by entrance into the host cells through receptor-mediated endocytosis (Acosta et al., 2008; Miller et al., 2008; van der Schaar et al., 2008), then fusion of the viral membrane with the endosome membrane, and subsequent delivery of the nucleocapsid into the cell cytoplasm (Heinz and Allison, 2003; Bressanelli et al., 2004). Following uncoating of the nucleocapsid in the cell cytoplasm, replication and translation of the viral RNA begin. Assembly of immature virions occurs on the surface of the host cell endoplasmic reticulum (ER), with newly formed nucleocapsids budding into the ER lumen (Welsch et al., 2009; Apte-Sengupta et al., 2014). Subsequently, the immature virions are transported through the trans-Golgi network (TGN), where they mature and form infectious particles (Zybert et al., 2008). Finally, the mature flaviviruses are released from the host cell by exocytosis.

Wolbachia are maternally transmitted intracellular symbiotic bacteria that are estimated to infect >65% of insect species and a large number of other arthropods, including ticks and mites, as well as filarial nematodes (Hilgenboecker et al., 2008). Through cytoplasmic incompatibility (Werren, 1997), *Wolbachia* can rapidly invade and become fixed in mosquito populations not already infected with the same *Wolbachia* stain (Xi et al., 2005; Hoffmann et al., 2011). Furthermore, different *Wolbachia* strains have been observed to induce resistance to DENV in mosquitoes (Moreira et al., 2009; Bian et al., 2010; Walker et al., 2011; Ford et al., 2019), with the strength of viral inhibition depending on the density of *Wolbachia* (Osborne et al., 2009, 2012; Lu et al., 2012; Chrostek et al., 2013). This *Wolbachia*-mediated pathogen interference also has a broad spectrum, being effective against a variety of RNA viruses, including ZIKV, West Nile virus (WNV), and yellow fever and chikungunya viruses, as well as eukaryotic parasites, such as *Plasmodium* and filarial nematodes (Kambris et al., 2009; Moreira et al., 2009; Glaser and Meola, 2010; van den Hurk et al., 2012; Bian et al., 2013; Hussain et al., 2013). Although the detailed mechanism(s) underlying viral interference are not well understood, it is believed that both immune priming and metabolic alterations of the host contribute to pathogen resistance (Pan et al., 2012; Caragata et al., 2013). For example, *Wolbachia* induces the production of reactive oxygen species in both naturally-infected and artificially-transinfected insect hosts, which can trigger either direct and/or indirect antiviral responses (Brennan et al., 2008; Pan et al., 2012; Wong et al., 2015). *Wolbachia* also perturbs host metabolic pathways/networks (Caragata et al., 2013; Melnikow et al., 2013), which may interfere with host factors required for completion of the viral life cycle (Guo et al., 2010). In addition, genetic variation in certain host factors has been observed to affect the strength of *Wolbachia*-mediated viral blocking in mosquitoes (Ford et al., 2019). However, the impact of *Wolbachia* on the flavivirus life cycle has not yet been fully characterized. Previous studies have found that the *Wolbachia* strain *w*Stri inhibited both ZIKV entry into *A. albopictus* cells and replication of the viral genome (Schultz et al., 2018). By contrast, the *Wolbachia* strain *w*Mel was not observed to inhibit DENV binding or entry to *A. aegypti* Aag-2 cells (Thomas et al., 2018). The *Wolbachia* strain *w*MelPop was reported to enhance replication of the WNV genome, but it reduced production of secreted virus in the Aag-2 cell line (Hussain et al., 2013). However, a reduction of WNV and DENV replication, rather than enhancement, was observed in both *w*Mel-infected and *w*AlbB-infected Aag-2 cells, respectively (Lu et al., 2012; Thomas et al., 2018). Thus, further studies are needed to clarify how the virus life cycle is affected by the presence of *Wolbachia* in host cells, and the universality of these impacts are among both different flaviviruses and *Wolbachia* strains. As different host factors participate in each stage of the virus life cycle (Kuadkitkan et al., 2010; Colpitts et al., 2011; Munoz Mde et al., 2013), knowledge of how the viral life cycle is affected by *Wolbachia* could provide important insights allowing further dissection of *Wolbachia* – flavivirus interactions in the mosquito host, thus facilitating the development of *Wolbachia*-mosquito symbioses with the greatest possible viral blocking.

We have previously shown that *w*AlbB induces strong resistance to the DENV serotype 2 (DENV-2) in the Aag-2 cell line (Lu et al., 2012) and that the strength of this viral inhibition depends on the density of *w*AlbB within host cells (Lu et al., 2012). DENV-2 is eliminated at a high density of *Wolbachia* in host cells, while both virus and the endosymbiont coexist in the cytoplasm of mosquito cells if *w*AlbB is present at a low density (Lu et al., 2012). In this study, in order to better understand how DENV is inhibited by *w*AlbB, we have focused on the impact of *w*AlbB on DENV-2 life cycle. Our results show that *w*AlbB prevents the intracellular accumulation of viral genome copies in Aag-2 cells by inhibiting the binding of both DENV-2 and ZIKV to Aag-2 cells and so prevents viruses from entering the next stage of their life cycle. Furthermore, we identify several mosquito host proteins bound by DENV, whose expression is downregulated by *w*AlbB and for which gene silencing is shown to interfere with viral binding in mosquito cells.

MATERIALS AND METHODS

Cell and Viral Culture

The *A. aegypti* W-Aag-2 cell line was generated by transinfecting Aag-2 cells (Peleg, 1968) with *Wolbachia* using the shell vial technique, as previously described (Lu et al., 2012). The R-Aag-2 cell line was generated from W-Aag-2 by treatment of the latter with the antibiotic rifampicin (Lu et al., 2012). The W-Aag-2 and R-Aag-2 cell lines were maintained at 25°C in Schneider's Drosophila Medium (Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin (Life Technologies) and were passaged at 1:5 dilution every 6–7 days.

The New Guinea C (NGC) strain of DENV-2 was grown in W-Aag-2 and R-Aag-2 cells, as previously described (Sim and Dimopoulos, 2010). Briefly, cells were seeded in a 48-well plate to a confluency of 80%. W-Aag-2 and R-Aag-2 monolayers were then infected with DENV-2 at the desired multiplicity of infection (MOI) of DENV-2. Plates were incubated at 25°C for the duration of the experiment. The ZIKV PRVABC59 strain, obtained from ATCC, was grown in Vero cells and cultured with Dulbecco's Modified Eagle Medium (10% FBS) at 37°C with 5% CO2, and the titer was measured by plaque assay.

DENV-2 and ZIKV Binding Assays

Binding assays were carried out to characterize the attachment of DENV and ZIKV to W-Aag-2 and R-Aag-2 cells. Prior to the initiation of viral binding, the culture medium was removed and cells were washed with cold Schneider's Drosophila Medium. Subsequently, viruses were overlain on the cell cultures and incubated with the cells for 1 h at 4°C, with either DENV-2 at an MOI of 1 or 10 or ZIKV at an MOI of 0.1. The cells were washed three times with cold phosphate-buffered saline (PBS) to remove any unbound virus, followed by the addition of 350 µl buffer RLT (QIAGEN) to each well for RNA extraction. The number of gene copies of bound DENV-2 and ZIKV were quantified by real-time PCR. For the DENV-2 assays, the incubation medium was collected for measurement of the titer of unbound viruses.

RNA Extraction, cDNA Synthesis, and Quantitative Reverse Transcription Polymerase Chain Reaction

Total RNA was extracted from the cell lines using the RNeasy Mini Kit (QIAGEN), and then the cDNA transcript was produced using the QuantiTect Reverse Transcription Kit (QIAGEN). Real-time PCR was conducted using the QuantiTect SYBR Green PCR Kit (QIAGEN) and an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). DENV-2 genomic RNA was measured by quantitative reverse transcription PCR (qRT-PCR) using primers directed to the *NS5* gene (Molina-Cruz et al., 2005). The copy numbers of both DENV-2 and ZIKV genomes were normalized using the host (*A. aegypti*) ribosomal protein S6 (*rps6*) gene. A standard curve was generated for each of the *NS5* and *rps6* genes by analyzing 10^1–10^8 copies/reaction of two different plasmids, containing a fragment of each gene (Lu et al., 2012). The number of genome copies of bound ZIKV was quantified by qRT-PCR using the primers ZIKV 835 and ZIKV 911c (Lanciotti et al., 2008). *Wolbachia*-regulated expression of mosquito DENV-binding proteins was assayed using the primers listed in **Supplementary Table S1**.

Tagged RT-PCR was used to specifically amplify the negative sense viral RNA by preventing false priming (Peyrefitte et al., 2003). The primer tagF 5'-CGGTCATGGTGGCGAATAAAC AAGTAGAACAACCTGGTCCAT-3' was designed to contain the DENV-targeting sequence in its 3'-end and a 19-mer-long non-DENV sequence in its 5'-end. After RNA extraction, the RNAs were denatured at 65°C for 3 min in the presence of 20 pmol of tagF primer for the negative strand-specific reverse transcription. cDNA was synthesized without addition of the RT primer mix. Real-time PCR was performed with a forward primer Tag 5'-CGGTCATGGTGGCGAATAA-3' and a DENV-targeting reverse primer, as previously described (Molina-Cruz et al., 2005). The host *rps6* gene was used to normalize the cDNA template.

Indirect Immunofluorescence Assay

Cells were seeded in an 8-well plate to a confluency of 80%. After the medium was removed, cells were washed with PBS, fixed with 4% formaldehyde solution for 15 min at room temperature, and then treated with 0.5% Triton X-100 in PBS for 5 min. Samples were incubated with 10% non-fat dry milk blocking solution at room temperature with gentle shaking for 1 h, followed by incubation with a rabbit anti-WSP primary antibody (GenScript) at 1:500 and Alexa Fluor 488-conjugated secondary antibody (Invitrogen) at 1:1000. After incubating with 0.1 µg/ml DAPI for 1 min, the samples were examined using an Olympus FluoView 1000 Laser Scanning Confocal Microscope.

Wolbachia Quantitative PCR

Quantitative PCR (qPCR) was performed to measure the density of *Wolbachia* in W-Aag-2 cells, as described previously (Tortosa et al., 2008). In brief, genomic DNA was extracted and *w*AlbB was amplified with the forward primer 183F (5'-AAGGA ACCGAAGTTCATG-3') and the reverse primer QBrev2 (5'-AGTTGTGAGTAAAGTCCC-3'), which are specific for the *Wolbachia* surface protein (*wsp*) gene. The *Wolbachia* genome copy was normalized with the host *rps6* gene.

DENV-2 RNA Transfection

W-Aag-2 and R-Aag-2 cells were seeded in a 48-well plate for 24 h prior to transfection and were at a confluency of 70–80% at the time of transfection (1×10^5 cells/well). DENV-2 RNA was extracted from virus-infected cell culture supernatant using RNeasy Mini Kit (QIAGEN). The infectious DENV RNA was transfected using TransIT-mRNA Transfection Kit (Mirus) according to the manufacturer's instructions. Briefly, DENV-2 RNA (0.5 µg) was incubated with 1 µl messenger RNA (mRNA) Boost Reagent and 1 µl *TranIT*-mRNA Reagent in 26 µl Schneider's Drosophila Medium for 5 min. The mixture was then transferred to the 48-well plate with W-Aag-2 or R-Aag-2 cells already grown to a 70–80% confluence (1×10^5 cells/well) in 260 µl complete medium. Four hours later, the medium containing transfection reagent was removed and replaced with normal fresh culture medium. This latter time point was designated as 0 h post-transfection. Cell lysates were collected at 0 h, 4 h, 3 days, and 7 days post-transfection to measure the levels of total DENV-2 RNA (0 h) and negative strand RNA (4 h, 3 days, and 7 days).

Plaque Assays for DENV-2 Virus Titration

DENV-2 titers were measured by plaque assays, as previously reported (Das et al., 2007; Bian et al., 2010). Briefly, C6/36 cells were seeded in the 48-well plate at a density of $4–8 \times 10^4$ cells/well and maintained for 2–3 days at 32°C in 5% CO_2. The virus-containing culture medium was serially diluted and inoculated into C6/36 cells. After incubation for 5 days, plaque forming units (PFUs) were measured in the plates by peroxidase

immunostaining, using mouse hyperimmune ascitic fluid (specific for DENV-2; CDC) as the primary antibody and a goat anti-mouse horseradish peroxidase (HRP) conjugate as the secondary antibody.

RNAi-Mediated Gene Silencing

Double-stranded RNA (dsRNA) was synthesized from PCR-amplified gene fragments using the MEGAscript T7 High Yield Transcription Kit (Ambion). The sequences of the primers are listed in **Supplementary Table S2**. Transfection of dsRNA was carried out using Attractene Transfection Reagent (Qiagen) according to the manufacturer's instruction. Briefly, cells were seeded in the 48-well plate for 24 h prior to transfection. One microgram of dsRNA was incubated with 3.5 μl Attractene Transfection Reagent in 50 μl Schneider's Drosophila Medium for 10–15 min at room temperature and then transferred to each well. Three days post-transfection, DENV-2 binding assays were then performed at 4°C on R-Aag-2 and W-Aag-2 cells with an MOI of 10. Gene silencing efficiency was determined by comparing the relative mRNA levels of the target gene after knockdown with its specific dsRNA and dsRNA of green fluorescent protein (dsGFP, the non-target control) using real-time PCR.

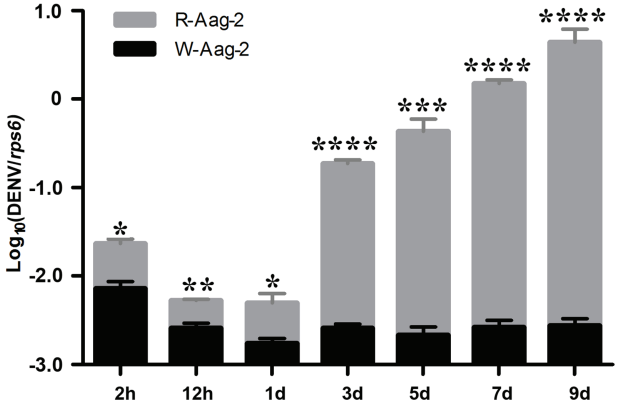

FIGURE 1 | Comparison of dengue virus serotype 2 (DENV-2) dynamics in W-Aag-2 and R-Aag-2 cells. The copy number of DENV-2 genomic RNA was measured by quantitative reverse transcription PCR (qRT-PCR) in *Aedes aegypti* W-Aag-2 and R-Aag-2 cells at different times post-infection. Ribosomal protein S6 (*rps6*) gene was used as a host gene to normalize the data. Error bars are standard errors of the mean of three biological replicates for each cell line. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$; Student's *t*-test.

RESULTS

*w*AlbB Inhibits Intracellular Accumulation of DENV-2 Genome Copies in Aag-2 Cells

We previously reported that the *Wolbachia* strain *w*AlbB induced density-dependent inhibition of DENV-2 in mosquito cells (Lu et al., 2012). In order to further investigate the dynamics of DENV suppression by *w*AlbB, we compared the number of genome copies of DENV-2 at various times post-infection in *w*AlbB-infected Aag-2 cells (W-Aag-2) and aposymbiotic cells (R-Aag-2, a cell line derived from W-Aag-2 cells through rifampicin treatment and used as a control). After both cells were infected with DENV-2 at an MOI of 1, the number of genome copies of DENV-2 was measured by qRT-PCR at seven different time points over the course of the 9-day experiment. Overall, the number of genome copies of DENV-2 was significantly lower in W-Aag-2 cells than R-Aag-2 cells at all seven of the time points assayed (**Figure 1**). At 2 h post-infection, the mean genome copy number of DENV-2 in R-Aag-2 cells was 3.2-fold higher than in W-Aag-2 cells, suggesting that *w*AlbB may interfere with early events in virus life cycle. The magnitude of variation in viral genome copies between two cell lines increased markedly from 3 dpi (i.e., when genome replication was first detectable in R-Aag-2 cells). Consequently, the viral copy number increased 72-, 200-, 574-, and 1,577-fold in R-Aag-2 cells relative to W-Aag2 cells at 3, 5, 7, and 9 dpi, respectively, indicating that *w*AlbB constantly and persistently inhibited intracellular accumulation of DENV genome copies in W-Aag-2 cells (**Figure 1**).

*w*AlbB Inhibits Binding of DENV-2 and ZIKV to Aag-2 Cells

The observed viral inhibition at 2 h post-infection indicated that the viral interference might occur as early as virus binding to and/or entry into host cells. Thus, we tested whether *w*AlbB prevented DENV-2 from binding to Aag-2 cells. Both W-Aag-2 and R-Aag-2 cells were incubated with DENV-2 at an MOI of either 10 or 1 for 1 h at 4°C to allow virus binding to – but not penetration of – host cells (Salas-Benito and del Angel, 1997; Wei et al., 2003). Immediately, the challenged host cells were then washed three times with ice-cold medium, and the number of bound RNA copies of DENV-2 was determined by qRT-PCR. At an initial MOI of 10, the amount of DENV-2 bound to W-Aag-2 cells (0.0033 RNA copies per RNA copy of host *rps6*) was 3.9-fold lower than the amount bound to R-Aag-2 cells (0.013 copies per copy *rps6*; **Figure 2A**). This represents a 75% reduction in binding of DENV-2 to Aag-2 cells. Similar results were observed when mosquito cells were exposed to lower levels of virus. At an MOI of 1, the viral genome copy number (0.0006 copies per copy of *rps6*) was 3.2-fold lower in the W-Aag-2 cell line than R-Aag-2 cells (0.0019 copies per copy of *rps6*; **Figure 2A**). For further validation, we also measured, using plaque assays, the titers of unbound DENV in the incubation medium. Consistent with an inhibition of viral binding to cells, the unbound viral titer in the incubation medium of W-Aag-2 cells (3.4×10^6 PFU/ml) was 2.1-fold higher than that of R-Aag-2 cells (1.6×10^6 PFU/ml) at an MOI of 10 (**Figure 2B**). At an MOI of 1, a 3.3-fold increase in the unbound viral titer was also observed in the incubation medium of W-Aag-2 cells (2.4×10^5 PFU/ml) compared to that of R-Aag-2 cells (7.2×10^4 PFU/ml; **Figure 2B**). In order to test how viral infection was affected by a temperature that allowed DENV-2 to both bind and penetrate into host cells, we performed the same assays at 25°C. At an initial MOI of 10, the genome copy number of DENV-2 was 6.1-fold lower in W-Aag-2 cells (0.0036 copies per copy of *rps6*) than R-Aag-2 cells (0.022 copies per copy of *rps6*; **Figure 2C**).

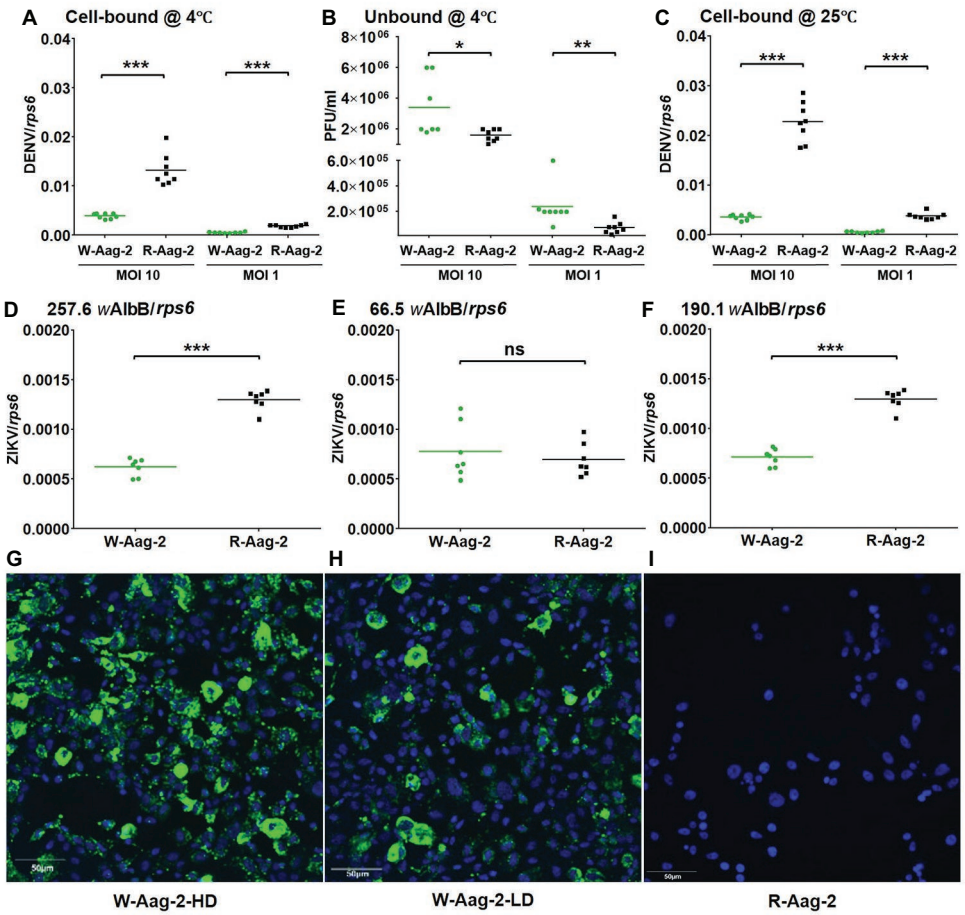

FIGURE 2 | Inhibition of binding of DENV-2 and Zika virus (ZIKV) to mosquito cells by *w*AlbB. **(A)** Binding of DENV-2 to W-Aag-2 and R-Aag-2 cells at 4°C. W-Aag-2 and R-Aag-2 cells were incubated with DENV-2 at either 10 MOI or 1 MOI for 60 min at 4°C. Virus-cell binding was measured by qRT-PCR and normalized using *rps6*. **(B)** Infectivity to C6/36 cells lacking *Wolbachia* infection of unbound DENV-2 in the culture medium from W-Aag-2 and R-Aag-2 cells. After incubation at 4°C with W-Aag-2 and R-Aag-2 cells, viral titer in the culture medium was measured by plaque assay to determine the amount of unbound virus. **(C)** W-Aag-2 and R-Aag-2 cells were incubated with DENV-2 at an MOI of either 10 or 1 for 60 min at 25°C. **(D–F)** Binding of ZIKV to Aag-2 cells at a 0.1 MOI for 60 min at 4°C, with different *w*AlbB densities in W-Aag-2 cells as indicated. **(G–I)** Representative indirect immunofluorescence assay (IFA) pictures showing: **(G)** high *Wolbachia* density (W-Aag-2-HD) in W-Aag-2 cells, **(H)** low *Wolbachia* density (W-Aag-2-LD) in W-Aag-2 cells, and **(I)** the absence of *Wolbachia* in R-Aag-2 cells. Viral genomic copies were measured by qRT-PCR and normalized by *rps6*. Lines indicate the median value of the eight biological replicates $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; ns, not significant; Mann Whitney *U* test.

A similar reduction was also observed at an MOI of 1, where the copy numbers of viral genomic RNA were 6.6-fold lower in W-Aag-2 cells (0.00058 copies per copy of *rps6*) than R-Aag-2 (0.0038 copies per copy of *rps6*; **Figure 2C**). The moderate variation in viral inhibition between 4 and 25°C does not support the conclusion that the viral internalization process is affected by *Wolbachia* as the low number of internalized viruses may be a simple consequence of binding inhibition.

In order to know whether *w*AlbB-mediated inhibition of DENV-2 binding to mosquito cells might apply to other flaviviruses, we repeated the above viral binding assays using ZIKV. At an initial MOI of 0.1, the amount of ZIKV bound to W-Aag-2 cells (0.000621 copies per copy of *rps6*) was 2.1-fold lower than the amount bound to R-Aag-2 cells (0.001296 copies per copy of *rps6*; **Figure 2D**). Quantitative PCR showed that the estimated relative density of *w*AlbB in the W-Aag-2 cells during this experiment was 257.6 copies of the *wsp* gene per copy of host cell *rps6*. In order to test whether the observed inhibition of ZIKV was influenced by the density of *Wolbachia*, we performed the same experiment again using cells with a lower *Wolbachia* density (66.5 *wsp*/*rps6*), which were random cultures from the same W-Aag-2 cell line but had different *Wolbachia* densities in a particular generation of culture. No significant difference was observed in the amount of ZIKV bound to W-Aag-2 cells as compared to R-Aag2 cells (**Figure 2E**). Interestingly, the density of *w*AlbB increased to 190.1 *w*AlbB/RPS6 after six passages of the low density *Wolbachia* culture of the above Aag-2 cells, and significant inhibition of viral binding to W-Aag-2 cells was observed again following this increase in *w*AlbB density (**Figure 2F**). The above high and

low densities of *w*AlbB in W-Aag-2 cells were also visualized using indirect immunofluorescence assay (IFA; **Figures 2G–I**). Overall, these results indicate that *w*AlbB needs a sufficiently high density to inhibit viral binding to mosquito cells.

*w*AlbB Inhibits DENV Replication in Aag-2 Cells

Given that the previous observation of *Wolbachia*-mediated viral interference is an accumulated outcome from viral binding and the other stages (Lu et al., 2012), we attempted to characterize the impact of *w*AlbB on virus replication alone. Thus, we delivered infectious DENV-2 RNA into host cells by transfection, in order to bypass the initial events in the life cycle of DENV infection – including binding, entry, nucleocapsid release, and uncoating – and then conducted tagged RT-PCR to measure negative-strand antigenomic RNA (Peyrefitte et al., 2003), a hallmark of active DENV replication (Tuiskunen et al., 2010). There was no significant difference in the amount of the viral genome (i.e., positive-strand RNA) in W-Aag-2 and R-Aag-2 cells at 0 h post-transfection (**Figure 3A**). However, we observed significantly lower copy numbers of viral negative-strand RNA at 4 h post-transfection in W-Aag-2 cells compared to R-Aag-2 cells (**Figure 3B**). This indicates that *w*AlbB inhibited virus genome replication by blocking synthesis of the viral negative-strand RNA. The copy number of viral negative-strand RNA was also measured at 3 and 7 days post-transfection. Again, there was a significantly lower copy number of viral negative-strand RNA in W-Aag-2 cells than R-Aag-2 cells at both time points (**Figure 3B**). In addition, the viral titer was also significantly lower in the supernatant of W-Aag-2 cells (2.8×10^3 PFU/ml) than R-Aag2 cells (1.0×10^7 PFU/ml) at 5 days post-transfection (**Figure 3C**). Overall, these observations indicate that *w*AlbB inhibits DENV-2 infection when the initial stages of the life cycle (i.e., binding, cell entry, and virion disassembly) are artificially by-passed using transfection.

*w*AlbB Regulates Expression of Host Cell Proteins Bound by DENV

In order to explore the molecular mechanism by which the DENV life cycle is inhibited by *w*AlbB in mosquito cells, we selected 21 mosquito host proteins that were previously reported as being bound by DENV (**Table 1**; Kuadkitkan et al., 2010; Colpitts et al., 2011; Munoz Mde et al., 2013) and tested whether *w*AlbB influenced the expression of the genes encoding these proteins. The 21 mosquito DENV-binding proteins were classified into two broad groups based on whether they were cell surface membrane proteins likely to be involved in viral binding to the host cell or non-cell surface membrane proteins putatively involved in the other (i.e., intracellular) stages of the DENV life cycle. We measured and compared their transcription in W-Aag-2 and R-Aag-2 cells by qRT-PCR. As a result, we found that – with the exception of prohibitin (AAEL009345) – seven out of the eight host membrane proteins were regulated by *w*AlbB in W-Aag-2 cells (**Figure 4**). Among them, dystroglycan (AAEL013147), laminin (AAEL001477), beta-tubulin (AAEL002851), and HSC70 (DQ440299) were downregulated by *w*AlbB, while cadherin (AAEL001196), enolase (AAEL001668), and BARK (AAEL006868) were upregulated in W-Aag-2 cells. In addition, 11 out of the 13 mosquito non-cell surface membrane proteins possibly involved in other stages of DENV life cycle were also regulated by *w*AlbB (**Figure 4**). Remarkably, histone 4 (AAEL003863) was downregulated more than 24.2-fold in W-Aag-2 cells compared to R-Aag-2 cells (**Figure 4**).

Silencing Membrane Binding Proteins Downregulated by *w*AlbB Results in Inhibition of DENV Binding to Aag-2 Cells

In order to examine how *w*AlbB inhibits DENV binding to Aag-2 cells, we used RNA interference (RNAi) to separately silence all eight of the mosquito host membrane proteins described above and then measured the copy number of DENV binding to host cells.

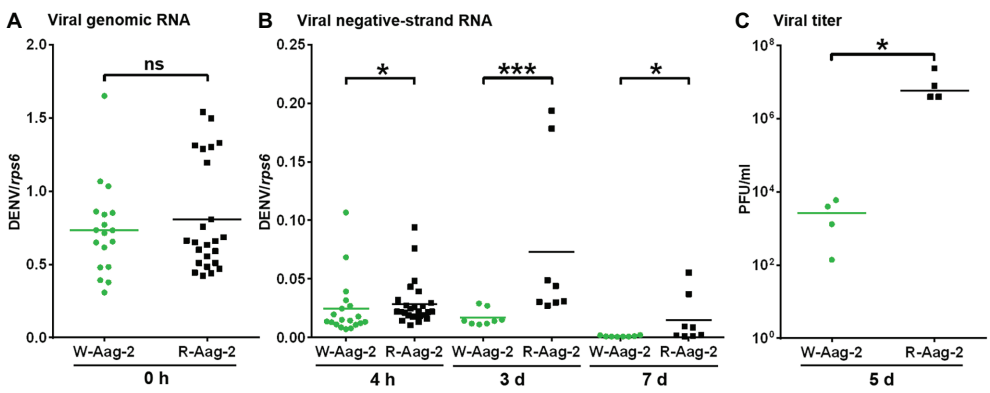

FIGURE 3 | Suppression of DENV RNA replication by *w*AlbB in mosquito cells. **(A)** The number of copies of DENV-2 genomic RNA in W-Aag-2 and R-Aag-2 cells at 0 h post-transfection. **(B)** The number of copies of DENV-2 negative-strand RNA in W-Aag-2 and R-Aag-2 cells at various times post-transfection. Equivalent amounts of purified DENV-2 RNA were transfected into cells. Cells were sampled at 4 h and 3 and 7 days post-transfection. Negative-strand RNA was measured by Tag-PCR. **(C)** The titer of DENV-2 in the supernatant of W-Aag-2 and R-Aag-2 cells at 5 days post-transfection. Viral titer was measured by plaque assay. *$p < 0.05$; ***$p < 0.001$; ns, not significant; Mann Whitney *U* test.

TABLE 1 | The 21 host proteins that were previously reported as being bound by DENV.

Gene ID	Gene name	Viral protein	Reference
AAEL003863	Histone 4	Capsid	(Colpitts et al., 2011)
AAEL015390	Histone 2A	Capsid	(Colpitts et al., 2011)
AAEL002851	Beta tubulin	E, NS2A	(Colpitts et al., 2011)
AAEL017096	EF-1 alpha	E, NS2A, NS4B	(Colpitts et al., 2011; Munoz Mde et al., 2013)
AAEL003670	Myelinprotein expression factor (MYEF)	NS2A	(Colpitts et al., 2011)
AAEL001928	Actin	Capsid, NS4B	(Colpitts et al., 2011)
AAEL001477	Laminin alpha-1, 2 chain	E	(Colpitts et al., 2011)
AAEL013147	Dystroglycan-like protein	E	(Colpitts et al., 2011)
AAEL015681	Histone 2B	Capsid	(Colpitts et al., 2011)
AAEL009994	60S ribosomal protein L4 (rpL4)	NS2A	(Colpitts et al., 2011)
AAEL006868	beta-adrenergic receptor kinase (BARK)	E	(Munoz Mde et al., 2013)
AAEL009345	Prohibitin	E	(Kuadkitkan et al., 2010)
AAEL000032	40S ribosomal protein S6 (*rpS6*)	NS2A	(Colpitts et al., 2011)
AAEL007439	Myosin light chain (MLC)	E	(Colpitts et al., 2011)
AAEL001668	Enolase	Capsid, E	(Colpitts et al., 2011; Munoz Mde et al., 2013)
AAEL001411	Myosin heavy chain (MHC)	E, NS2A	(Colpitts et al., 2011)
AAEL001196	Cadherin	E	(Colpitts et al., 2011; Munoz Mde et al., 2013)
AAEL000386	PI3 kinase	E	(Colpitts et al., 2011)
AAEL003594	Kinectin	Capsid	(Colpitts et al., 2011)
AAEL003827	Histone 3	Capsid	(Colpitts et al., 2011)
DQ440299	HSC70	E	(Paingankar et al., 2010)

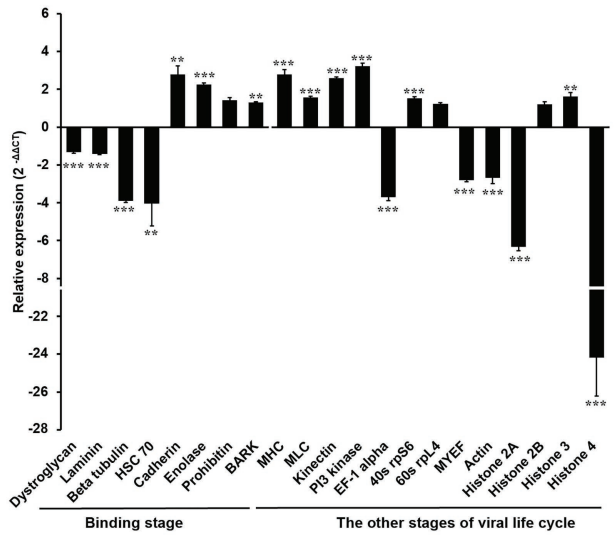

FIGURE 4 | Differential expressions in W-Aag-2 cells compared to R-Aag-2 cells of 21 host genes encoding proteins bound by DENV. The 21 host proteins bound by DENV were grouped into those involved in either the binding stage or other stages of viral life cycle. qRT-PCR was used to quantify the expression of each gene relative to the *rps6* gene, which was used as an internal reference control to normalize the data. The $2^{-\Delta\Delta CT}$ method was used to calculate the fold-change for each gene, and significance was determined based on comparison of ΔCT of each gene in W-Aag-2 and R-Aag-2 cells. Each gene has eight biological replicates. Mean ± SEM; $^{**}p < 0.01$; $^{***}p < 0.001$; Mann Whitney *U* test.

For those genes that were upregulated by *w*AlbB, we knocked them down in both W-Aag-2 and R-Aag-2 cells using their respective dsRNAs and tested whether the inhibition of viral binding was compromised compared to the control groups in which RNAi was performed using dsGFP. Individual silencing of cadherin, enolase, BARK, and prohibitin had no effect on *w*AlbB-induced inhibition of viral binding to Aag-2 cells, and difference in viral binding between W-Aag-2 and R-Aag-2 cells stayed the same, regardless of which of these four genes was silenced (**Figure 5A**). These observations indicate that they were not involved in *w*AlbB-mediated viral binding interference even if they were upregulated by *w*AlbB. However, cadherin and prohibitin silencing resulted in significant reduction in the number of viruses binding to both W-Aag-2 and R-Aag-2 cells as compared to the control group (**Figure 5A**). A similar reduction was also observed in R-Aag-2 cells but not in W-Aag-2 cells, after enolase was knockdown. The above indicate that these genes regulate binding of DENV to Aag-2 cells, although unrelated to *w*AlbB-mediated binding inhibition effects. A similar and high knockdown efficiency was observed for all four of these genes in both W-Aag-2 and R-Aag-2 cells (**Figure 5B**). For the four membrane proteins that were downregulated by *w*AlbB, we individually knocked them down only in R-Aag-2 cells, in order to mimic the impact of *w*AlbB in the W-Aag-2 cells, and then tested whether the RNAi resulted in a similar inhibition of viral binding to host cells. Knockdown of dystroglycan and beta-tubulin led to a significant reduction in DENV-2 binding compared to the control using dsGFP treatment, while silencing of HSC70 and laminin had no effect (**Figure 6A**). Over 70% knockdown efficiency was achieved for all four genes encoding the *w*AlbB-downregulated membrane proteins (**Figure 6B**). These results suggest that downregulation of the transcription of dystroglycan and beta-tubulin by *w*AlbB may explain inhibition of DENV binding to Aag-2 cells.

DISCUSSION

Wolbachia has shown a great potential to be used as a biocontrol agent to prevent transmission of flaviviruses due to its ability both to suppress mosquito populations and to render them resistant to viruses. Understanding the mechanisms underlying

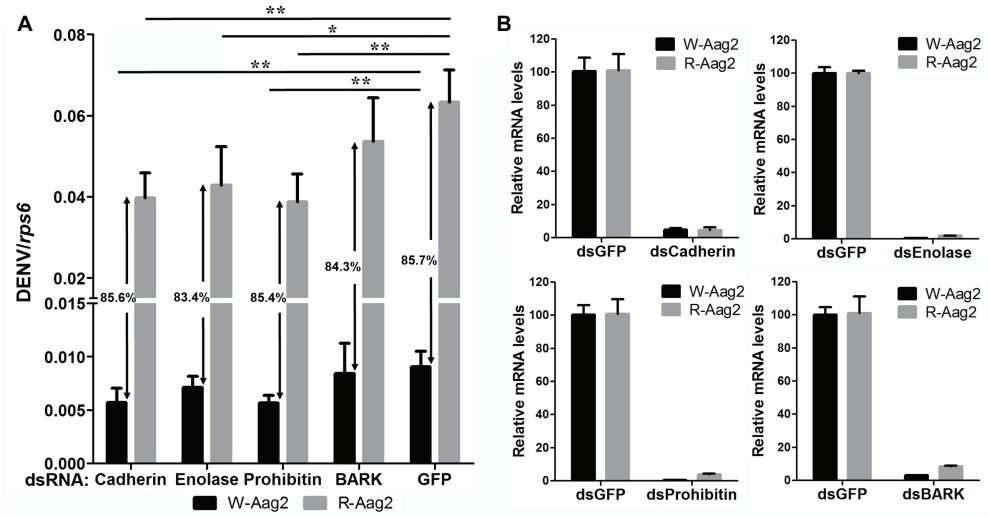

FIGURE 5 | The contribution of *w*AlbB upregulating putative DENV mosquito receptors to viral binding interference. **(A)** Each gene was knocked down individually in both W-Aag-2 and R-Aag-2 cells, and its impact on viral binding was measured through comparison with their respective control groups (the dsGFP treatment). An 83–86% reduction in viral binding was consistently observed for W-Aag-2 cells compared to R-Aag-2 cells, regardless of which of these four genes was silenced. **(B)** Knockdown efficiency was measured by the relative messenger RNA (mRNA) levels of the target gene after its RNA interference (RNAi) silencing in both W-Aag-2 and R-Aag-2 cells as compared to the dsGFP treatment. Each treatment has six biological replicates. Mean ± SEM; $^{*}p < 0.05$, $^{**}p < 0.01$; ANOVA and Dunn's multiple comparisons test.

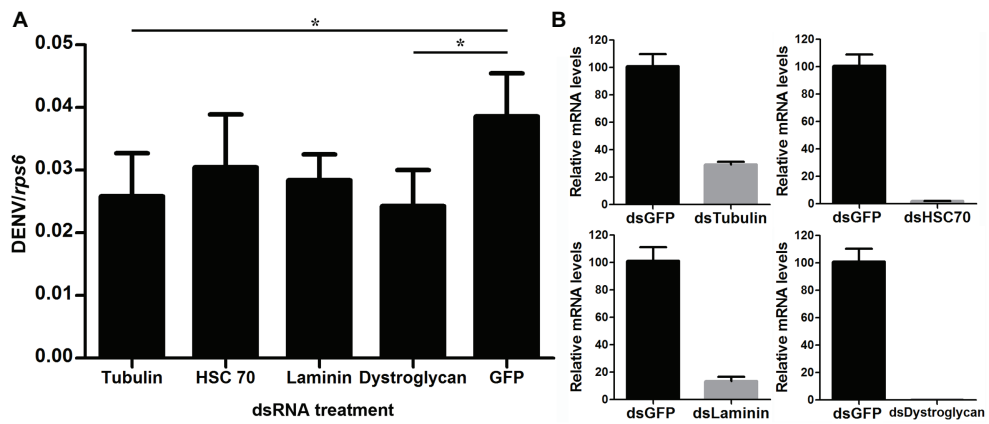

FIGURE 6 | The role of *w*AlbB downregulating putative DENV mosquito receptors in viral binding interference. **(A)** Each gene was knocked down individually in R-Aag-2 cells, and its impact on viral binding was measured through comparison with the control group (with the dsGFP treatment). **(B)** Knock-down efficiency was measured by the relative mRNA levels of the target gene after its RNAi silencing as compared to the dsGFP treatment. Each treatment has six biological replicates. Mean ± SEM; $^{*}p < 0.05$; ANOVA and Dunn's multiple comparisons test.

this viral interference will help facilitate the development and improvement of *Wolbachia*-based strategies for disease control. In this work, we showed that the *Wolbachia* strain *w*AlbB was able to persistently inhibit the intracellular accumulation of DENV RNA in W-Aag-2 cells. *w*AlbB not only significantly inhibited the synthesis of viral negative-strand RNA but also decreased the number of DENV-2 virions binding to Aag-2 cells, thus preventing attachment of the virus to host cells. In addition, *w*AlbB also inhibited binding of ZIKV to Aag-2 cells, an effect that was dependent on *Wolbachia* density. Lastly, we provided evidence to show that *w*AlbB-induced downregulation of two potential mosquito dengue receptors – dystroglycan and tubulin – might contribute to inhibition of viral binding to mosquito cells.

The first step of DENV life cycle is binding to host cells before entry into them. Direct binding assays at a low temperature (4°C) have been previously used to prevent subsequent viral entry so that the binding step can be studied without consideration of the impact of the downstream steps (Salas-Benito and del Angel, 1997; Wei et al., 2003). Based on this approach, we found that the number of DENV attached to W-Aag-2 cells was significantly less than that to R-Aag-2 cells, at both high and

low MOI. The magnitude of viral binding inhibition did not depend on the viral dose used to challenge the cells, and a 3–4 fold (or 68–75%) reduction in the number of virus binding to cells was observed at an MOI of both 10 and 1. A similar inhibition of ZIKV binding to Aag-2 cells, with a 2-fold (46%) reduction, was also observed when *w*AlbB density was high. Previous studies reported that *w*Stri inhibited entry of ZIKV into *A. albopictus* cells, while a similar inhibition was not observed in *w*Mel-infected Aag-2 cells (Schultz et al., 2018; Thomas et al., 2018). One of the potential reasons for these inconsistent observations is that the cell lines used in the two former studies may have had different *Wolbachia* densities, similar to the different results that were observed here in the ZIKV binding assays, using the same cell line but with either a low or high density of *w*AlbB. Consistent with a reduction in viral binding to W-Aag-2 cells, more viral particles were present in the culture medium of W-Aag-2 compared to that of R-Aag-2 cells. Furthermore, although they did not effectively bind to W-Aag-2 cells, using a plaque assay, we were able to demonstrate that unbound DENV-2 virions exposed to W-Aag-2 cells remained infective to mosquito cells lacking *Wolbachia* infection (**Figure 2B**). This latter result would seem to refute the hypothesis that antiviral effectors secreted into the culture medium from W-Aag-2 cells inactivate of DENV and thereby inhibit its binding to W-Aag-2 cells. There was a ~6-fold reduction in the copy number of DENV-2 at 1 h post-infection in W-Aag-2 compared to R-Aag-2 cells at 25°C, while only ~3-fold reduction was observed at 4°C. As the level of viral infection was similar in W-Aag-2 cells at both temperatures, this variation might be due to initiation of viral RNA replication following the binding stage in R-Aag2 cells at 25°C. This is consistent with the previous observations that DENV enters into mosquito cells within 5–7 min, and its replicative intermediate RNA could be detected as soon as 20 min post-infection in mosquito cells (Vaughan et al., 2002; Mosso et al., 2008).

In order to understand the molecular mechanism by which *w*AlbB inhibited the binding of DENV to mosquito cells, we determined the relative expression and knocked-down host genes, previously reported as being bound by DENV. Our results indicate that binding of DENV to cells may be inhibited by *Wolbachia* through its suppression of the expression of two membrane proteins: dystroglycan and tubulin, but it did not support the involvement of those *w*AlbB-induced host membrane proteins, including cadherin or enolase, in inhibition of viral binding to Aag-2 cells. The former is consistent with previous evidence of direct interaction between these putative DENV receptors. Alpha-dystroglycan is an extra-cellular protein which binds to laminin, a component of the extracellular matrix, and to beta-dystroglycan, a transmembrane protein which binds to components of the cytoskeleton, including tubulin and actin. Direct binding of the laminin receptor to tubulin and actin was also reported previously (Venticinque et al., 2011). Interestingly, actin is also downregulated by *Wolbachia*, and previous studies have showed that actin is involved with viral endocytosis and replication (Acosta et al., 2008). In both filarial nematodes and *Drosophila*, *Wolbachia* has been shown to interact with the cytoskeletal proteins, actin and tubulin, with potential functions involving facilitation of bacterial migration, distribution, and maternal transmission (Ferree et al., 2005; Melnikow et al., 2013). Thus, interaction of *Wolbachia* with a molecular complex comprising dystroglycan, tubulin, and actin may decrease the binding of DENV to mosquito cells.

It should be noted that the magnitude of viral binding inhibition is lower in RNAi-mediated silencing of either dystroglycan or tubulin than observed in the W-Aag-2 cells, although the degree of silencing produced by dsRNA is higher than the downregulation produced by *w*AlbB. This is probably caused by silencing of only one single gene in the dsRNA treatment, whereas *w*AlbB suppresses numerous host genes simultaneously, which can produce additive or synergistic effects on inhibition of viral binding to host cells. Viruses may utilize multiple redundant host membrane proteins to mediate the binding such that silencing of a single host gene may cause only a subtle effect on inhibition. In addition, transient effects induced by dsRNA-mediated gene silencing may also limit the robustness of this approach in recapitulating the ability of *Wolbachia* to affect those viral host factors during its persistent intracellular infection. However, it is possible that there are other unknown factors involved in this *Wolbachia*-mediated inhibition of viral binding to host cells. Lack of inhibition of ZIKV binding to Aag-2 cells at a low level of *w*AlbB infection suggests that the intracellular *Wolbachia* titer should be above a threshold to interfere with viral binding to the cells. Although further studies are needed to fully elucidate the impact of *Wolbachia* on each stage in the virus life cycle, the previous observation of viral inhibition without impact on viral binding (Thomas et al., 2018) suggests that *Wolbachia*-mediated viral inhibition may be mainly exerted in intracellular replication with the binding as an additional step.

The presence of negative-strand RNA is a hallmark of DENV replication within host cells. In order to study the impact of *Wolbachia* on viral replication, we used a transfection assay to directly introduce the DENV-2 genome into the cytoplasm of host cells and so bypass the initial life cycle stages of viral binding, entry, and uncoating. We then measured the copy number of viral negative-strand RNA. Even though an equivalent amount of DENV-2 genome was introduced into both W-Aag-2 and R-Aag-2 cells, we observed significantly lower copy numbers of viral negative-strand RNA in W-Aag-2 cells as compared to R-Aag-2 cells. Since it takes about 1 day for DENV to start *de novo* virion production in Aag-2 cells (Sim and Dimopoulos, 2010), the negative-strand RNA at 4 h post-transfection should come only from the initial round of replication of the primarily infecting virus. Thus, this result provides direct evidence that *w*AlbB can inhibit viral replication even after viral entry into host cells. Furthermore, we observed an increase in viral inhibition with increasing time after transfection. At days 3 and 7 post-transfection, there was significantly less negative-strand RNA in W-Aag-2 cells than in R-Aag-2 cells. The viral titer was also significantly lower in the supernatant of W-Aag-2 cells than in that of R-Aag2 cells at 5 days post-transfection. However, this difference at later time points could be caused by both inhibition of binding and replication because progeny viruses can be subject to interference at both stages.

Overall, our findings highlight several important aspects for understanding both the mechanism and practical application for disease control of *Wolbachia*-mediated viral interference. First, *Wolbachia*-mediated viral inhibition occurs at multiple stages of the DENV life cycle, including binding and replication, resulting in a high efficacy of blocking viral propagation. With up to 75% reduction in DENV binding to host cells, this could be one of the important factors contributing to the overall outcome of viral interference. Targeting DENV at multiple stages of its life cycle would also make it more difficult for DENV to evolve resistance to *Wolbachia* than other antiviral agents, which target only a single stage in the viral life cycle. It is worth noting that *w*AlbB also induced inhibition of ZIKV binding to mosquito cells, indicating that the viral interference associated with *w*AlbB is a broad spectrum. Second, like antiviral drugs, *Wolbachia* does not appear to destroy either the viral genome or assembled infectious virions; instead, *Wolbachia* inhibits progression through the viral life cycle preventing the formation of new virus. Given our current lack of anti-dengue drugs, understanding the mechanism of *Wolbachia*-mediated viral inhibition may provide insights into the rational design and development of new drugs for medical therapy. Finally, *Wolbachia*-mediated viral interference occurs through alteration of host factors that are required for viral growth. Future studies should continue focusing on identification and characterization of the host factors that interact with *Wolbachia* to inhibit viral binding and replication. This knowledge may contribute toward and facilitate the development of novel strategies for the control of mosquito-borne diseases.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

PL and ZX conceived the idea, designed the experiments, and supervised the project. PL and QS performed the majority of the experiments and analyzed the data. PF, KL, and XL performed partial experiments. PL, QS, and ZX wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01750/full#supplementary-material.

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frontiers in Microbiology

ORIGINAL RESEARCH
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Effects of Mosquito Microbiota on the Survival Cost and Development Success of Avian *Plasmodium*

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Both intrinsic and extrinsic factors affect the capacity of mosquitoes for the transmission of vector-borne pathogens. Among them, mosquito microbiota may play a key role determining the development of pathogens in mosquitoes and the cost of infections. Here, we used a wild avian malaria-mosquito assemblage model to experimentally test the role of vector microbiota on the cost of infection and their consequences for parasite development. To do so, a cohort of *Culex pipiens* mosquitoes were treated with antibiotics, including gentamicin sulfate and penicillin-streptomycin, to alter their microbiota, and other cohort was treated with sterilized water as controls. Subsequently, both cohorts were allowed to feed on *Plasmodium* infected or uninfected house sparrows (*Passer domesticus*). The antibiotic treatment significantly increased the survival rate of mosquitoes fed on infected birds while this was not the case of mosquitoes fed on uninfected birds. Additionally, a higher prevalence of *Plasmodium* in the saliva of mosquitoes was found in antibiotic treated mosquitoes than in mosquitoes of the control group at 20 days post exposure (dpe). Analyses of the microbiota of a subsample of mosquitoes at 20 dpe suggest that although the microbiota diversity did not differ between individuals of the two treatments, microbiota in control mosquitoes had a higher number of unique features and enriched in biochemical pathways related to the immune system than antibiotic treated ones. In sum, this study provides support for the role of mosquito microbiota on mosquito survival and the presence of parasite DNA in their saliva.

Keywords: ***Culex pipiens*, ecology-diseases, malaria, microbiome, parasite transmission, vector-borne pathogens, virulence**

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INTRODUCTION

The vectorial capacity of mosquitoes, that describes the potential of a vector to transmit a pathogen, is driven by four major parameters including the blood feeding behavior of the insects, the ability of the pathogen to develop in the insects, the latency time, and the cost induced by pathogens in vector longevity (Macdonald, 1955; Rund et al., 2016). Both intrinsic and extrinsic factors, including behavioral, ecological and environmental variables, affect the capacity of mosquitoes

for the transmission of mosquito-borne pathogens (Lefèvre et al., 2013). Among them, mosquito microbiota has been identified as a key component (Weiss and Aksoy, 2011) being involved in many biological processes of mosquitoes (Huang et al., 2020), finally determining the development of pathogens in vectors and the cost of infections (Guégan et al., 2018; Martínez-de la Puente et al., 2018). Mosquito microbiota affects the development of pathogens in the mosquitoes throughout different processes including the production of metabolites directly impairing parasite survival in the midgut and by stimulating the insect immunological responses (Dong et al., 2009; Romoli and Gendrin, 2018; Huang et al., 2020). Mosquito microbiota may reduce the success of parasite development, at least, in the *Anopheles*-human *Plasmodium* assemblages studied (Romoli and Gendrin, 2018), but contradictory results could be expected depending of pathogen-mosquito's microbiota assemblages tested (Mideo, 2009; Romoli and Gendrin, 2018; Guégan et al., 2018). However, contrary to the case of parasites affecting humans, the role of mosquito microbiota on the transmission of vector-borne pathogens affecting wildlife has been traditionally neglected, as in the case of avian *Plasmodium* (Martínez-de la Puente et al., 2018).

Avian malaria parasites of the genus *Plasmodium* are mosquito-borne parasites naturally circulating between birds and mosquitoes. These parasites are considered excellent models for studies on the determinants of the ecology and evolution of the transmission dynamics of malarial parasites (Rivero and Gandon, 2018). The life cycle of avian *Plasmodium* parasites includes different phases in the vertebrate and invertebrate hosts. To be efficiently transmitted, a competent mosquito vector needs to feed on blood of an infected bird and, after parasite development in the mosquito, the insect may inoculate the parasite infective forms, the sporozoites, to a new host (Valkiūnas, 2005). Mosquitoes of different genera are competent vectors of avian malaria parasites, with those of the *Culex* genus playing a central role in their transmission (Santiago-Alarcón et al., 2012; Gutiérrez-López et al., 2020). Avian malaria infections in wild birds reduced survival and fitness of infected individuals (Merino et al., 2000; Asghar et al., 2015). In some cases, avian *Plasmodium* has dramatically affected bird populations. For instance, the widespread *Plasmodium relictum* is featured on the widely cited list of "100 of the World's Worst Invaders" (Lowe et al., 2000) being considered a major cause of the decline of the populations of many avian species after its introduction in Hawaii (van Riper et al., 1986). Contrary to the case of their effects on vertebrate hosts, factors determining the interaction between mosquitoes and avian *Plasmodium* and their consequences for parasite amplification have been poorly investigated.

Here, we used a wild mosquito-*Plasmodium* assemblage to experimentally test the role of *Culex pipiens* microbiota on the mosquito survival and parasite development, two components determining the vectorial capacity of mosquitoes. Most studies conducted in this topic have used mosquito colonies to analyze the role of parasite microbiota. This may simplify the insect microbiota with respect to those present in wild mosquitoes, thus alternative models for the study of the mosquito and its microbiota are required (Romoli and Gendrin, 2018). We used *Cx. pipiens* mosquitoes raised in the laboratory from field collected larvae. This mosquito species was selected based on its ornithophilic behavior, frequently interacting with avian *Plasmodium* parasites infecting wild birds (Martínez-de la Puente et al., 2016, 2020; Rivero and Gandon, 2018). In addition, this mosquito is considered a competent vector for the transmission of different avian *Plasmodium* species (Santiago-Alarcón et al., 2012; Gutiérrez-López et al., 2020), thus playing a central role in the epidemiology of these pathogens under natural conditions.

MATERIALS AND METHODS

Mosquito Sampling and Antibiotic Treatment

During 2018, mosquito larvae were collected in two close localities of the province of Huelva (Spain) according to the availability of mosquito breeding areas. Mosquito larvae were transferred to the laboratory where they were kept in plastic trays with water from the original breeding sites and fed with Mikrozell (20 ml/22 g; Hobby Mikrozell; Dohse Aquaristik GmbH & Co. KG, D-53501, Gelsdorf, Germany). Insects were maintained in a climatic chamber at constant conditions (temperature: 28°C, relative humidity (RH): 65–70%, light/dark cycle: 12:12 h). After emergence, adult mosquitoes were placed in insect cages (BugDorm-43030F, 32.5 × 32.5 × 32.5 cm) and fed *ad libitum* with sterilized 10% sugar solution. Two to five days later, female *Cx. pipiens* mosquitoes were identified by morphology (Schaffner et al., 2001) and mosquitoes of the same age and breeding area were assigned to each of the treatments: control and antibiotic-treated mosquitoes. Control mosquitoes were fed with sterilized 10% sugar solution, while experimental mosquitoes were fed with sterilized 10% sugar solution with antibiotics, which were 15 μg gentamicin sulfate (Sigma-Aldrich, Stockholm, Sweden) and 10 units/10 μg of penicillin-streptomycin (Invitrogen, Carlsbad, CA, United States) per ml of water solution (Dong et al., 2009). Mosquitoes were allowed to feed on the antibiotic treated or the control sugar solution during seven days prior to their exposure to vertebrate hosts. The sugar solution with or without antibiotics were replaced by sterilized water 24 h prior to each blood feed trial (see below) and access to water was removed 12 h before blood feed trials began.

Bird Sampling and Experimental Assays

Eighteen juvenile house sparrows (*Passer domesticus*) were captured using mist nets in San Juan del Puerto (Huelva, Spain) and were individually ringed. We only included in this study those birds with single infections by *Plasmodium* parasites or uninfected birds. All birds with evidence of infection by *Haemoproteus*, *Leucocytozoon* parasites or mixed infections were removed from the experiment to avoid any confounding effect on mosquito survival (Valkiūnas et al., 2014). Each bird was immobilized and placed in an insect cage containing ≈50–100 mosquitoes. Each bird was exposed to control and antibiotic treated mosquitoes of the same age and geographical (breeding area) origin. Birds were exposed to mosquitoes of each treatment during 45 min directly in the field under dark conditions. The

order of exposure of each bird to mosquitoes from each control or experimental treatments was randomly assigned. After the experiment, birds were blood sampled from the jugular vein using sterile syringes (never exceeding 1% of body mass) and released in the same area. Back to the laboratory, mosquitoes with a recent blood meal in their abdomen were separated in a new box and maintained in a climatic chamber with *ad libitum* access to sterilized 10% sugar solution during the following 20 days. Each box contained only engorged mosquitoes from the same treatment and fed on the same individual bird. The mortality rate of mosquitoes was daily monitored. A subsample of 65.90% (n = 315) fed mosquitoes that survived until the end of the experiment were used to molecularly identify the presence of avian *Plasmodium*. All mosquitoes from boxes containing less than 21 alive mosquitoes at the end of the experiment were analyzed. However, we only analyzed between 20 and 21 individuals in those cases where a higher number of mosquitoes survived until the end of the experiment. This subsample was selected based on the impossibility to handle additional mosquitoes in the same day and to reduce the cost of molecular analyses, while provide reliable estimates of parasite prevalence (Jovani and Tella, 2006). From these mosquitoes, we isolated the saliva following Gutiérrez-López et al. (2019), and the head-thorax of each mosquito (containing the salivary glands) was separated from the abdomen using sterile tips. Samples were kept in the freezer at −80°C.

Molecular Analyses

The MAXWELL® 16 LEV Blood DNA Kit was used to extract the genomic DNA from blood samples and from the head-thoraxes of mosquitoes. The Qiagen DNeasy® Kit Tissue and Blood (Qiagen, Hilden, Germany) was used to extract the DNA from mosquito saliva. Detection and lineage identification of parasites were conducted following Hellgren et al. (2004). The presence of amplicons was verified in 1.8% agarose gels and positive samples were sequenced using the Macrogen Inc. facilities (Madrid, Spain). Sequences were edited using the software Sequencher™ v 4.9 (Gene Codes Corp.© 1991–2009, Ann Arbor, MI 48108, United States) and assigned to parasite lineages/morphospecies after comparison with those deposited in GenBank (National Center for Biotechnology Information) and Malavi databases (Bensch et al., 2009).

To detect bacterial species in mosquito midguts, we analyzed the microbiota profile from 16 blood-fed mosquitoes 20 days after blood-feeding (days post exposure, dpe), including eight antibiotic-treated mosquitoes and eight control mosquitoes. Mosquitoes of both treatments (controls and antibiotic treated mosquitoes) from the two larval collection localities and feed on four bird individuals were included in this study. None of these 16 mosquitoes were infected by avian *Plasmodium* (i.e., absence of parasite DNA in the head-thorax). Mosquito surface was sterilized in 70% ethanol, then rinsed in sterile PBS solution, and midguts were dissected with sterilized forceps and tips on clean smears and, subsequently stored individually in sterile water at −80°C. DNA extraction from each midgut was done using the QIAamp DNA stool Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA concentration and purity were estimated with a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, United States). Libraries from midguts were built with the Ion 16S Metagenomics kit (Thermofisher), consisting of primer pools to amplify multiple variable regions (V2, 3, 4, 6–7, 8 and 9) of the 16S rRNA. After generating amplicons, the Ion PlusTM Fragment Library Kit (Thermofisher) was used to ligate barcoded adapters and synthesize libraries. Barcoded libraries from all the samples were pooled and templated on the automated Ion Chef system (Thermofisher) followed by a 400 bp sequencing on the Ion S5 (Thermofisher). Three samples from the antibiotic-treated mosquitoes and one sample from the control group were discarded for posterior analyses due to the low number of sequences obtained (<15,000 reads).

Statistical Analyses

Cox's proportional hazards mixed−effect models by maximum likelihood were used to assess the effect of antibiotic treatment on mosquito survival until 20 dpe. The exposure order of birds (birds exposed first to antibiotic-treated mosquitoes and later to control mosquitoes, or vice versa) were included as a fixed factor to control for potential effects of mosquito bites on *Plasmodium* development. Independent models were used for those mosquitoes fed on *Plasmodium* infected and uninfected birds. We used this approach in order to statistically control for the bird identity in the analyses (i.e., birds were either infected or uninfected). In addition, this procedure allows us to control for additional factors linked to bird identity but not considered in the analyses, which could affect the results (e.g., the phase of infection, the parasite intensity in the bird or the immunological / nutritional status of birds). Differences in the presence/absence of *Plasmodium* in the head-thorax or saliva of mosquitoes were analyzed using Generalized Linear Mixed Models (GLMMs) with binomial error and logit link function including the antibiotic treatment as a fixed factor and bird identity and exposure order as random terms. Statistical analyses were performed in R software 3.2.5 (R Core Team, 2016) with the package *lme4* (Bates et al., 2015).

The bacteria sequences obtained from mosquito midguts were translated into amplicon sequence variants (ASVs) using DADA2 (Callahan et al., 2016) within the microbiome analysis package QIIME2 2019.1[1]. The same package was used for diversity analysis and subsequent taxonomic analysis through clustering with VSEARCH function (Rognes et al., 2016) and the reference base Greengenes version 13_8 at 97% of identity. Weighted Unifrac distance was used for diversity analysis (Lozupone et al., 2011). Differential abundance analysis was assessed with ANCOM within QIIME2 (Mandal et al., 2015) and core features were compared with a Venn diagram with Venny 2.1.0 (Oliveros, 2007). PICRUSt 1.1.1 was used to infer the functional profiles of the microbial communities (Langille et al., 2013). We inferred the biochemical pathways of the microbiota found in mosquitoes of each treatment through the Kyoto Encyclopedia of Genes and Genomes (KEGG). This procedure allows to search for the potential role of identified

[1]www.qiime2.org

microbiota affecting the physiological pathways on the host (i.e., mosquitoes). The KEGG ortholog predictions were calculated, and subsequently translated into KEGG Pathways. The Statistical Analysis of Metagenomic Profiles (STAMP) tool was used for the analysis of the KEGG Pathways (Parks et al., 2014).

RESULTS

Eight uninfected and ten *Plasmodium* infected birds corresponding to the *P. relictum* lineages SGS1 (n = 8) and GRW11 (n = 1) and the *Plasmodium* sp. lineage COLL1 (n = 1) were exposed to 2,250 mosquitoes. At the beginning of the experiment, 1,066 of these mosquitoes took a blood meal, including 632 mosquitoes fed on birds infected with *Plasmodium* and 434 mosquitoes fed on uninfected birds. For mosquitoes fed on *Plasmodium* infected birds, we found a higher survival to the 20 dpe of antibiotic-treated (271 out of 327; 82.87%) than control mosquitoes (207 out of 305; 67.87%) (**Figure 1A**; Cox model, treatment: Z = 4.76, P < 0.001; exposure order: Z = 0.68, P = 0.49). A similar trend was found for the case of mosquitoes fed on uninfected birds, although the effect of the antibiotic treatment on the survival of mosquitoes did not reach significance (**Figure 1B**; treatment: Z = 1.95, P = 0.052; exposure order: Z = −1.15, P = 0.25; antibiotic treated mosquitoes: 176/273, 64.47%; control mosquitoes: 83/161, 51.55%).

The head-thorax of 315 out of 478 mosquitoes fed on infected birds that survived until 20 dpe were tested for the presence of avian *Plasmodium*. These analyses included 153 mosquitoes of the control group and 162 mosquitoes of the antibiotic-treated group. Of them, 150 (47.62%) were positive, including 73 mosquitoes treated as controls and 77 mosquitoes treated with antibiotics. The infection status of the head-thorax of mosquitoes fed on infected birds did not differ between treatments (**Figure 2**; Z = −0.69, P = 0.49). Of these mosquitoes with positive head-thoraxes, 20 individuals treated with antibiotics (n = 77; 25.97%) showed *Plasmodium* parasites in their saliva, while this was the case of only 8 (n = 73; 10.96%) mosquitoes treated as controls. Thus, a higher infection rate was found in the saliva of mosquitoes treated with antibiotics with respect to control ones (**Figure 2**; Z = −2.08, P = 0.037).

Microbiota (core) features shared by at least 85% of the samples of a group were calculated to find the shared microbiome between experimental groups. A total of 18 out of 39 features, which were classified at genus level, were shared by both groups while 19 genera were exclusively found in control mosquitoes and only two in antibiotic-treated mosquitoes (**Figure 3B**). The complete list of bacteria found in mosquitoes is shown in **Supplementary Table S1**. However, no statistical differences were found neither in alpha (Shannon index; H = 0.798, p = 0.372) nor in beta diversity (weighted unifrac distance; pseudo-F = 0.764, p = 0.498) in mosquitoes of both treatments (**Figure 3A**). Moreover, the strict ANCOM analysis did not detect any compositional statistical difference between groups (data not shown). In spite of that, KEGG Pathways analysis revealed that microbiota from control mosquitoes was enriched in pathways related to energy metabolism, immune system and folding, sorting and degradation (**Figure 3C**).

DISCUSSION

Different studies have shown that microbiota affects the development of different pathogens on their vectors (Gendrin et al., 2015; Kalappa et al., 2018), although information on the impacts of mosquito microbiota on the development of protozoans of wild animals is scarce. To the best of our knowledge, we have tested for the first time the role of mosquito

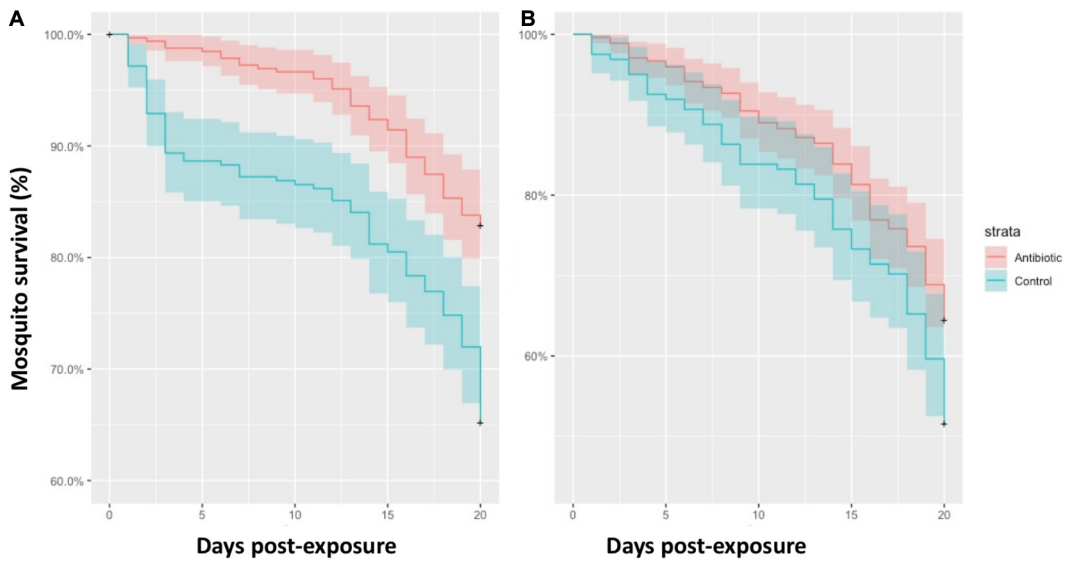

FIGURE 1 | Survival rate of mosquitoes treated with antibiotics (red lines) and control mosquitoes (blue lines) fed on *Plasmodium* infected birds **(A)** and uninfected birds **(B)**. Colored areas represent standard errors.

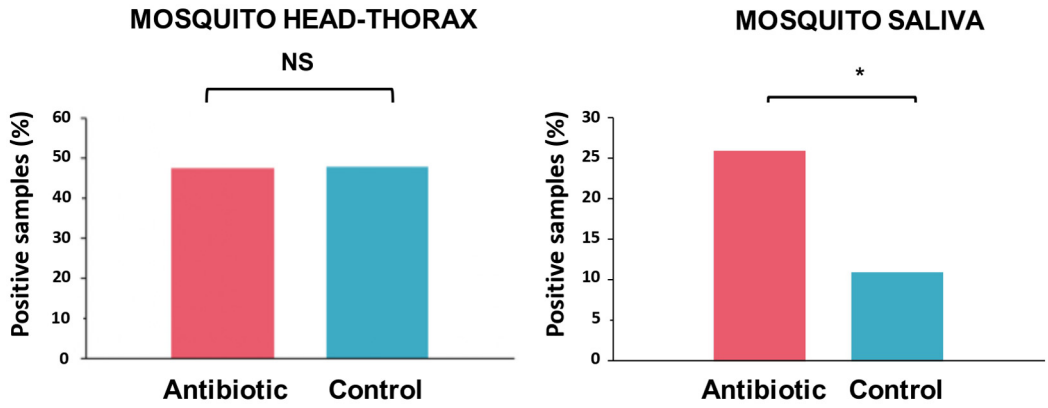

FIGURE 2 | Percentage of the head-thoraxes and saliva samples with presence of *Plasmodium* DNA according to their experimental treatment: mosquitoes treated with antibiotics (red) and control mosquitoes (blue). Statistically significant differences are indicated with an asterisk (*). NS means non-significant differences.

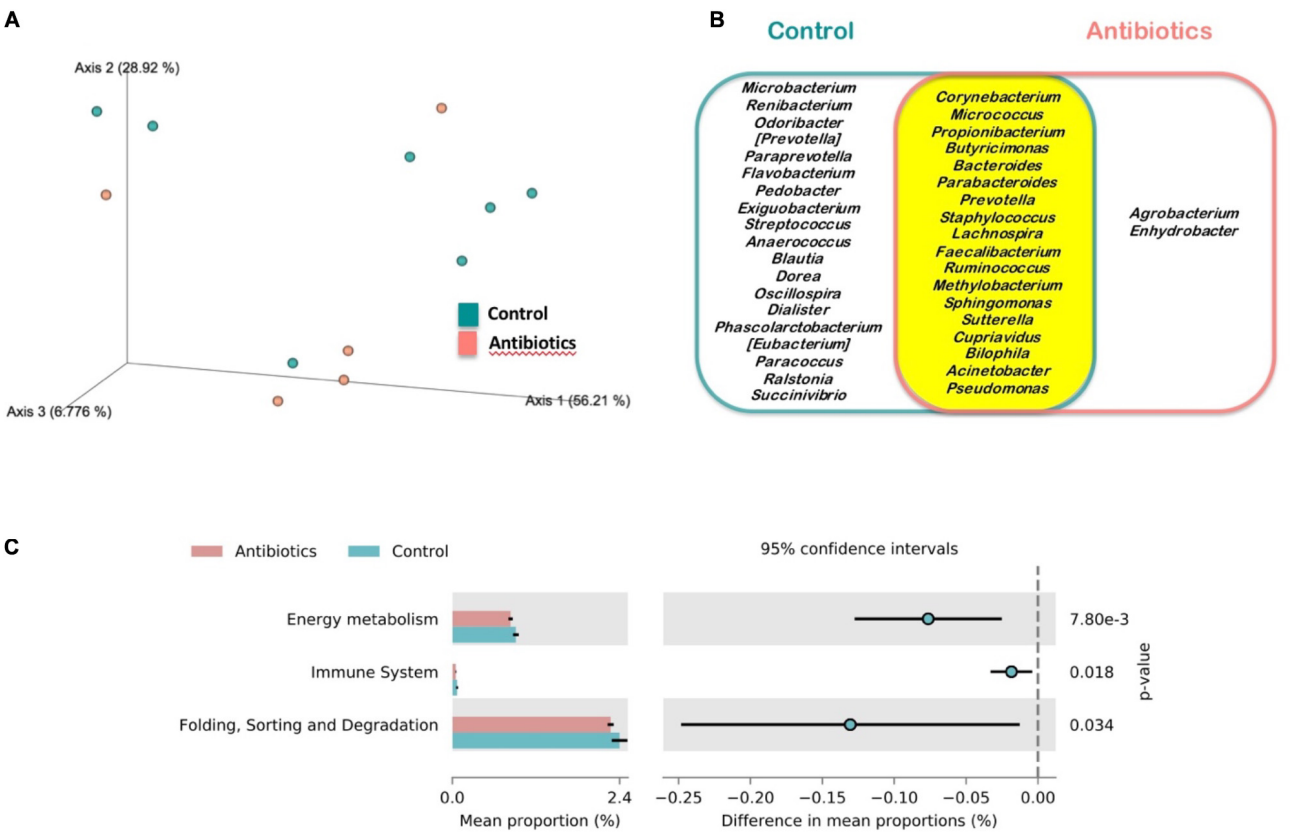

FIGURE 3 | Microbiota analysis of mosquitoes treated with antibiotics (red) and control mosquitoes (blue). **(A)** Principal Coordinates Analysis (PCoA) of bacterial communities. **(B)** Venn diagram of the core microbiomes at genus level of the analyzed mosquitoes for the case of bacteria identified in control mosquitoes (blue), mosquitoes treated with antibiotics (red) and those shared by mosquitoes of both experimental groups (yellow). **(C)** Differentially abundant KEGG pathways identified at level 2 in both sampled groups.

microbiota on the development and cost of infection (i.e., survival rate) of avian *Plasmodium* in its main vector *Cx. pipiens*. We found that antibiotic administration increased both the survival probability of mosquitoes and the presence of parasite DNA in their saliva after biting on naturally *Plasmodium*-infected birds.

Plasmodium infections may impact mosquito survival, although this effect may depend on the vector–parasite combinations studied or the methodological approaches used (e.g., the duration of the studies) (Ferguson and Read, 2002). In the case of mosquitoes infected by avian *Plasmodium*

contradictory results have been reported (Martínez-de la Puente et al., 2018). For instance, while Gutiérrez-López et al. (2019) found experimental support for the negative effect of parasite infections on mosquito survival, other authors have found the opposite pattern (Vézilier et al., 2012) or, even, non-significant associations between mortality rate and parasite infection (Delhaye et al., 2016). Different intrinsic, including genetic differences between mosquitoes, and extrinsic factors may modulate the cost of avian malaria parasites in the vectors. These discrepancies could be partially explained by differences in the sugar concentration provided to the mosquitoes or the parasite species used or, even, to other factors including the parasite load of the bird donors (Martínez-de la Puente et al., 2018; Gutiérrez-López et al., 2020). These factors merit further research in order to identify their relevance on the effects of mosquito microbiota on parasite development and the cost of infections in mosquitoes. Our results indicate that mosquito microbiota also determine the cost imposed by the parasites on their vectors, a factor that has been traditionally neglected in studies on avian malaria parasites. Mosquito microbiota may have protective effects against parasite infections such as the inhibitory bioactivity of secreted enzymes or toxins and the mosquito physiological responses against parasites induced by their microbiota (Weiss and Aksoy, 2011; Smith et al., 2014). Thus, it could be expected a lower survival rate of mosquitoes fed on infected birds treated with antibiotics than controls. However, similarly to our case, Gendrin et al. (2015) found a positive effect of antibiotics on mosquito survival after feeding on blood infected with the rodent parasite *Plasmodium berghei*. Authors from this experimental study argued that the increase of the microbial populations following a mosquito blood meal might determine the observed pattern. This could also explain the results reported here and the lack of significant differences between the microbiota profiles of the respective groups. In addition, the immunological responses against the bacterial population grown could induce important costs for mosquitoes (Ahmed et al., 2002), finally affecting their longevity. Indeed, a higher immune system response, as well as a higher energy metabolism and activities related to the processing of genetic information, have been inferred within the control group from its microbiota KEGG pathways. Additionally, it is possible that the presence of particular bacteria in control mosquitoes increase their mortality rate, as in the case of mosquitoes exposed to *Chromobacterium* (Ramirez et al., 2014), although this genus was not found in the mosquitoes studied here. The antibiotic treatment did not significantly affect the survival probability of mosquitoes exposed to uninfected birds, although the same trend was found. Thus, the significant effects of the treatment on mosquito survival found in mosquitoes exposed to *Plasmodium* infected birds suggest a parasite-mediated effect of mosquito microbiota on survival. In this respect, in spite that we did not analyzed the presence of parasites in dead mosquitoes, it could be expected that infected mosquitoes were more likely to die during the course of the experiment (Valkiūnas et al., 2014).

We identified the presence of parasite DNA in the saliva of mosquitoes, and found that the antibiotic treatment affected the prevalence of avian *Plasmodium* DNA. In particular, we found a higher prevalence of parasites in mosquitoes supplemented with antibiotics than those treated as controls, suggesting that mosquito microbiota affected negatively the development of the parasites in their vectors. These results provide support to previous studies on other *Plasmodium*-vector assemblages using antibiotic alterations of mosquito microbiota (Romoli and Gendrin, 2018). For instance, the human malaria vector *Anopheles gambiae* fed on hosts treated with antibiotics were more susceptible to *Plasmodium* infections (Gendrin et al., 2015). More recently, Kalappa et al. (2018) found a higher prevalence of oocysts in *Anopheles stephensi* treated with antibiotics after their exposition to *P. berghei* infected mice. Our results support an effect of mosquito microbiota on parasite transmission in a novel study model. However, these differences were only evident when considering the presence of parasite DNA in the saliva of mosquitoes, but not in their head-thoraxes. Most studies on the vector capacity of mosquitoes for the transmission of avian malaria parasites are based on the amplification of parasite DNA in the head-thorax of individuals, whereas it is in the salivary glands of the mosquitoes where the infective forms of the parasites are accumulated. This method, although useful, may overestimate the capacity of vectors to transmit the parasites because DNA could be amplified from non-infective parasite forms present in the body even of non-competent insects (Valkiūnas, 2011).

Studies on the effects of mosquito microbiota on parasite transmission have largely used antibiotic treatments. These studies have found that mosquitoes supplemented with antibiotics reduce the bacterial load to undetectable levels (Dong et al., 2009), although antibiotics may fail to completely eliminate all the bacteria (Guégan et al., 2018). We failed to identify any significant effect of the antibiotic treatment in the diversity of the microbiota of mosquitoes at 20 dpe. However, mosquitoes treated with antibiotics had a lower number of unique features than those of the control group suggesting a simplification of their microbiota. In our study, mosquito larvae from both treatments were bred in the water from their breeding areas and were fed with the same diet, thus being colonized by similar bacteria (Linenberg et al., 2016; Guégan et al., 2018). In addition, the antibiotic treatment was provided to adult mosquitoes only prior to the bird exposure while mosquitoes of both treatments were supplemented with the same diet (i.e., sugar solution) after the blood meal. These could potentially affect the mosquito microbiota during this period and the absence of significant differences in the microbiota of mosquitoes of each treatment at the end of the experiment (20 dpe). It is also possible that the treatment had a homogeneous effect across taxa of the mosquito microbiota, explaining the absence of significant effects. However, our results suggest some simplification of the mosquito microbiota due to the antibiotic treatment as 19 bacteria genera appeared only in control mosquitoes while two were only found in antibiotic treated mosquitoes. Nevertheless, it is important to clarify that we are only looking at long-term effects of the treatment on the mosquito microbiota in a subset of only 12 mosquitoes. This is a limitation of our study that does not allow us to obtain further conclusions.

In summary, results from this study provide support for the importance of mosquito microbiota affecting two major parameters (survival rate and parasite development, measured as the presence of parasite DNA in the mosquito saliva) of models of vector transmission (i.e., Ross-MacDonald models). Differences in the microbiota exist between mosquito species and populations (Coon et al., 2016; Muturi et al., 2017; Duguma et al., 2019), which may affect their vector competence. These differences could explain, at least in part, the geographical differences found in the infection patterns between populations of wild birds, where a proportion of the variance is explained by the mosquito community present in the area and landscape configuration (Ferraguti et al., 2018). In addition, these results suggest that pollution of rivers by antibiotics used in human and animal health, which represent a worldwide problem especially in undeveloped countries (Danner et al., 2019), could also affect the epidemiology of mosquito-borne pathogens, such as avian *Plasmodium*. However, the antibiotic concentration used here is 500 times larger than those found in freshwaters, and consequently further experiments using antibiotic concentrations in the range found in antibiotic polluted areas is necessary to test this effect under more realistic conditions.

DATA AVAILABILITY STATEMENT

16S raw data was stored at the public repository SRA database (NCBI) with the BioProject PRJNA634467.

ETHICS STATEMENT

The animal study was reviewed and approved by CSIC Ethics Committee.

AUTHOR CONTRIBUTIONS

JM-P, RS, and JF conceived and designed the study. JM-P, RG-L, and AD-F performed the experiments. JM-P, RG-L, and IM-I analyzed the samples and the data. JM-P led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.562220/full#supplementary-material

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Wickerhamomyces anomalus in Mosquitoes: A Promising Yeast-Based Tool for the "Symbiotic Control" of Mosquito-Borne Diseases

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The ascomycete yeast *Wickerhamomyces anomalus* is a mutualistic symbiont of different insects, including diptera vectors of diseases. Although fungal symbioses have been so far poorly characterized, the topic is gaining attention as yeast-insect interactions can provide pivotal information on insect biology, such as their environmental adaptation or vectorial capability. We review the symbiosis between *W. anomalus* and mosquitoes, which implies nutritional and protective functions. Furthermore, we focus on antiplasmodial effects of *W. anomalus* in malaria vectors and discuss the yeast potential for the "symbiotic control" (SC) of mosquito-borne diseases (MBDs).

Keywords: *Wickerhamomyces anomalus*, yeast, insect, mosquito, malaria, symbiosis, mycobiota, symbiotic control

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INTRODUCTION

Mosquitoes comprise different species that are vectors of pathogens to humans and other animals. The most important mosquito-borne diseases (MBDs) are transmitted by three mosquito genera: *Aedes*, *Anopheles*, and *Culex*. *Aedes* transmit several arboviruses including Dengue, Yellow Fever, Chikungunya, Zika, and West Nile; *Anopheles* spread malaria parasites (*Plasmodium* spp.); *Culex* is a major vector of West Nile virus and filarial nematodes. In the last decades, climate changes, anthropization of new habitats, and international trade have favored the global expansion of mosquito vectors and emergence of MBDs causes major public health concerns in new geographic areas (Brugueras et al., 2020). Since vaccines against most mosquito-borne pathogens are not available, their prevention relies mainly on insecticides. However, insecticides become less effective as vectors develop resistance and the overuse of chemical insecticides increases the costs besides having deleterious effects on non-target species and the environment. Thus, innovative eco-friendly alternatives are requested. New control methods are focusing on the rising knowledge of the mosquito microbial community (microbiota) and its effect on the mosquito-pathogen relationship. Understanding the biology of symbionts in insect vectors is essential for the development of biological control strategies like the "symbiotic control" (SC; Bourtzis et al., 2014). The SC is a promising non-chemical method for the control of vectors and diseases they transmit. This approach exploits symbiotic microorganisms, bacteria or fungi, with the aim of reducing the vector capability (Niang et al., 2018). One of the strategies

for the SC of vector-borne pathogens is based on the exploitation of antagonism between symbionts and pathogens, as in the case of the bacteria *Wolbachia* that is applied for the control of mosquito-borne viruses (Frentiu, 2017).

The analysis of the mosquito-associated microbiota (bacteria, fungi, protists, viruses, and nematodes) is gaining attention since microbes are recognized to influence many traits of the mosquito biology, such as development, physiology, immunity, and vector competence (Guégan et al., 2018). While an increasing number of studies have focused on bacteria, the fungal community (mycobiota) has been largely neglected, but recent studies show the presence of an important fungal diversity in mosquitoes (Malassigné et al., 2020). Mosquito-mycobiota is mainly composed of Ascomycota, which comprise mostly species of Pezizomycotina and Saccharomycotina (subphyla); Pezizomycotina include species of filamentous fungi, whereas Saccharomycotina are basically represented by yeasts, such as *Candida*, *Meyerozyma*, *Pichia*, and *Wickerhamomyces*, that adapt to survive in the insect gut and different mutualistic yeast-insect symbioses based on trophic interdependence have been described (Malassigné et al., 2020). Yeasts generate signals of sugar resources through metabolic pathways that produce compounds, such as fermentative volatiles, that attract insects. On the other hand, insects disperse yeasts over a variety of sugar sources and provide them with food and a habitat (the gut) to mate or generate sexual forms, increasing yeast biodiversity (Madden et al., 2018). Yeasts are important not only for attraction to food, they influence oviposition sites and larval development, but also supply diet integration of adults providing organic nitrogen, essential vitamins, and lipids (Stefanini, 2018). In summary, insects are an essential component of the ecology of ascomycetous yeasts, and the latter influence many traits of the insects' biology.

Wickerhamomyces anomalus (class: saccharomycetes) is a budding yeast identified in the gut of diverse insects, in which it is suggested as a mutualist symbiont with nutritional and protective functions (Toki et al., 2013; Steyn et al., 2016). The presence of *W. anomalus* is reported in different orders of insects, such as mosquitoes (Diptera: Culicidae), sand flies (Diptera: Psychodidae), honey bees (Hymenoptera), plant hoppers (Hemiptera), and beetles (Coleoptera). Several studies focused on the yeast association with mosquitoes, in which *W. anomalus* is identified in *Anopheles*, *Aedes*, and *Culex* species (Ricci et al., 2011a; Muturi et al., 2016; Steyn et al., 2016). Interestingly, the strain of *W. anomalus* identified in the malaria vector *Anopheles stephensi* produces a killer toxin (KT) with wide antimicrobial activities that may protect the host from entomopathogenic microbes (Ricci et al., 2011b). KTs are glycoproteins with exo-β-1,3-glucanase enzymatic activity that hydrolyze glucans on the surface of different microbial targets, such as viruses, bacteria, fungi, and protozoa (Walker, 2011). Additional studies demonstrated a strong KT-based effect against the protozoa *Plasmodium berghei* that prevent the parasite development in the midgut of *An. stephensi* (Cappelli et al., 2019). Here, we review the *W. anomalus* symbiotic association with mosquitoes and focus on the antiplasmodial effect, discussing the potential application of a new yeast-based approach for the SC of malaria and possibly other MBDs.

GENERAL FEATURES OF *Wickerhamomyces anomalus*

Wickerhamomyces anomalus, formerly *Pichia anomala* and *Hansenula anomala*, was renamed following phylogenetic studies (Daniel et al., 2012). The biotechnological potential of this yeast and its use in different industrial applications derive from capability of *W. anomalus* to secrete antimicrobials factors acting on a broad spectrum of pathogens (Walker, 2011). In the last decade, the yeast has drawn special attention and the genome sequences of two environmental strains have been published (Schneider et al., 2012; Cunha et al., 2020). The European Food Safety Authority (EFSA) classifies *W. anomalus* at biosafety level one and different "killer strains" (yeast isolates that produce KTs) are used as biocontrol agents with inhibitory effects against mold and bacteria in the agro-food sector (Sundh and Melin, 2011). Specific properties make *W. anomalus* a suitable product for different biotechnological applications: (i) adaptation to a wide range of growth conditions in terms of temperature (3–37°C), pH value (2–12), and osmolarity; (ii) robustness and competitiveness in different habitats; (iii) ability to produce biomass on a large scale; (iv) stress-tolerance, survival, and maintenance of biocontrol activity in different formulations and after desiccation (Passoth et al., 2011).

Wickerhamomyces anomalus has been isolated from different environmental matrices: flowers and leaves, food and feed systems, and waters and insects (Walker, 2011). Moreover, opportunistic strains have been detected in immunocompromised patients and, even though few outbreaks have been reported, they should be taken into consideration (Dutra et al., 2020). Thus, distinguishing pathogenic from non-pathogenic strains is crucial for future applications of *W. anomalus*. Since it was identified in hematophagous insects (mosquitoes and sand flies), a study investigated whether *W. anomalus* infects humans: screening immunocompromised and malaria patients, and healthy volunteers (exposed to mosquito bites) revealed that *W. anomalus* is not relevant to human, consistently with the rare reports of fungemia (Epis et al., 2015). Safety tests showed that *W. anomalus* does not harm mammalian cells, suggesting that they are not sensible to the yeast killer activity (Cappelli et al., 2019).

Wickerhamomyces anomalus-MOSQUITO SYMBIOSES AND THEIR IMPACT ON THE INSECT BIOLOGY

On the background that *W. anomalus* has been isolated from different environmental matrices such as flowers and water, mosquitoes larvae can acquire the yeast from the aquatic breeding sites, whereas adults by feeding on nectars. First identifications of *W. anomalus* in mosquitoes were in *Anopheles stephensi* (lab-reared colony), and *Anopheles gambiae*,

Aedes aegypti, and *Aedes albopictus* (lab-reared colonies and wild samples; Ricci et al., 2011a,b). An in-depth screening of the mycobiota in *Culex* spp. revealed the presence of *W. anomalus* in *Culex pipens* (wild samples; Steyn et al., 2016). More recent investigations by metagenomic analyses of invasive species identified the yeast in *Aedes japonicus* and *Aedes triseriatus* (wild samples; Muturi et al., 2016). However, mosquitoes are not the only host of *W. anomalus* since it is found and suggested as mutualist symbiont in *Phlebotomus perniciosus* (sand fly), *Apis mellifera* (honeybee), *Laodelphax striatellatus* (planthopper), and *Doubledaya bucculenta* (beetle; **Table 1**).

Transmission Routes and Influence on Larval Development

Investigations in *An. stephensi* revealed the presence of *W. anomalus* in all the developmental stages of the mosquito (larvae, pupae, and adults). *Wickerhamomyces anomalus* is supposed to be associated with pre-adult stages also in *Ae. japonicus* and *Ae. triseriatus*, since it has been isolated from the midgut of adult females hatched in the laboratory from wild-caught pupae. Different results have been reported in *Cx. pipens*, which seems to host *W. anomalus* only at the larval stages, in fact, adult mosquitoes caught near the larval breeding sites tested negative for the yeast detection. As observed for most bacteria, structure and abundance of fungal communities vary during the mosquito life-cycle with a significant reduction of fungal diversity in the midgut of newly emerged adults (Steyn et al., 2016) as well as in females after blood ingestion (Muturi et al., 2016). Conversely, the presence of *W. anomalus* in newly emerged adults reared under controlled conditions supports the hypothesis of a trans-stadial transmission in *An. stephensi*, *Ae. japonicus*, and *Ae. triseriatus*. Except for the observations reported in *Cx. pipiens*, *W. anomalus* bypasses the fungal reduction during the mosquito metamorphosis from larva to adult, in different species. This is a prerogative of few microbes, which implies robustness and competitiveness in different habitats, since the pupal gut meet physiological adaptations that are needed for molting as hypoxia (Valzania et al., 2018).

Wickerhamomyces anomalus has been detected both in the midgut and gonads (male and female) of adults *An. stephensi*, suggesting its possible involvement in the mosquito reproduction and vertical transmission routes. Indeed, experiments performed using a monoclonal antibody targeting KTs demonstrated that *W. anomalus* is not affected by the blood meal and it is transmitted to the offspring (Cappelli et al., 2014). *Wickerhamomyces anomalus* maternal transmission through the egg surface in newly hatched larvae has been shown in the non-social beetle *D. bucculenta*, whose eggs acquire the yeast from the ovipositor-associated fungal pocket (mycangium) of adult females (Toki et al., 2013). This mechanism ensures an immediate acquisition of *W. anomalus* that is an essential food source for the larval development of *D. bucculenta*. A similar mutualism is proposed also in the planthopper *L. striatellatus*, in which *W. anomalus* favors the larval development and localizes the fat body of adult females, like other symbionts that are transmitted to the progeny through the ovary (Cao et al., 2015). Concerning mosquitoes, a positive

TABLE 1 | *Wickerhamomyces anomalus* in mosquitoes and other insects.

Insect host (order)	Species	Symbiotic functions described (bold) or proposed (?)	Developmental stages (organs)	References
Mosquito (Diptera: Culicidae)	*Anopheles stephensi* (laboratory reared colonies)	**Protective role: killer strain with antifungal and antiplasmodial effects (β-1,3-glucanase activity);** Nutritional role in larvae and adults (?)	Larvae, pupae, adult male, and female (midgut, gonads)	Ricci et al., 2011b; Cappelli et al., 2014, 2019; Valzano et al., 2016; Cecarini et al., 2019
	Anopheles gambiae (wild samples and laboratory reared colonies)	Nutritional/protective role (?)	Adult male and female	Ricci et al., 2011a
	Aedes albopictus (wild samples and laboratory reared colonies)	Nutritional/protective role (?)	Adult male and female	Ricci et al., 2011a
	Aedes aegypti (wild samples and laboratory reared colonies)	Nutritional/protective role (?)	Adult male and female	Ricci et al., 2011a
	Aedes japonicus (wild samples)	Nutritional/protective role (?)	Adult female (midgut)	Muturi et al., 2016
	Aedes triseriatus (wild samples)	Nutritional/protective role (?)	Adult female (midgut)	Muturi et al., 2016
	Culex pipiens (wild samples)	**Nutritional role: larval development;** protective role (?)	Larvae	Steyn et al., 2016
Sand fly (Diptera: Psychodidae)	*Phlebotomus perniciosus*	**Protective role: killer strain with antifungal effect;** nutritional role (?)	Larvae (gut), adult male and female	Martin et al., 2016; Giovati et al., 2018
Honeybee (Hymenoptera)	*Apis mellifera*	**Protective role: immunomodulatory effect;** nutritional role (?)	Adult female (midgut)	Tauber et al., 2019
Plant hopper (Hemiptera)	*Laodelphax striatellus*	**Nutritional role: larval development;** protective role (?)	Nymphs, adult female (fat body)	Cao et al., 2015
Beetle (Coleoptera)	*Doubledaya bucculenta*	**Nutritional role: larval development;** protective role (?)	Eggs, larvae, and adult female (putative mygangium)	Toki et al., 2012, 2013

influence of *W. anomalus* on larval survival and pupation has been shown in *Cx. pipiens*, using the yeast as an exclusive food source (Steyn et al., 2016).

Role in Digestive Processes of Adult Insects

In adult mosquitoes, *W. anomalus* is supposed to establish trophic interactions useful to integrate a sugar restricted diet. This support has been described in *L. striatellatus*, in which *W. anomalus* provides nutrients to compensate the adult planthopper diet for the unbalanced composition of amino acids in plant phloem (Cao et al., 2015). Additionally, as floral nectar-residing yeast *W. anomalus* may support nutritional functions by acting on sugar digestibility. For instance, it may participate by fermentation to the breakdown of sugar while still residing on the flowers, as well as in the insect gut after the nectar collection, as suggested in *A. mellifera* (Tauber et al., 2019).

Another contribution of *W. anomalus* in digestive processes may be specifically exerted in adult female mosquitoes after the blood ingestion, since the yeast is among the few microbes that persist in the midgut after blood ingestion (Cappelli et al., 2019). This peculiarity is likely due to the ability of *W. anomalus* to adapt to different growth conditions, as a wide range of pH values and osmolarity. Indeed, chemical setup in the female midgut sensibly varies depending on the meal (sugar or blood), and during the blood digestion it changes from acid to basic pH values (del Pilar Corena et al., 2005). Moreover, *W. anomalus* withstands high concentrations of uric acid likely implying complete degradation pathways, thus it has potential to participate in the urate degradation and in the removal of nitrogenous wastes deriving from the blood digestion. Martin and collaborators demonstrated this contribution in females of the haematophagous diptera *P. perniciosus* by another insect-associated yeast, *Meyerozyma guilliermondii* (Martin et al., 2018).

Antimicrobial Properties and Defense of the Host

In addition to nutritional support for larvae and adults, *W. anomalus* has been suggested for protective functions thanks to antimicrobial properties. It is demonstrated that yeasts can selectively shape the insect microbial community inhibiting the development of entomopathogenic fungi and increasing the development of mutualistic fungi (Davis et al., 2011). For example, *W. anomalus* defends larva-inhabiting bamboo internodes of *D. bucculenta* from the invasion of microbial contaminants, so that the inner surface of larval sites is covered with a fungal layer that represents a *W. anomalus* monoculture (Toki et al., 2012). However, the molecular basis of the antimicrobial activity in *D. bucculenta* has not yet been clarified. Instead, protective functions based on killer activities have been proposed in mosquitoes and the related mechanisms of action have been characterized (Cappelli et al., 2014). A strain of *W. anomalus* (F17.12) isolated from *An. stephensi* produces a KT with a strong antimicrobial activity likely exerting defense from entomopathogens. F17.12 secretes KT in specific cultural conditions as well as *in vivo* in the mosquito body, both in midguts and gonads (female and male). The KT-antimicrobial activity has been demonstrated *in vitro* against different susceptible yeast strains (e.g., of *Candida albicans*) and at diverse acidity conditions. Indeed, F17.12 KT activity assays showed that the yeast killer property spans a wide range of pH values (4.5–8). This finding fits well possible antimicrobial competition of *W. anomalus* in the mosquito body, since different physiological conditions occur in different organs (midgut and gonads). Likely in mosquitoes, a killer strain of *W. anomalus* displaying a killer phenotype and candidacidal activity has been isolated also in larvae and adults (male and female) of the sand fly *P. pernicious* (Martin et al., 2016; Giovati et al., 2018).

INFLUENCE OF *Wickerhamomyces anomalus* ON THE MOSQUITO' VECTOR COMPETENCE

Antiplasmodial Effects

On the basis that *W. anomalus* releases active KT in the female mosquito's midgut even after the blood meal, the possible killer effect against the parasite in the mosquito midgut (early sporogonic stages) has been investigated. A strong antiplasmodial effect of KT has been demonstrated *in vitro* against early sporogonic stages (ookinetes) of the malaria rodent parasite *P. berghei*, with an inhibition of the parasite survival by 90%, and several morphological/structural alterations revealed by microscopy analysis (Valzano et al., 2016). These findings prompted additional *in vivo* studies highlighting that *W. anomalus* inhibits the development of *P. berghei* in the midgut of the malaria vector *An. stephensi* (Cappelli et al., 2019). Dietary supplementation of adult females with F17.12 reduced ookinetes in the midgut by 65%. *In vitro* and *in vivo* inhibition rates cannot be compared because of substantial differences between the two experimental systems. However, the result obtained *in vivo* is very important considering that the number of ookinetes that complete their development in malaria experimental models (e.g., *An. stephensi*/*P. berghei*) is considerably higher than those observed in wild mosquitoes (100-folder plus; Sinden, 1997). Therefore, *W. anomalus* has potential to significantly reduce the vector capability of mosquitoes in the field. Likely in mosquitoes, the yeast may also affect *Leishmania* (protozoan parasite) and phleboviruses in *P. perniciosus*, though further studies are necessary to evaluate anti-pathogen effects in the sand fly.

Direct and Indirect Mechanisms of Action

Valzano et al. (2016) showed that effects on *P. berghei* ookinetes are due to direct implication of the KT-enzymatic activity. In particular, treatment with castanospermine, which is an inhibitor of exo-β-1,3-glucanase-mediated activities, reduced the killer effect on the parasite and the membrane damage. These data suggest that *P. berghei* death is induced by the hydrolysis of β-glucans located in the cell-wall of the parasite. The biochemical characterization of the KT purified from F17.12 identified a glycoprotein of 140 kD and limited electrophoretic mobility,

corresponding to a high molecular weight β-glucosidase, as confirmed by activity tests in presence of castanospermine and the analysis of the predictive three-dimensional structure (Cecarini et al., 2019). Besides direct killer effects, yeasts can act by indirect mechanisms modulating the host immune system. For instance, *W. anomalus* has been reported as an active component of the gut with immunomodulatory effects in *A. mellifera* (Tauber et al., 2019). As regard mosquitoes, interesting results have been obtained by a dietary supplementation of *An. stephensi* with a KT non-producer strain of *W. anomalus* (UM3; Cappelli et al., 2019). The study demonstrated a reduction of the intensity of *P. berghei* infection by 30% (around half that by F17.12). Since UM3 is not able to induce any effect *in vitro* on cultivated parasites, indirect effects on the mosquito immune response are supposed. By the combination of direct KT-mediated activity and immune modulation of the host system, *W. anomalus* may sensibly affect the mosquito vector competence.

Wickerhamomyces anomalus IS A PROMISING TOOL FOR "SYMBIOTIC CONTROL" OF MBDs

Among a wide number of mosquito-associated yeasts (Malassigné et al., 2020), *W. anomalus* is a promising candidate for SC strategies against malaria and possibly other MBDs. We summarize main features that support this finding: (i) it is a mutualistic symbiont in *Anopheles*, *Aedes*, and *Culex* mosquitoes (impling a stable association with vectors); (ii) it bypasses the fungal reduction during the mosquito metamorphosis and after the blood ingestion; (iii) it spreads by trans-stadial and vertical transmission routes (impling self-spreading among mosquitoes and reducing efforts and costs of delivery); (iv) it inhibits the malaria parasite in the vector mosquitoes suggesting that, thanks to the wide killer activity, it has potential to affect a broad spectrum of mosquito-borne pathogens (e.g., arboviruses); (v) it is considered safe for humans and the environment (EFSA authorizes use of killer strains); (vi) it is clinically not relevant and its killer effect is based on exo-β-1,3-glucanase enzymatic activities (which targets microbes but is harmless in mammalian cell); (vii) it can be ingested by larvae and adults as a food source (advantage of yeasts over intracellular symbionts such as *Wolbachia*); and (viii) it can be formulated at low costs of production and released in the environment by dried-yeasts containing tablets, as recently carried out with fungal-larvicides used against *Aedes* spp. (Stewart et al., 2020).

CONCLUDING REMARKS

Evidence of the ability of *W. anomalus* to inhibit the malaria parasite multiplication and survival in the mosquito, pose the bases for an exploitation of this killer yeast as an environmental-friendly and safe tool to interfere with vector competence. Since *W. anomalus* has been isolated in different vector species, further investigations are worthy for understanding whether it is able to interfere with different mosquito-borne pathogens. Its biological characteristics and the possibility of a large scale manifacture of a practical product both for storage and release, make *W. anomalus* a new yeast-based tool for "symbiotic control" strategies of MBDs.

AUTHOR CONTRIBUTIONS

AC and IR: conceptualization, original draft preparation, and editing. GF: manuscript reviewing. IR: project administration and supervision. All authors contributed to the article and approved the submitted version.

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Exploring Changes in the Microbiota of *Aedes albopictus*: Comparison Among Breeding Site Water, Larvae, and Adults

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The mosquito body hosts highly diverse microbes, which influence different physiological traits of both larvae and adults. The composition of adult mosquito microbiota is tightly linked to that of larvae, which are aquatic and feed on organic detritus, algae and prokaryotic microorganisms present in their breeding sites. Unraveling the ecological features of larval habitats that shape the structure of bacterial communities and their interactions with the mosquito host is still a poorly investigated topic in the Asian tiger mosquito *Aedes albopictus*, a highly invasive species that is vector of numerous arboviruses, including Dengue, Chikungunya, and Zika viruses. In this study, we investigated the composition of the bacterial community present in the water from a natural larval breeding site in which we separately reared wild-collected larvae and hatched eggs of the Foshan reference laboratory strain. Using sequence analysis of bacterial 16S rRNA gene amplicons, we comparatively analyzed the microbiota of the larvae and that of adult mosquitoes, deriving information about the relative impact of the breeding site water on shaping mosquito microbiota. We observed a higher bacterial diversity in breeding site water than in larvae or adults, irrespective of the origin of the sample. Moreover, larvae displayed a significantly different and most diversified microbial community than newly emerged adults, which appeared to be dominated by Proteobacteria. The microbiota of breeding site water significantly increased its diversity over time, suggesting the presence of a dynamic interaction among bacterial communities, breeding sites and mosquito hosts. The analysis of *Wolbachia* prevalence in adults from Foshan and five additional strains with different geographic origins confirmed the described pattern of dual *w*AlbA and *w*AlbB strain infection. However, differences in *Wolbachia* prevalence were detected, with one strain from La Reunion Island showing up to 18% uninfected individuals. These findings contribute in further understanding the dynamic interactions between the ecology of larval habitats and the structure of host microbiota, as well as providing additional information relative to the patterns of *Wolbachia* infection.

Keywords: breeding site, water, larvae, 16S rRNA gene, *Wolbachia*

INTRODUCTION

The microbiota of *Aedes* mosquitoes is known to play a significant role in host physiology, including egg production, blood digestion (Gaio Ade et al., 2011; Coon et al., 2016a), immunity regulation (Xi et al., 2008), host–pathogen interaction and vector competence (Ramirez et al., 2012; Charan et al., 2013; Goncalves et al., 2014; Dickson et al., 2017; Souza-Neto et al., 2019).

The microbiota of both laboratory and wild *Aedes* mosquitoes has been investigated and shown to be dominated by Proteobacteria (Wang et al., 2018). However, despite the overall composition of the microbiota was similar between laboratory-reared and wild mosquitoes (David et al., 2016), the diversity of midgut bacterial communities was found to be higher in field-caught mosquitoes (Osei-Poku et al., 2012). In *Aedes aegypti*, different bacterial communities were detected between domestic and sylvatic habitats (Dickson et al., 2017). Moreover, when *Ae. aegypti* populations from the field were reared in the laboratory, they were shown to display a similar midgut microbiota (Dickson et al., 2018). These findings indicate the importance of the environment in shaping mosquito microbiota.

Mosquitoes are holometabolous insects with larval and adult stages occupying different ecological niches, thus exploiting different resources. Larvae develop in aquatic habitats, while adults are terrestrial. Larval stages acquire their symbionts primarily through feeding in their breeding site water, with their microbiota representing a subset of the bacteria found in the water (Coon et al., 2014, 2016b; Dada et al., 2014; Dickson et al., 2017; Wang et al., 2018). Adults can introduce bacteria through feeding on nectar (Merritt et al., 1992), imbibing water from their breeding sites at emergence, as occurring in *Anopheles gambiae*, or *trans*-stadially from larval gut bacterial during metamorphosis (Lindh et al., 2008). Thus, larval and adult stages are not independent from each other and biotic and abiotic features of the larval environment can influence adult microbiota.

The Asian tiger mosquito *Aedes albopictus* (Skuse, 1894) is a highly invasive species of growing public health concern due to its ability to transmit at least 22 arboviruses, including Chikungunya, Dengue, and Zika viruses (ISSG Invasive Species Specialist Group, 2020; Lwande et al., 2020). The worldwide expansion of this species was favored both by anthropogenic factors such as increased mobility and trades, and biological characteristics of this species, such as its ability to undergo photoperiodic diapause and use natural and artificial breeding sites. A number of studies have described the microbiota of *Ae. albopictus* in recent years, including the prevalence of *Wolbachia* (Zouache et al., 2009; Chouaia et al., 2010; Minard et al., 2013; Valiente Moro et al., 2013; Minard et al., 2014, 2015; Yadav et al., 2015, 2016; Coon et al., 2016b; Park et al., 2016; Ahmad et al., 2017; Muturi et al., 2017; Mancini et al., 2018; Rosso et al., 2018; Thongsripong et al., 2018; Wang et al., 2018; Guegan et al., 2018; Hu et al., 2020; Mancini et al., 2020).

Wolbachia is a genus of Gram-negative bacteria infecting about 40% of arthropod species (Zug and Hammerstein, 2012) and almost 30% of mosquito species (Ricci et al., 2002). *Wolbachia* is transovarially transmitted and is able to exert a number of reproductive manipulations, such as cytoplasmic incompatibility, that can be exploited for the control of mosquito populations (Floate et al., 2006; Bourtzis et al., 2014). Moreover, the generation of novel *Wolbachia* transinfections in *Ae. aegypti* showed to have an impact on host susceptibility for several pathogens (see for example Moreira et al., 2009; Bian et al., 2010; Walker et al., 2011; van den Hurk et al., 2012; Frentiu et al., 2014; Ye et al., 2015; Ant et al., 2018). *Aedes albopictus* transinfected with the *w*Mel *Wolbachia* strain is unable to transmit Dengue (Blagrove et al., 2012) or Chikungunya (Blagrove et al., 2013) viruses in laboratory assays. *Aedes albopictus* is naturally superinfected with two *Wolbachia* strains, *w*AlbA and *w*AlbB (Dutton and Sinkins, 2004). Thus, to develop effective *Wolbachia*-based strategies for controlling *Ae. albopictus*, it is essential to assess the stability and population invasion potential for *Wolbachia* newly generated infections. Because this is most likely linked to *Wolbachia* inter-strain interactions (Ant and Sinkins, 2018), it important to determine *w*AlbA and *w*AlbB prevalence across *Ae. albopictus* populations and strains.

In *Ae. albopictus*, most studies focused on the characterization of adult endosymbionts, and little is known about larval microbiota and how it can shape adult biological traits. Additionally, the complexity of the interactions between the mosquito host and the bacteria in breeding sites and the relationships between *Wolbachia* infection and other components of the microbiota are still poorly understood.

In this exploratory study, we aimed at expanding the current understanding of the dynamic interactions between the ecology of larval habitats and the structure of host microbiota, as well as the patterns of *Wolbachia* infection. To do so, we addressed three main questions: (i) to what extent is the breeding site microbiota affecting endosymbiont community assemblage in larval and adult *Ae. albopictus*? (ii) Is the bacterial community of the breeding site water changing over time during mosquito development? (iii) What is the frequency of *Wolbachia* in adult samples reared as larvae in the wild-collected water?

To answer these questions, we compared bacterial community composition among (i) water from natural larval breeding sites, (ii) mosquito larvae, and (iii) adult individuals. In water from a natural breeding site, we reared both wild-collected larvae and larvae of the Foshan reference strain to derive information about the relative impact of the environment and the genetic background of mosquitoes on shaping mosquito microbiota. Finally, we analyzed *Wolbachia* presence in Foshan and five additional laboratory strains with different geographic origins.

MATERIALS AND METHODS

Water Sampling

Water from *Ae. albopictus* larval breeding sites was collected in a private garden in Crema, Italy (45°21′51.2″N 9°40′57.7″E), which was an accessible site characterized by high population density, at the end of August 2018, when climate conditions are optimal for mosquito development. Domestic collection of water, eggs, and larvae originated from two plastic buckets of a maximum volume of 500 ml that were placed in the same garden

next to ornamental plants. Environmental water collected in these buckets derived from dew and rain and was monitored for its level and clarity every day for 4 days. No sedimentary layer was observed. Water and eggs/larvae were then collected in sterile 50-ml Falcon tubes and transferred to the insectary of the University of Pavia, Italy. The collected water was divided into three aliquots and used as follows: (i) an aliquot of 200 ml was brought to the ZooPlant laboratory in Milano, Italy, to characterize its microbial composition (samples hereafter called W Start); (ii) 300 ml were aliquoted in two different pans (150 ml/pan) and daily monitored for natural larval hatching in the Pavia insectary (samples named CR); (iii) 600 ml were used to hatch eggs from the Foshan strain (Chen et al., 2015; Palatini et al., 2020), after having verified the absence of wild eggs using a stereomicroscope. The Foshan strain is an established laboratory colony derived from wild mosquitoes from South-East China and reared in the Pavia insectary since 2013 (Palatini et al., 2017). A total of 400 Foshan eggs were hatched, in batches of 100, each in 150 ml of breeding site water (samples named FO). To avoid overcrowding, the amount of water was determined based on the average hatching rate of the Foshan strain in our insectary conditions. No food was added in any pan to allow larvae to grow based on the nutrients present in the breeding site water.

For both the CR and FO samples, the developing individuals were collected separately as fourth instar larvae (in pools of eight individuals) and adults (individually collected the day of their emergence). In the case of adult collections, pupae developed in the larval rearing pans were individually transferred to cups containing 10 ml of the same wild water until adult eclosion. When mosquito development was completed and all adults had eclosed, the remaining water from each rearing pan was transferred into sterile 50 ml Falcon tubes for the analysis of the microbiota (samples hereafter called W End).

All mosquito life stages were maintained in the Pavia insectary at 28°C and 80% RH, with a photoperiod of 12:12 hrs light:dark.

Chemical Analyses of Water Samples

For each water sample, total nitrogen (N_tot), nitrites (NO_2), nitrates (NO_3), ammoniacal nitrogen (NH_4-N), and phosphate (P_tot) were measured with a spectrophotometer (Spectroquant Pharo 300, Merck). Moreover, pH and conductivity were also recorded. Analyses were performed using the following kits, according to the manufacturer's instructions: Ammonium Test Photometric Method NH_4-N, Nitrate Test Photometric Method NO_3-N, Nitrite Test Photometric Method NO_2-N, Total Nitrogen Test Photometric Method, Phosphate Test Photometric Method PO_4-P, Merck Spectroquant®.

Sample Pre-processing and DNA Extraction

Genomic DNA was extracted from (i) water samples at the two time-points described above; (ii) pooled larval (L) samples; (iii) individual adult (A) mosquitoes collected immediately after eclosion.

Water samples were processed according to a modified version of a previously described protocol (Bruno et al., 2017a). First water samples were filtered by a serial vertical (orthogonal) filtration using nitrocellulose membrane filters with a pore size of 8 μm, followed by a filtration with a membrane with pores of 3 μm. Vacuum was generated by a vacuum pump (ME 2 NT VacuubrandTM) connected to a filtering apparatus. The filtrate was collected and concentrated using tangential flow filtration (TFF) to recover as much biological material as possible. The TFF system involved a peristaltic pump (Masterflex L/S Economy Drive), Tygon® tubing, sterile reservoirs and filtration modules. The used tangential flow filter was a VivaFlow® 200 cassette (Sartorius) made of polyethersulfone (PES) with a nominal pore rating of 10000 MWCO and a surface area of 200 cm^2. The system was scaled up with an additional unit connected in parallel to increase the filtration surface area and the flow speed. The TFF system was run at a transmembrane pressure of 1.5 bar. The initial water samples were concentrated to a final retentate volume of 100 mL, which was further reduced to 1 mL through the Vivaspin® 20 ultrafiltration unit (Sartorius) made of PES with a nominal pore rating of 10000 MWCO. The final volume was allowed to be adsorbed onto a membrane filter before DNA extraction.

All tubing, tubing connections, and containers were sterilized with sodium hypochlorite or autoclaved prior to each experiment and among samples. Every step was conducted in the laminar flow cabinet in a pre-amplification dedicated laboratory.

Environmental DNA was extracted separately from each of the filters obtained by water filtration using DNeasy® PowerSoil® Kit (Qiagen) with the QIAcube (Qiagen) automated system, following the manufacturer protocol. DNA was eluted in 75 μL of warmed (40°C) elution buffer, to increase DNA concentration. DNA extraction negative controls were included.

A total of 25 *Ae. albopictus* samples were processed, namely 11 larval pools (four CR and seven FO samples, respectively) and 14 individual adults (five CR, of which four males and one female; nine FO, of which six males and three females, respectively). The finding that sequencing pools of six mosquitoes allowed to capture a level of bacterial diversity comparable to that of single mosquitoes (Bennett et al., 2019), together with our aim to achieve the maximum information level, prompted us to process larvae and adults differently. Larval samples were treated as pools of eight individuals, similarly to other studies (e.g., Wang et al., 2018) to minimize the biological variability of the microbiota among individuals and, at the same time, avoid overcycling in amplicon PCR. Individual adults were also used to obtain data related to *Wolbachia* infection at the single-mosquito level. Prior to DNA extraction from both larval pools and adults, each individual was surface-washed twice with 1X PBS, after washing in ethanol 70% (following Seabourn et al., 2020). DNA extraction was performed using the Wizard Genomic DNA Purification Kit (Promega) following the manufacturer's instructions. DNA extraction negative controls were included.

All the procedures were carried out in a laminar flow cabinet, in order to avoid contamination with exogenous DNA and inter-samples contamination, and in separate rooms for the pre- and post- amplification steps, with dedicated personal protective equipment.

16S Metagenomic Sequencing of Mosquito and Water Samples

Illumina MiSeq 16S libraries were generated following the standard protocol "16S Metagenomic Sequencing Library Preparation, Part # 15044223 Rev B." Amplicon PCR was performed using PCR primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′) with Illumina library adaptors, for 25 cycles of amplification. DNA extraction negative controls (Bianco_1EXT, Bianco_2EXT) and amplicon PCR negative controls were included in library preparation. Libraries quantification through TapeStation 4100 (Agilent) showed no amplicon signal for amplicon PCR negative controls, which were excluded from the sequencing. In the case of *Ae. albopictus* samples, amplicon sequencing was run by Macrogen, Inc. using an Illumina MiSeq platform and the Herculase II Fusion DNA Polymerase Nextera XT Index Kit V2. Water samples were sequenced by the Center for Translational Genomics and Bioinformatics – San Raffaele Scientific Institute (Milan, Italy) with Illumina MiSeq 2 × 300 paired-end chemistry (MiSeq Reagent Kit v3).

Illumina Data Processing and Analyses of the Microbiota Composition

The raw paired-end FASTQ reads were imported into the Quantitative Insights Into Microbial Ecology 2 program (QIIME2, ver. 2017.9.01) (Caporaso et al., 2010) and demultiplexed using native plugin. The Divisive Amplicon Denoising Algorithm 2 (DADA2) (Callahan et al., 2016) was used to quality filter, trim, denoise, and mergepair the data and remove chimeric sequences.

The resulting Amplicon Sequence Variants (ASVs) with less than a 50x coverage were discarded from further analyses. The classification of the obtained ASVs was run using the feature-classifier plugin implemented in QIIME2 against the SILVA SSU non-redundant database (138 release), adopting a consensus confidence threshold of 0.8. The analysis on the bacterial diversity as well as the corresponding figures were done using the *phyloseq* R package (McMurdie and Holmes, 2013).

Any reads assigned to *Wolbachia* were filtered using the filtered taxa plugin of QIIME2.

Microbiota diversity was described in terms of within (alpha) and between (beta) sample diversities. The Shannon index and Observed Features alpha diversity metrics were calculated to estimate the variation of bacterial diversity in the water, larvae and adult samples. Values were compared using the pairwise Kruskal–Wallis test.

To explore the bacterial diversity of our samples, we used a relative abundance bar plot to show phyla and family distribution between our three sample types (water, larvae, and adults). The distribution of the 100 most abundant ASVs was studied with a heatmap plot. Both visualizations were obtained with the *phyloseq* R package.

Rarefaction is used to subsample and calculate distances among samples. Beta diversity was estimated with quantitative distance metrics using the diversity QIIME2 plugin based on the rarefied dataset with a sampling depth of 10,000 sequences. Samples with lower depth were automatically discarded from beta diversity analysis.

We estimated the unweighted UniFrac and Bray–Curtis dissimilarity indexes by sampling 10,000 reads per sample (Lozupone et al., 2007). Statistical significance among groups, including sampling site and developmental stage, was determined by a permutation-based ANOVA (PerMANOVA) test using ADONIS (Anderson, 2005) and a 999 permutation-based UniFrac distance metrics. PerMANOVA Pairwise contrast was performed by the beta-group-significance command of *diversity* plugin. The structure of microbial communities was explored by Non-Multidimensional Scaling (NMDS), an ordination approach (Kruskal, 1964). Sequences representative of each community were aligned with MAFFT and used for phylogenetic reconstruction in FastTree (Price et al., 2010).

The list of bacterial genera, as derived from ASVs, in each sample was compared using Venn diagrams following two criteria. We considered ASVs showing more than 50 reads and occurring in at least two tested samples as 'general' ASVs. Then, among these ASVs, we looked for those occurring in at least 70% of all tested samples; we considered these ASVs as the "conserved" mosquito microbiota. This analysis allowed us to explore the flux of bacterial symbionts acquired from the water environment by the mosquito larvae and maintained until the adult stage. Venn diagrams were created using an online tool[1].

qPCR Amplification of 16S rRNA Gene in Water Samples

Quantitative Real Time PCR (qPCR) assays were performed targeting the 16S rRNA gene to verify the sensitivity of our approach in detecting the microbiota of water samples and to compare relative bacterial abundances between W Start and W End, according to a protocol previously described (Bruno et al., 2017a). Water samples, samples deriving from TFF filtrate, DNA extraction negative controls and amplification negative controls were tested in triplicates.

Cycling conditions adopted were as follows: an initial denaturation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s and annealing-elongation at 55°C for 1 min. A final dissociation stage was performed. Amplification reaction consisted of 5.0 μl SsoFast EvaGreen Supermix with Low ROX (Bio-Rad S.r.l., Segrate, Milan, Italy), 0.1 μl each 10 μmol l^{-1} primer solution, 2 μl DNA sample, and 2.8 μl of Milli-Q water. Assays were performed on an AB 7500 thermocycler (Applied Biosystem) and results analyzed as previously described (Bruno et al., 2017a).

Estimates of *Wolbachia* Prevalence by PCR

To obtain a qualitative validation of *Wolbachia* infection status in the 25 adult samples used for metagenomic analysis, PCR reactions for *Wolbachia* detection were performed in 15 μl reaction volumes consisting of 7.5 μl DreamTaq Green Master

[1]http://bioinformatics.psb.ugent.be/webtools/Venn/

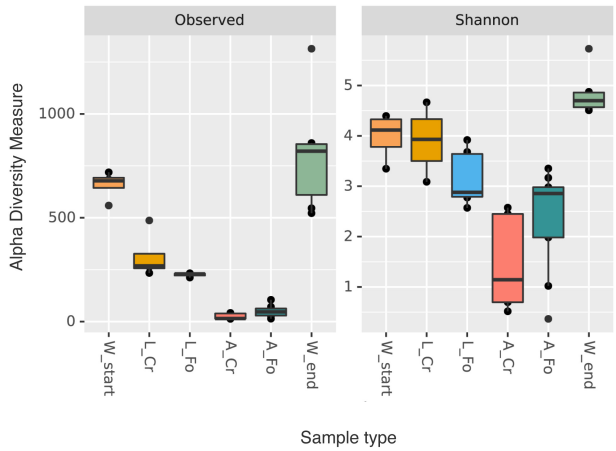


FIGURE 1 | Alpha diversity indices (richness and Shannon). Box plots of alpha diversity values distribution for both indices (richness and Shannon diversity) of the water (W Start, orange; W End, light green), larval (CR, golden; FO, blue), and adult (CR, coral; FO, dark green) samples are shown. The line inside each box represents the median value. Outliers are shown as dots.

Mix (Thermo Fisher Scientific, Eugene, OR, United States), 4 μl of autoclaved Milli-Q water, 1 μl primers (10 μM), and 1.5 μl of DNA (∼20 ng). The PCR cycling conditions were as follows: 3 min at 95°C for the initial denaturation step, followed by 35 cycles of 1 min at 95°C, 1 m at 55°C, 1 m at 72°C and 10 min at 72°C for the final extension. The published *wsp* primers were used (Zhou et al., 1998): 328F (5′-CCAGCAGATACTATTGCG-3′) and 691R (5′-AAAAATTAAACGCTACTCCA-3′) for the detection of *w*AlbA, and 183F (5′-AAGGAACCGAAGTTCATG-3′) and 691R for *w*AlbB. PCRs reactions were run on the same samples also using the primer set Aealbo18S_F1 (5′-TGCCATGGATGCTTTCATTA-3′) and Aealbo18S_R1 (5′-GTACAAAGGGCAGGGACGTA-3′) to test for DNA quality. All PCR products were visualized on 1% agarose gels and sequenced.

Moreover, the presence of *Wolbachia* was assessed by PCRs in Foshan (generation 37) and five additional long-established laboratory strains deriving from Canton (China, G20), Recife (Brasil, G25), Tapachula (Mexico, G31), Tampon (La Reunion, G26), and Crema (Italy, G33). These strains were selected for several reasons. First, to compare *Wolbachia* prevalence estimated through metagenomics in CR and FO samples (reared in wild-collected water) with estimates of *Wolbachia* presence in the Foshan and Crema laboratory strains. The Crema strain was established in 2016 from mosquito eggs collected in the same breeding site used in this study. Second, to determine whether insectary rearing for several generations have led to a 100% *Wolbachia* frequency in adult individuals.

RESULTS

The microbiota of breeding site water (W), larval (L), and adult (A) *Ae. albopictus* samples was examined by sequencing of the bacterial 16S rRNA gene. Sequences of a total of 37 libraries (*i.e.*, 10 water samples; four CR and seven FO larval samples; five CR and nine FO adults, respectively; two DNA extraction negative controls) resulted in 8,191,150 million sequence reads, ranging from 14,277 to 264,318 (with an average of 126,018). No reads were reported in negative controls. Total number of reads per sample before and after *Wolbachia*-associated reads removal is reported in **Supplementary Table S1**. Identified ASVs per sample ranged between 19 and 3410 (**Supplementary Table S2**).

W End samples showed higher variability in the number of ASVs than W Start samples; larval samples showed a much higher variability than adults, irrespective of their origin (**Figure 1**). These differences are statistically supported, as shown by the results of the Kruskal–Wallis test (**Table 1**). Quantity of bacterial DNA in water samples was also estimated by qPCR (**Supplementary Figure S1A** and **Supplementary Table S3**) confirming a significantly higher number of bacterial DNA copies in W End in comparison to W Start (ANOVA, Tukey *post hoc* test: $p < 0.05$).

Water chemistry at mosquito breeding sites has been shown to have a key impact on mosquito survival and abundance (Chen et al., 2009; Rajesh et al., 2013; Onchuru et al., 2016). Additionally, specific physical and chemical parameters (*i.e.*, oxygen and conductivity) were found to be associated with microbiota composition in *Ae. aegypti* (Hery et al., 2021). Thus, in an attempt to provide a qualitative analysis that could support the metagenomic results, a set of physical and chemical parameters were measured for both W Start and W End samples. Both pH and conductivity significantly increased in W End samples (*t*-test, $P < 0.05$) (**Supplementary Figure S1B** and **Supplementary Table S4**). Moreover, nitrates, ammoniacal nitrogen and phosphate values were higher (at least > 2.5

TABLE 1 | Pairwise comparison (Kruskal–Wallis test) of diversity indices between water, larval, and adult samples.

		Water		Crema		Foshan
		W Start	W End	L_CR	A_CR	L_FO
Water	W End	**6.54 (0.010)**				
Crema	L_CR	0.083 (0.773)	3.682 (0.05)			
	A_CR	**6.00 (0.014)**	**7.50 (0.006)**	**6.00 (0.014)**		
Foshan	L_FO	**4.321 (0.038)**	**9.00 (0.003)**	2.893 (0.089)	**7.180 (0.007)**	
	A_FO	**7.384 (0.007)**	**9.60 (0.02)**	**5.654 (0.017)**	1.371 (0.242)	**1.929 (0.165)**

H and p-values (in brackets) are shown. Significant comparisons ($P < 0.05$) are indicated in bold. The number of samples within each group were: W Start (n = 4). W End (n = 6), L_CR (n = 4), L_FO (n = 7), A_CR (n = 5), A_FO (n = 9).

folds) in W End than in W Start, providing further support to the presence of differences between the two considered samples, despite measurements were often close to detection limits (**Supplementary Table S4**).

Microbiota Composition and Distribution

A total of 28 bacterial phyla and 262 families were identified across all samples. Taxonomic analysis showed that most of the sequences are associated with the phylum Proteobacteria (59.7%), followed by Bacteroidota (16.4%), Firmicutes (3.7%), and Spirochaetota (3.6%).

In W Start samples, the main bacterial phyla were Proteobacteria (>95%), followed by Bacteroidota (0.5%) and Verrucomicrobiota (0.3%). In addition to these phyla, in W End samples we also identified Patescibacteria, Dependentiae, Cyanobacteria, and Acidobacteria (**Figure 2A**). The trend of increasing diversity from W Start to W End samples is also evident at the family taxonomic level (**Figure 2B**). Proteobacteria and Bacteroidota dominated the microbiota of larvae; the bacterial community of adults displayed a much lower level of diversity, with Proteobacteria representing almost the totality of the microbiota of adult mosquitoes (95% in A_CR and 71% in A_FO) (**Supplementary Table S5**).

The trend of decreasing ASVs variability from water to adult samples is also depicted in a heatmap generated using the 100 most abundant ASVs assigned at the taxonomic level of Family (unweighted UniFrac distance) (**Figure 3**). The heatmap shows that the 100 most abundant ASVs are differently distributed in the water, larvae, and adult samples. Differences are evident also between W Start and W End, as well as between CR and FO samples, independently of the developmental state. At the family taxonomic level, W Start includes Sphingobacteriaceae, Spirosomaceae, Chitinophagaceae and, to a lesser extent, Nocardiaceae. W End samples include Cellvibrionaceae, Burkholderiaceae, Caulobacteraceae, Planococcaceae, Cytophagaceae, Blastocatellaceae, and several unculturable unidentified bacteria. Larval samples are characterized by Weeksellaceae, Spirosomaceae and Chitinophagaceae (phylum Bacteroidetes), Spirochaetaceae (phylum Spirochaetes), Sphingomonadaceae and Rhodobacteraceae (phylum Proteobacteria) (**Figures 2B**, **3**).

Wild and laboratory larval samples differentiated by the presence of Verrucomicrobiaceae, Chitinophagaceae, Pirellulaceae, Cellvibrionaceae, and Terrimicrobiaceae exclusively in laboratory samples. The only bacterial family uniquely present in wild larval samples was Microbacteriaceae, with the *Herbiconiux*, *Leifsonia*, and *Leucobacter* genera.

In addition to bacteria already known to be part of the microbiota of *Aedes* mosquitoes, such as *Sphingomonas* and *Chryseobacterium* (see Scolari et al., 2019 for a review), in Foshan larval samples we also identified bacteria of the *Paenibacillus* genus, which were previously associated only with *Ae. aegypti* (Scolari et al., 2019). In Crema samples, genera reported for both *Ae. aegypti* and *Ae. albopictus*, such as *Bacillus, Pseudomonas,* and *Escherichia–Shigella*, were also found.

In the case of adult samples, the families Verrucomicrobiaceae, Acetobacteracea, Planococcaceae, Weeksellaceae, Crocinitomicaceae, Cytophagaceaea, Corynebacteriaceae, Blastocatellaceae, and Bryobacteriaceae were typical of the Foshan laboratory samples, with Weeksellaceae and Planococcaceae being particularly abundant.

Based on these results, beta diversity metrics were computed to further explore differences among samples. For more reliable results we used a rarefaction process to resample the data. Obtained rarefaction curves are shown in **Supplementary Figure S2**.

Samples separated into clusters in a non-metric multidimensional scaling (NMDS) ordination plot (**Figure 4**). Using pairwise weighted UniFrac distance matrix, the obtained stress value was <0.1. W Start and W End samples clustered together, although into two identifiable groups. All larval samples clustered together, suggesting a limited contribution of the genetic background in shaping larval microbiota. On the opposite, adult samples showed greater intra and inter sample variability, with a portion of them not being clearly separated from the larvae. PerMANOVA pairwise comparison based on the unweighted UniFrac distance metrics revealed statistically significant differences among the groups (**Table 2**).

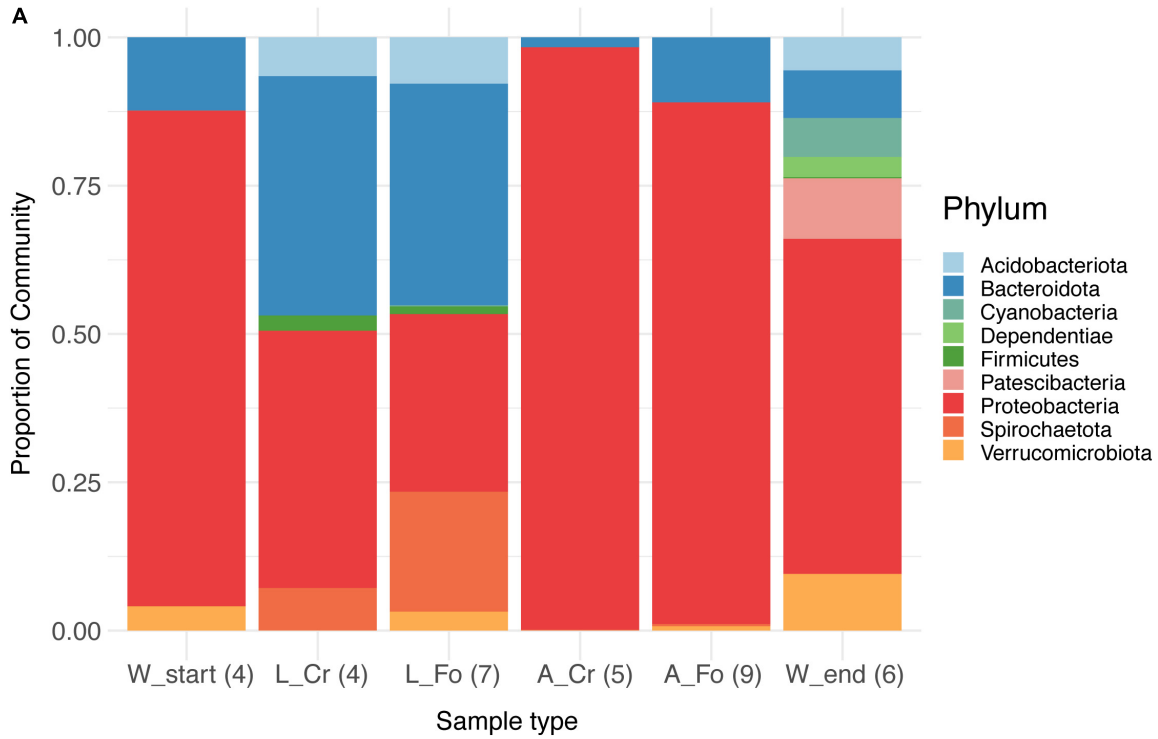
Sample-Unique Bacteria

The intersections among the list of bacterial genera found in analyzed samples were calculated to identify the components of the microbiota that are unique of each sample (**Figure 5**). As expected according to the previously described data, the number of genera shared between larvae and water samples were higher than those shared between larvae and adults, both in the case of Crema and Foshan samples (**Figure 5**). Wild larvae unique genera included *Dietzia*, *Blautia*, and *Leifsonia*; in wild adults, unique genera were *Tepidimonas*, *Cloacibacterium*, *Haemophilus,* and *Neisseria*. An unidentified uncultured bacterium from the family Rhodobacteraceae was found to be unique of Foshan larvae; *Dietzia*, *Meiothermus*, *Porphyromonas*, *Blautia*, *Micrococcus,* and *Fructobacillus* were the unique genera in Foshan adults.

When focusing on comparison between W Start and W End, and in both larvae and adults between FO and CR samples, the most striking result was the number of ASVs uniquely detected in adults of Foshan, and including genera such as *Prosthecobacter*, *Solimonas*, *Ancylobacter*, *Anaerococcus*, *Schlesneria,* and *Micrococcus* (**Supplementary Figure S3**).

Conserved Microbiota

We define "conserved" microbiota the bacterial genera detected in at least 70% of all our mosquito samples. Mosquito conserved microbiota includes 102 genera (**Figure 6** and **Supplementary Tables S6, S7**). A total of 81 genera were also detected in water samples (*i.e.*, W Start), emphasizing the role of the breeding site in shaping mosquito microbiota. Among the bacteria absent in water samples (both W Start and W end), *Vulcaniibacterium* was the only present in both larvae and adults, of both CR and FO samples. *Escherichia–Shigella* was instead found only in CR adult samples. Several unclassified bacteria were found to be conserved in larval samples and/or adults, absent from the W Start samples but detected in the W End samples (**Supplementary Table S7**).



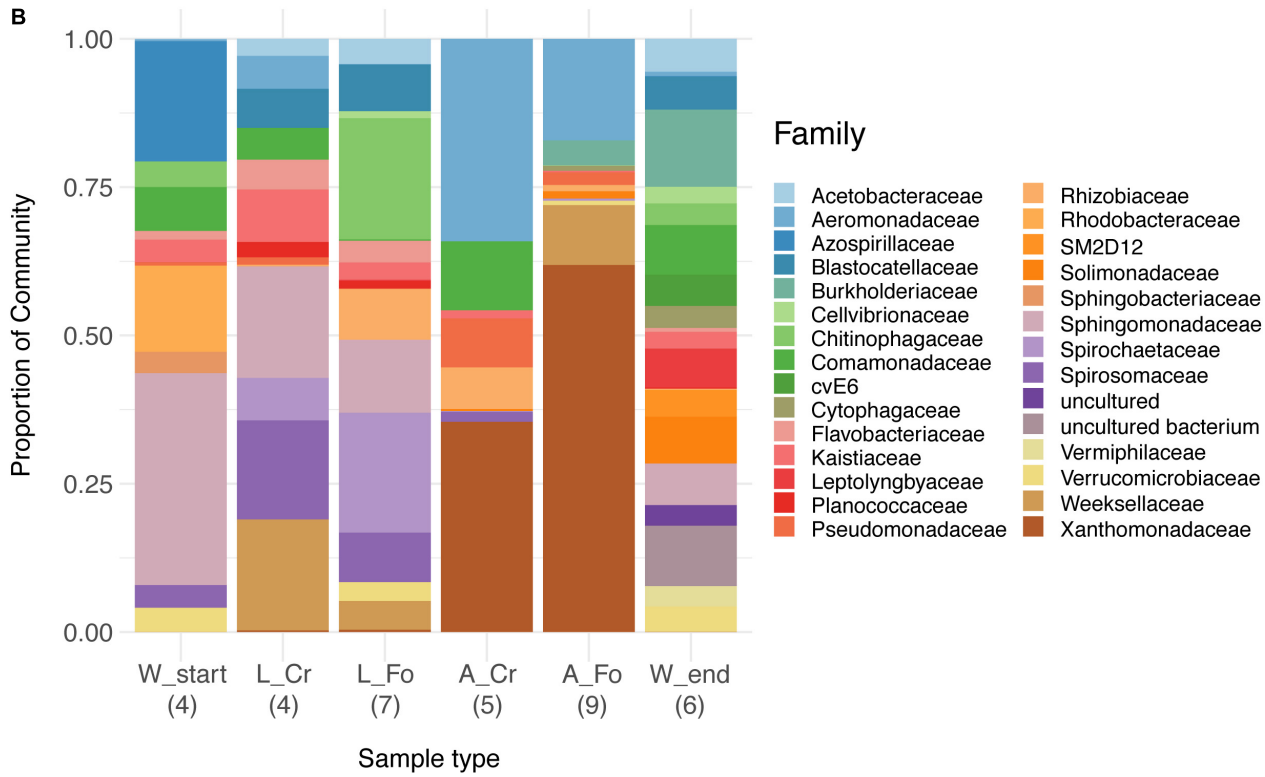


FIGURE 2 | Relative abundance bar plot of bacteria at phylum **(A)** and family **(B)** taxonomy level in water, larval, and adult samples from wild-collected and laboratory mosquitoes. Each bar represents the proportion of sequencing reads (relative abundance expressed as percentage) assigned to a given bacterial taxon. Only the 50 most abundant Amplicon Sequence Variants were considered and assigned to corresponding taxa. The legend lists the most abundant taxonomic categories (9 in **A** and 29 in **B**). The number of samples analyzed for each category is reported in parentheses.

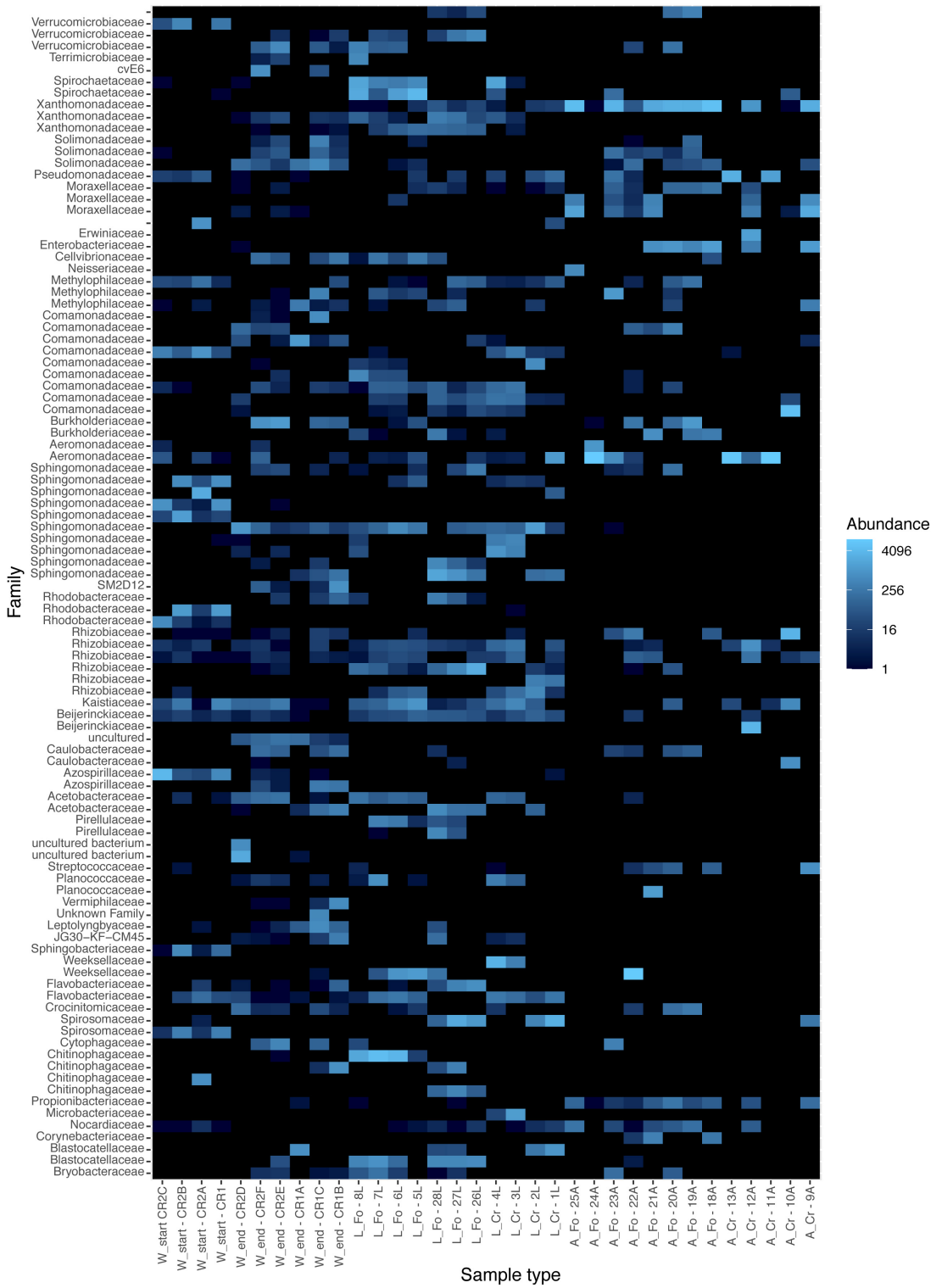


FIGURE 3 | Heatmap showing the relative abundance of the components of the microbiota in the breeding site water, larvae, and adult samples from laboratory and wild-collected mosquitoes. The distribution of the 100 most abundant Amplicon Sequence Variants was explored in each analyzed sample with a heatmap plot based on rarefied tables. Each sample is shown on the *X*-axis; W refers to water samples, L and A to larvae and adult samples, respectively. Start and End refers to water collected at the moment of mosquito collection and after all adults had emerged, respectively. FO and CR refer to mosquitoes of the Foshan laboratory strain or collected in the wild, respectively. Heatmap colors (from dark to light blue) indicate increasing abundance of each microbiota component. The heatmap was generated with the *phyloseq* R package.

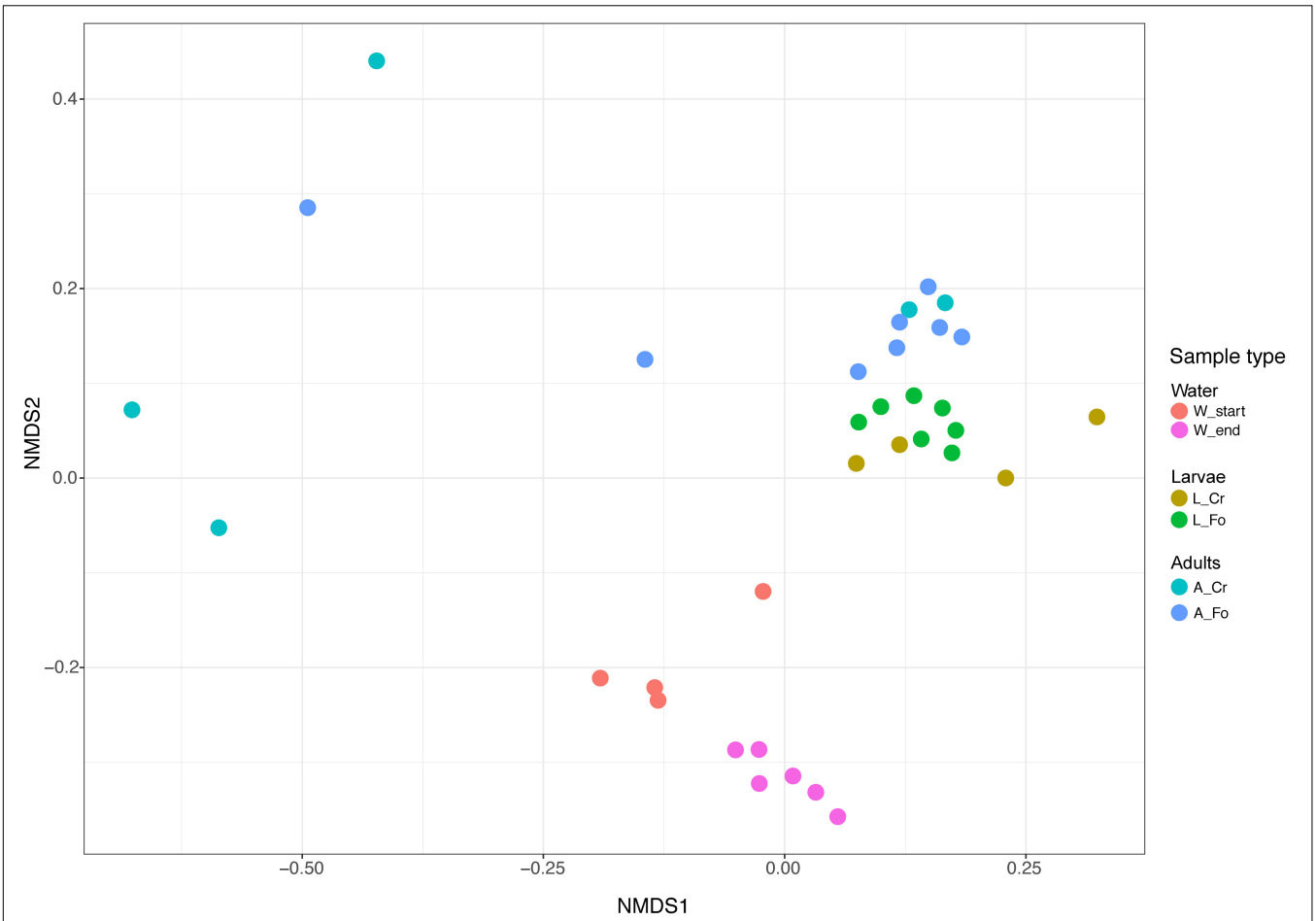


FIGURE 4 | Non-metric multidimensional scaling (NMDS) of water, larval and adult samples. Colors in the bidimensional NMDS plot are used according to the different sample origin as shown in the legend. Start and End refers to water collected at the moment of mosquito collection and after all adults had emerged, respectively. FO and CR refer to mosquitoes of the Foshan laboratory strain or collected in the wild, respectively.

TABLE 2 | PerMANOVA pairwise comparison based on unweighted UniFrac distance metrics between water, larval, and adult samples.

		Water		Crema		Foshan
		W Start	**W End**	**L_CR**	**A_CR**	**L_FO**
Water	W End	**2.884 (0.006)**				
Crema	L_CR	**2.718 (0.023)**	**3.497 (0.007)**			
	A_CR	**3.230 (0.013)**	**4.540 (0.003)**	**2.421 (0.048)**		
Foshan	L_FO	**3.700 (0.008)**	**4.870 (0.001)**	1.330 (0.094)	**3.279 (0.002)**	
	A_FO	**3.760 (0.003)**	**5.621 (0.001)**	**2.483 (0.002)**	1.537 (0.163)	**2.9351 (0.001)**

Pseudo-F and p-values (in brackets) are shown. Significant comparisons (P < 0.05) are indicated in bold. The number of samples within each group were: W Start (n = 4), W End (n = 6), L_CR (n = 4), L_FO (n = 7), A_CR (n = 5), A_FO (n = 9).

Wolbachia Abundance Impacts Microbial Richness in *Ae. albopictus* Adults

Shannon diversity index was calculated for each sample resulting into two groups. One group included five samples (11A_wild_CR, 13A_wild_CR, 22A_FO, 10A_Wild_CR, and 24A_FO) with Shannon diversity index < 1.5; all the other samples had a Shannon diversity index higher or equal to 2 (**Supplementary Figure S4**). Interestingly, samples from the first group had lower frequency of *Wolbachia* reads (ranging from 0% in 11A_wild_CR and 13A_wild_CR to 41.2% in 22A_FO) than samples of the second group, thus we called these two groups "*Wol*_low" and "*Wol*_high," respectively, and we compared their alpha diversity after excluding *Wolbachia* reads. *Wol*_high samples displayed a significantly higher number of ASVs than *Wol*_low samples (*t*-test, $P < 0.05$) (**Figure 7**).

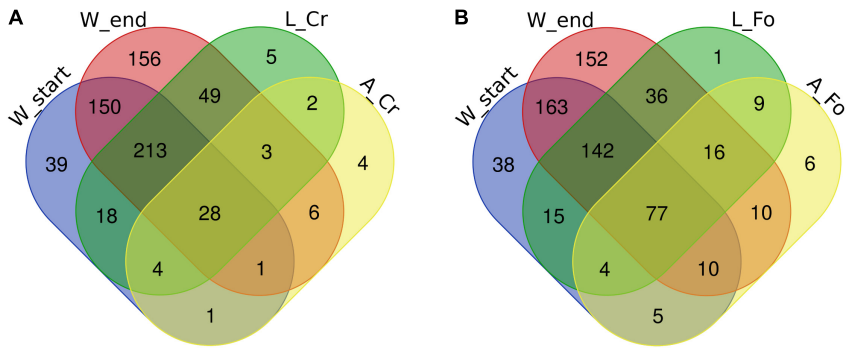


FIGURE 5 | Number of unique bacterial genera in breeding site water (W), larval (L), and adult (A) samples from wild (CR) or laboratory (FO) samples. Venn diagrams show the number of shared genera in **(A)** Crema (CR) and **(B)** Foshan (FO) samples with respect to what observed in breeding site water collected at the beginning (start) or after all adults had emerged (end). The Venn diagrams were created using an online tool (http://bioinformatics.psb.ugent.be/webtools/Venn/).

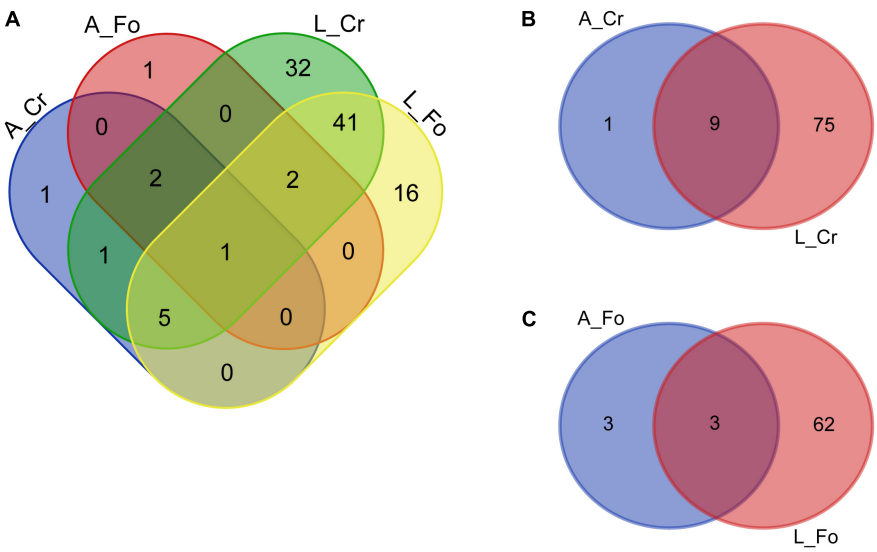


FIGURE 6 | Number of unique bacterial genera from the conserved microbiota of larval (L) and adult (A) samples from wild (CR) or laboratory (FO) mosquitoes. Venn diagrams show the number of conserved genera in **(A)** adults and larvae from Crema (CR) and Foshan (FO), **(B)** within CR mosquito samples, and **(C)** within FO mosquito samples. The Venn diagrams were created using an online tool (http://bioinformatics.psb.ugent.be/webtools/Venn/).

Wolbachia Presence in Different Laboratory Strains

The absence of *Wolbachia* in four adult samples prompted us to further investigate *Wolbachia* prevalence in six long-established laboratory strains. The prevalence of *w*AlbA and *w*AlbB strains was also determined. In all mosquito strains, both *w*AlbA and *w*AlbB were detected. However, while *Wolbachia* was present in all tested individuals in Foshan, Canton, and Recife strains, some individuals were shown to not carry *Wolbachia* in Crema, Tapachula, and Tampon (**Table 3**). The percentage of *Wolbachia*-negative mosquitoes ranged between 3% in Crema to 18% in Tampon. Absence was more prevalent for *w*AlbA than *w*AlbB. Patterns of *Wolbachia* infections were also evaluated in relation to mosquito sex (**Table 3**). Dual infection was more common in females than in males in Foshan, Canton, and Recife strains, while in Crema and Tampon, males had the highest values of infection. Furthermore, *w*AlbA-only infection was never detected in males, while *w*AlbB-only infection appeared to be mostly limited to males.

DISCUSSION

Aedes albopictus Larvae Acquire Microbiota From Breeding Site Water

The analysis of the diversity of bacterial communities showed that *Ae. albopictus* larvae contain a subset of the ASVs present in breeding site water, supporting the idea that larvae are colonized by a fraction of the bacteria ingested through feeding (Minard et al., 2013; Coon et al., 2016b; Strand, 2018). Higher ASVs richness in water than in mosquito samples has been already

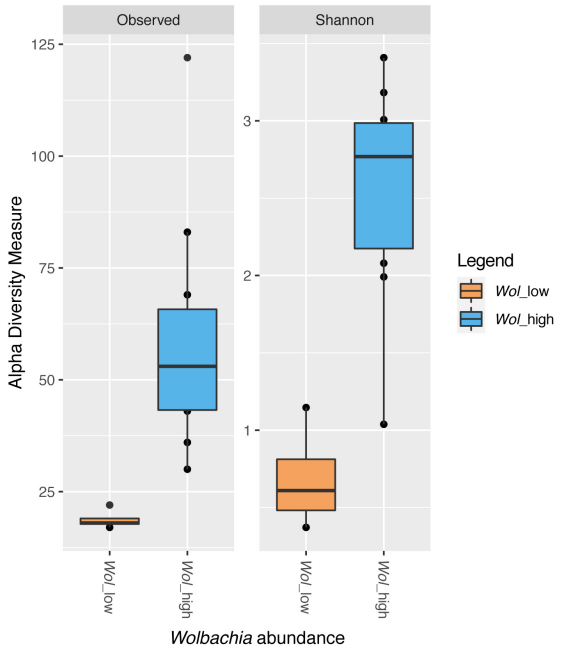


FIGURE 7 | Alpha diversity indices (richness and Shannon) in samples with low (*Wol*_low, in orange) or high (*Wol*_high, in blue) *Wolbachia* abundance. *Wol*_low grouped five samples (11A_wild_CR, 13A_wild_CR, 22A_FO, 10A_Wild_CR, and 24A_FO), which had individually a Shannon diversity index < 1.5, as shown in **Supplementary Figure S4**, and a frequency of *Wolbachia* reads up to 41.2%. *Wol*_high grouped all the other samples, which had a frequency of *Wolbachia* reads > 86.8% and an individual Shannon diversity index > 2 (**Supplementary Figure S4**). The line inside each box represents the median value. Outliers are shown as dots. *Wol*_high samples displayed a significantly higher number of ASVs than *Wol*_low samples (*t*-test, $P < 0.05$).

described in *Aedes* spp. (Dada et al., 2014; Dickson et al., 2017; Wang et al., 2018; Alfano et al., 2019). Among the bacterial genera present in the breeding site water and acquired by both CR and FO larvae, it is worth mentioning *Chryseobacterium*, which is known to localize in the larval gut (see Scolari et al., 2019 for a review). We also observed differences between CR and FO larvae in bacterial genera shared with W Start samples, although all larval samples grouped together in the NMDS plot, suggesting a limited contribution of the genetic background in shaping larval microbiota. For example, Foshan larval samples shared with W Start bacteria such as *Roseococcus, Paenibacillus,* and *Ferruginibacter*, which were not detected in Crema larvae. The microbiota of Crema larvae showed *Bacillus*, which was previously detected in *Ae. aegypti* (Koneman et al., 1992) and *Ae. albopictus* field-collected larvae (Coon et al., 2016b), and *Ensifer*, *Sphingobium,* and *Aeromicrobium*.

Microbial Community of Breeding Site Water Changes Over Time

Despite the intrinsic impossibility to provide a comparison with respect to breeding site prior to mosquito oviposition (*i.e.*, a 'natural' control, characterized by total absence of mosquito eggs and larvae), the comparison between W Start and W End water samples suggests the presence of a dynamic interaction between larvae and their breeding site. Such a difference was also detected by qPCR amplification of the 16S rRNA gene, which confirmed a significantly higher number of bacterial DNA copies in W End in comparison to W Start. Although unculturable candidate phyla are likely present in our water samples, the procedure of relying on 16S rRNA quantification to infer bacterial load in breeding site water and mosquito samples is routinely used (e.g., Charan et al., 2013; Wang et al., 2018).

Future studies comprising water samples collected in breeding sites before mosquito oviposition will provide important data to clarify the impact of mosquito larvae on shaping the water bacterial communities. Such studies will require the possibility to robustly predict the likelihood of a water collection to become a mosquito breeding site. Moreover, additional studies comprising water controls collected at breeding sites and maintained without larval samples over time to cover the mosquito developmental

TABLE 3 | *Wolbachia* prevalence in six *Ae. albopictus* laboratory strains.

		***Wolbachia* prevalence (%)**				
Strain (generation)	**N. samples**	***w*AlbA**	***w*AlbB**	***w*AlbA + *w*AlbB**	**Total *Wolbachia*+**	**Total *Wolbachia* –**
Foshan (G37)	40	0	22	78	100	0
	(22M, 18F)	(0M, 0F)	(22M, 0F)	(33M, 45F)		
Canton (G20)	40	0	10	90	100	0
	(20M, 20F)	(0M, 0F)	(10M, 0F)	(40M, 50F)		
Recife (G25)	40	5	10	85	100	0
	(20M, 20F)	(0M, 5F)	(10M, 0F)	(40M, 45F)		
Crema (G33)	40	0	0	97	97	3
	(20M, 20F)	(0M, 0F)	(0M, 0F)	(50M, 47F)	(50M, 47F)	(0M, 3F)
Tampon (G26)	40	5	5	72	82	18
	(20M, 20F)	(0M, 5F)	(2.5M, 2.5F)	(42M, 30F)	(45M, 37F)	(5M, 13F)
Tapachula (G31)	40	5	22	65	92	8

Patterns of infection in relation to the sex were evaluated in positive samples for Foshan, Canton, Recife, Crema, and Tampon. M, males; F, females.

window will provide further ground to investigate the actual modifications induced by larval development to the water microbial communities.

Mosquitoes are able to modify their aquatic habitat through larval feeding and excretion of bacteria from the digestive tract, an effect that can be magnified in small volume containers (Coon et al., 2016b), as adopted in our experiments. Mosquitoes are impacting their breeding sites through excretion of relatively high levels of ammonium (NH_4^+) from the larval anal papillae, as shown in *Ae. aegypti* (Donini and O'Donnell, 2005; Weihrauch et al., 2012). The chemical analysis of the W End samples is in line with this finding, since we detected fourfold higher levels of ammonia than in the W Start samples.

Competition among larvae may impact breeding site microenvironment and consequently affect the composition of the water microbiota over time. Competition driven by excreted chemicals has indeed been shown to occur in *Ae. aegypti* (Bédhomme et al., 2005). Larval feeding and excretion in the breeding water may contribute to developing the optimal conditions for the growing of bacteria that, in the initial water samples, could not find the ideal environment for proliferation and may thus have gone undetected. The composition of detritus-associated microfauna is indeed known to be altered over time as a consequence of larval grazing in *Aedes triseriatus* (Walker et al., 2010). Moreover, similarly to what occurs for *Wolbachia*, which is transmitted vertically from mother to offspring via the egg cytoplasm (Werren, 1997), other bacteria can be vertically transmitted (Moran et al., 2008). This can be the case of certain bacterial genera identified in our study that are not present in the water at the breeding sites, thus not being acquired through feeding. For example, *Escherichia–Shigella* was found to colonize ovaries of *An. gambiae* (Mancini et al., 2018). In *Ae. albopictus*, the reported wide dominance of *Wolbachia* in the ovaries (94% according to Mancini et al., 2018), could have masked the identification of other bacteria, which may have an importance in the biology of the species. Further studies aimed at localizing the larval- and/or adult-specific bacteria in mosquito tissues will be essential to clarify this aspect.

When considering the composition of the W End samples, it is worth mentioning the presence of Patescibacteria, which were previously shown to be associated with different developmental stages of *Ae. albopictus* (Qing et al., 2020) and adult *Culex nigripalpus* females (Duguma et al., 2019). Patescibacteria are prevalent in water environments (Tian et al., 2020), especially in groundwater (Bruno et al., 2017b; Schwab et al., 2017), and due to a reduced genome and consequent limited biosynthetic capabilities, the presence of members of this phylum may depend on nutrient uptake from other members (autotroph) of the microbial community (Brown et al., 2015; Herrmann et al., 2019). Members of this phylum have been associated with oligotrophic environments (Herrmann et al., 2019). This feature could explain the presence of members of this phylum in our water, since we allowed the larvae to grow solely based on the nutrients present in the water collected at the breeding site, which may have become depleted as a consequence of feeding. Another phylum that characterized W End samples is Dependentiae that, similarly to Patescibacteria, are widespread across different environments, including wastewater (McLean et al., 2013; Yeoh et al., 2015), and have limited metabolic capacities, suggesting their dependence on other aquatic autotrophic and heterotrophic microorganisms (Deeg et al., 2019). The abundance of Cyanobacteria is in general not surprising, since these bacteria are known to be present in mosquito breeding sites and have been previously isolated from mosquito guts (Thiery et al., 1991; Vazquez-Martinez et al., 2002). Members of this phylum are particularly abundant in environments with high phosphate concentrations (Roldán and Ramírez, 2008), which is the case of the water collected at the end of our experiments. Moreover, the growth of cyanobacteria has been shown to be favored by increase concentrations of nitrates and ammonia (Kim et al., 2017), which in our experiments could be related to mosquito development. Acidobacteria have been previously found in mosquito breeding sites (Onchuru et al., 2016), as well as adult individuals [e.g., *Culex nigripalpus* (Duguma et al., 2019), *An. coluzzii* (Mancini et al., 2018), *An. gambiae* (Mwadondo et al., 2017), *An. stephensi* (Kalappa et al., 2018), but also *Ae. albopictus* (Wang et al., 2018)].

We also identified bacteria, such as Nocardiaceae, that are shared between W Start samples and all tested mosquito samples (*i.e.*, both CR and FO larvae and adults) but they are absent in the W End sample. This pattern may indicate that members of this family are acquired and digested. In triatomines, Nocardiaceae play the role of nutritional symbionts able to provide the host with essential nutrients (Salcedo-Porras et al., 2020). Conversely, Burkholderiaceae were present in larval and adult individuals, as well as the W End, but not in W Start. Burkholderiaceae were previously found to be associated with *Ae. albopictus* breeding sites (Shelomi, 2019) as well as adults (Seabourn et al., 2020). Similarly, larval and pupal samples from *Ae. koreicus* were found to be dominated by this bacterial family (Alfano et al., 2019).

The presence of mosquito larvae has been previously shown to have different impacts on the composition of the bacterial community of breeding site water. For example, microcosmos-based experiments involving *Cx. restuans* and *Ae. triseriatus* showed that larvae affect the composition of the bacterial community in breeding sites and reduce bacterial abundance, diversity and richness (Walker et al., 1991; Muturi et al., 2020); a similar trend was seen in natural breeding sites in tree holes for *Ae. triseriatus* (Walker et al., 1991). These results contrast with those obtained by Kaufman et al. (1999) in the same mosquito species, in a microcosms-based set-up, as they found that larval presence increase total bacterial numbers, similarly to the increased bacterial abundance detected in water columns in small container habitats reported for the pitcher plant mosquito *Wyeomyia smithii* (Heard, 1994; Cochran-Stafira and von Ende, 1998).

Larvae can contribute to the development of enriched and more anoxic conditions, which favor the growth of facultative anaerobe bacteria.

Newly Emerged *Ae. albopictus* Adults Display a Simplified Microbiota

The data obtained in this study show a decrease in microbiota complexity from larval to adult stage. This trend, evident in both

wild and laboratory samples, is most likely due to the dramatic changes the gut epithelium undergoes during metamorphosis. In particular, the formation of two meconial peritrophic matrices (MPM1 and MPM2) was shown to contribute to *Ae. aegypti* adult midgut sterilization by sequestering microorganisms ingested during the larval stage, which are excreted after emergence (Moll et al., 2001; Moncayo et al., 2005). This process is not fully understood in *Ae. albopictus*.

After such loss of components, adults shape a new microbiota and diet plays a key role. Mosquito hosts, such as plants and animals, are a source of bacteria, as well as viruses and other microorganisms. Indeed, it is known that the midgut of the two sexes harbors different microbial communities: the microbiota of females is typical of blood feeding insects, and it is mostly colonized by Gammaproteobacteria; males, instead, display a midgut mainly colonized by Firmicutes (Minard et al., 2013).

Developmental Stage Affects Microbiota Composition

Our results suggest that the developmental stage influences microbiota composition in mosquitoes. Only one genus, *i.e.*, *Vulcaniibacterium*, was found to be shared by both Foshan and Crema samples. This bacterium has been previously found in the gut of both sexes of *Bactrocera oleae* (Koskinioti et al., 2019) but it was never detected in a mosquito species so far.

Microbacteriaceae, the only family found to be uniquely present in wild CR larval samples, was previously isolated from *Ae. albopictus* larvae and reported to be absent from the other developmental stages (Yadav et al., 2016). Similarly, in *Ae. aegypti*, Microbacteriaceae were found to be lost during metamorphosis (Frankel-Bricker et al., 2020). In our work, we confirmed the larval-specificity of this bacterial family in *Ae. albopictus*. While *Herbiconiux* was previously identified in *Ae. aegypti* larvae (Hery et al., 2021), and it is present also in our wild sampled larvae, *Leifsonia* was found in *Ae. albopictus* for the first time. This bacterium has been identified in the breeding site water of *A. darlingi* in Brazil, and in the midgut of the sandfly *Leishmania major* (Louradour et al., 2017), but to the extent of our knowledge it was never identified in *Ae. albopictus* before. The role of this bacterium in the microbiota of the tiger mosquito will require further investigation, extending the sampling to other natural breeding sites. *Leucobacer* was previously detected in the larvae of *Ae. aegypti* and were rare in breeding site water and nearly absent in adults (Coon et al., 2014), mirroring our results in *Ae. albopictus*.

Wolbachia Abundance Contributes to Bacterial Community Structure

In addition to the effects of the breeding site environment, also interactions between members of the microbiota within a host can induce changes in the structure of the microbial communities and in the relative abundance of its components (Brinker et al., 2019). Recent studies began to show that the history of *Wolbachia* colonization has an impact on the physiological changes mediated by this bacterium in the host, with effects on the resident microbiota. After a stable transinfection, *Wolbachia* was shown to lead to a decrease in microbial diversity in *Ae. aegypti* (Audsley et al., 2018). This decrease in microbial diversity has been suggested to be mediated by immune system modulation, resource competition and the pH (Simhadri et al., 2017; Audsley et al., 2018). *Wolbachia*-mediated immune regulation appears to be lost in hosts developing long-term co-evolutionary relationships with this bacterium (Shi et al., 2018). Differently from what occurs in *Ae. aegypti*, *w*AlbA and *w*AlbB are native *Wolbachia* infections of *Ae. albopictus* with a long history of co-association (Dutton and Sinkins, 2004). Accordingly, here we show that *Ae. albopictus* adults displaying a high *Wolbachia* prevalence have a more diverse microbiota than mosquitoes with no or low *Wolbachia* reads. Our study did not focus on investigating whether and to what extent *Wolbachia* prevalence is linked to features of water at mosquito breeding sites. Further studies involving an extended sample size are needed to confirm results from our exploratory study as well as to further clarify the complex interactions between mosquito host, the environment and the different members of the bacterial communities residing in different tissues and organs, as mentioned above. The identification of *Wolbachia* also in somatic tissues of insects, including mosquitoes (see Pietri et al., 2016 for a review), led to formulate the hypothesis that this bacterium can also be acquired from the environment and/or host sharing, as suggested for ants and triatomines (Espino et al., 2009; Andersen et al., 2012; Frost et al., 2014).

Wolbachia Prevalence in Laboratory Strains Varies

This study contributed to further support that *Wolbachia* is highly prevalent in *Ae. albopictus*. However, we found that in one strain from La Reunion Island (*i.e.*, Tampon), the prevalence was particularly low with respect to what detected in the other five strains and previously reported (Noor Afizah et al., 2015). Moreover, in the Crema strain derived from eggs collected in 2016 in the same site used for this study, we found that 3% of the mosquitoes were uninfected. Given that *Wolbachia* is transmitted vertically and this strain has been reared in our insectary for 33 generations, this result was unexpected and provides ground for further analyses.

Our results also confirmed the prevalence of dual *Wolbachia* infection in *Ae. albopictus* strains derived from different geographic populations. In the field, the prevalence of double infection by *Wolbachia* has been reported to be over 99.41% (Kittayapong et al., 2002a) and in Korean populations more than 98.8% (Park et al., 2016). We found dual infection ranging from 65 to 97%. As reported by previous studies, dual infection is mainly present in females (Joanne et al., 2015; Noor Afizah et al., 2015; Ahmad et al., 2017), but in two of the strains we analyzed, namely Crema and Tampon, both *Wolbachia* were present with higher frequency in males. A previous study that investigated *w*AlbA density in wild-sampled *Ae. albopictus* males from two Italian localities in the Central and Southern regions (*i.e.*, Central Italy: Crevalcore, Bologna; Southern Italy: Anguillara Sabazia, Rome) showed that *w*AlbA titer was very low in about half of the collected males from both sites (Calvitti et al., 2015), in agreement

with the findings of Tortosa et al. (2010). However, density of both *Wolbachia* strains was confirmed to be impacted by adult age, population geographic origin and environmental conditions such as temperature and food availability in larval breeding sites (Calvitti et al., 2015).

Our data suggest that an extensive survey of *Wolbachia* prevalence in wild populations is extremely important not only to better understand the role of this bacterium in contributing to shape the microbial community in *Ae. albopictus*, but also to provide essential basic-biology information to inform current and future mosquito control programs based on the Incompatible Insect Technique approach, especially given that cytoplasmic incompatibility level has been correlated with *w*AlbA density (Calvitti et al., 2015).

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the European Nucleotide Archive (ENA) under the BioProject: PRJEB41031. The codes used for our analyses are available at the following link: https://gitlab.com/anna.sandionigi/metamosquito.

AUTHOR CONTRIBUTIONS

MB and MCas conceived and designed the experiment. FS, AS, MCar, and AB performed the experiments. FS, AS, and AB analyzed the data. FS, AS, AB, and MB wrote the manuscript. All the authors provided critical comments on the manuscript, read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.624170/full#supplementary-material

Supplementary Figure 1 | Water samples analyses. **(A)** 16S rDNA-based bacterial quantification in water samples. On the *X*-axis, water samples are reported, colored by sampling period (W Start – orange – and W End – green – refers to water collected at the moment of mosquito collection and after all adults had emerged, respectively). Values are expressed as log2(DNA counts)/mL. **(B)** Physical analysis of W Start and W End water samples. Shannon alpha diversity index, pH, and conductivity values are shown. Both pH and conductivity significantly increased in W End samples (*t*-test, $P < 0.05$).

Supplementary Figure 2 | Rarefaction curves used to discover all the microbial 'species'/ASVs in each sample where *Wolbachia* reads were removed.

Supplementary Figure 3 | Bacterial genera shared between breeding site water, and wild and laboratory larvae and adults. Venn diagrams show the number of genera shared in **(A)** water (W Start vs. W End), **(B)** larval (CR vs. FO), and **(C)** adult (CR vs. FO) samples.

Supplementary Figure 4 | Alpha diversity indices of adult individuals. Alpha diversity (Shannon) values distribution for adults (CR and FO) samples are shown. Each sample is indicated with a different color.

Supplementary Table 1 | Number of sequencing reads per sample before and after removal of *Wolbachia*-associated reads. DNA extraction negative controls are included. Samples with no *Wolbachia* reads are indicated in bold.

Supplementary Table 2 | List of ASVs identified in each larval, adult, and water sample.

Supplementary Table 3 | Bacteria quantification through qPCR assays.

Supplementary Table 4 | Physio-chemical analyses of water samples.

Supplementary Table 5 | Proportion of sequencing reads assigned to a given bacterial taxon at the phylum and family level in water, larval, and adult samples. Only 50 most abundant ASVs were considered.

Supplementary Table 6 | List of bacterial genera identified in the conserved microbiota for each larval, adult, and water sample.

Supplementary Table 7 | List of unique and conserved bacterial genera from the microbiota of larvae and adults from wild or laboratory samples.

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Novel *Asaia bogorensis* Signal Sequences for *Plasmodium* Inhibition in *Anopheles stephensi*

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Mosquitoes vector many pathogens that cause human disease, such as malaria that is caused by parasites in the genus *Plasmodium*. Current strategies to control vector-transmitted diseases are hindered by mosquito and pathogen resistance, so research has turned to altering the microbiota of the vectors. In this strategy, called *paratransgenesis*, symbiotic bacteria are genetically modified to affect the mosquito's phenotype by engineering them to deliver antiplasmodial effector molecules into the midgut to kill parasites. One paratransgenesis candidate is *Asaia bogorensis*, a Gram-negative, rod-shaped bacterium colonizing the midgut, ovaries, and salivary glands of *Anopheles* sp. mosquitoes. However, common secretion signals from *E. coli* and closely related species do not function in *Asaia*. Here, we report evaluation of 20 native *Asaia* N-terminal signal sequences predicted from bioinformatics for their ability to mediate increased levels of antiplasmodial effector molecules directed to the periplasm and ultimately outside the cell. We tested the hypothesis that by increasing the amount of antiplasmodials released from the cell we would also increase parasite killing power. We scanned the *Asaia bogorensis* SF2.1 genome to identify signal sequences from extra-cytoplasmic proteins and fused these to the reporter protein alkaline phosphatase. Six signals resulted in significant levels of protein released from the *Asaia* bacterium. Three signals were successfully used to drive the release of the antimicrobial peptide, scorpine. Further testing in mosquitoes demonstrated that these three *Asaia* strains were able to suppress the number of oocysts formed after a blood meal containing *P. berghei* to a significantly greater degree than wild-type *Asaia*, although prevalence was not decreased beyond levels obtained with a previously isolated siderophore receptor signal sequence. We interpret these results to indicate that there is a maximum level of suppression that can be achieved when the effectors are constitutively driven due to stress on the symbionts. This suggests that simply increasing the amount of antiplasmodial effector molecules in the midgut is insufficient to create superior paratransgenic bacterial strains and that symbiont fitness must be considered as well.

Keywords: malaria, secretion, paratransgenesis, *Anopheles*, *Plasmodium*, *Asaia*

INTRODUCTION

In 2019 there were an estimated 229 million cases and 409,000 deaths caused by human malaria (World Health Organization, 2020). This vector-borne disease continues to burden many countries in sub-Saharan Africa and India (World Health Organization, 2020). Malaria in humans is caused by several parasitic protist species belonging to the genus *Plasmodium* and vectored by *Anopheles* sp. mosquitoes (Wang and Jacobs-Lorena, 2013). Major control strategies include the use of insecticides to manage vector populations, insecticide treated bed nets to manage biting behavior, and antimalarial drugs to target the parasites in infected humans (Birkholtz et al., 2012; Benelli, 2015). Despite a decline in the number of malaria cases and deaths in recent years, the effectiveness of these measures has begun to decrease. Parasites have evolved resistance to common drug treatments, especially to artemisinin-based and partner drugs (Haldar et al., 2018). Mosquitoes, too, have evolved resistance to insecticides and adapted biting behaviors to avoid physical barriers (Sokhna et al., 2013). Clearly, additional strategies are needed in order to combat this deadly disease.

Plasmodium has a complex life cycle that requires an *Anopheles* mosquito vector and a human host. Newly proposed strategies target mosquitoes through population size reduction and alteration. One approach is to genetically modify the mosquito genome to create transgenic mosquitoes for release that reduce population size through gene drive mechanisms. Studies have shown success at engineering these mosquitoes to introduce genes that cause sterility or death in natural populations (Kitzmiller, 1972; Alphey, 2014; Adelman and Tu, 2016). Others have altered the genome to make the mosquitoes resistant to infection from the *Plasmodium* parasite (Wang and Jacobs-Lorena, 2013). However, these may prove difficult for field applications since a large number of *Anopheles* mosquito species can vector the pathogen, with some living in reproductively isolated populations (Favia et al., 1997; Raghavendra et al., 2011).

Paratransgenesis is another promising strategy for malaria control. Mosquitoes are hosts to a variety of microbial communities, and these symbiotic microorganisms can be used to affect the mosquito phenotype, specifically by engineering them to express antiplasmodials to kill the parasites in the mosquito. Strains of *Wolbachia pipientis* have been used for field applications within *Aedes aegypti* mosquitoes to inhibit the spread of dengue viruses (Moreira et al., 2009; Frentiu et al., 2014). While successful, these studies also highlight problems that can arise when using non-native microbes, such a deleterious impact on population dynamics or transmission issues due to the native symbionts in mosquito species that *Wolbachia* does not naturally inhabit (Hughes et al., 2014; Telschow et al., 2017). Researchers have explored multiple symbiotic microbes in mosquitoes for both vector control and pathogen transmission control. A densonucleosis virus found in *Anopheles* sp. was engineered to express green fluorescent protein in the mosquito host as proof of concept that viruses can be used for paratransgenesis (Ren et al., 2008). Two fungal species, *Beauveria bassiana* and *Metarhizium anisopliae* not only naturally infect and spread through mosquito populations, but have been shown to innately cause progressive mosquito death (Scholte et al., 2005; Bukhari et al., 2011). Furthermore, engineering the *Metarhizium anisopliae* fungi to express antimalarials showed a significant reduction in *Plasmodium* sporozoite counts within the mosquito host (Fang et al., 2011). However, roadblocks exist for these organisms since mosquito-specific viruses are host-specific and fungal survival in the midgut environment is low. The midgut provides the greatest bottleneck for the number of parasites, resulting in just 0–5 oocysts in field-caught mosquitoes even though thousands of parasites are typically ingested in the blood meal (Sinden, 1999; Shahabuddin and Costero, 2001; Sinden and Billingsley, 2001). This illustrates the need to use microbes that are native to the mosquito midgut for paratransgenesis since they are already adapted to surviving in the midgut conditions and would likely have little to no impact on midgut microbiota.

Several symbiotic bacterial species isolated from mosquitoes have been explored as potential paratransgenesis candidates for malaria. *Pantoea agglomerans* was engineered to secrete different antiplasmodial proteins and was able to inhibit *Plasmodium falciparum* development within the *Anopheles gambiae* mosquito host up 98% (Wang et al., 2012). While these results were promising, *P. agglomeran*s has no drive mechanism to spread throughout mosquito populations. Another candidate, *Serratia marcescens AS1*, is able to propagate through mosquito populations and can significantly reduce *Plasmodium falciparum* development when engineered to secrete antiplasmodials (Wang et al., 2017). However, strains of *Serratia marcescens* are major opportunistic pathogens in humans, causing many nosocomial infections every year (Grimont and Grimont, 1978; Mahlen, 2011; Samonis et al., 2011; Khanna et al., 2013; Sridhar et al., 2015; Fernández et al., 2020). An ideal paratransgenesis candidate would not only possess the ability to spread from one mosquito to another but would also lack human pathogenicity. *Asaia bogorensis* SF2.1 is a promising candidate that colonizes the ovaries, testes, salivary glands, and the midgut of the mosquito, and spreads both horizontally and vertically through mosquito populations (Favia et al., 2007; Damiani et al., 2010; Mancini et al., 2016). *Asaia* was first identified in the flowers of the orchid tree (*Bauhinia purpurea*) and of plumbago (*Plumbago auriculata*), and has even been found associated as spoilers of still natural product drinks and organic product enhanced packaged waters (Yamada et al., 2000; Horsáková et al., 2009). This bacterium colonizes a number of arthropods, especially those that feed on the nectar of plants, including *Anopheles stephensi*, *An. gambiae, An. maculipennis, Aedes aegypti*, *Ae. albopictus*, *Culex pipiens*, *Scaphoideus titanus*, and *Sogatella furcifera*, most of which can vector human diseases (Favia et al., 2007; Crotti et al., 2009; Chouaia et al., 2010; Ricci et al., 2012; De Freece et al., 2014; Li et al., 2020). Importantly, bacteria in the genus *Asaia* apparently cause very few human infections. Only a handful of rare cases have been reported, occurring in either severely immunocompromised patients or when it was directly injected into the bloodstream (Snyder et al., 2004; Tuuminen et al., 2006, 2007; Alauzet et al., 2010; Juretschko et al., 2010; Epis et al., 2012; Carretto et al., 2016).

A major challenge in developing paratransgenic strains of bacteria is the release of antiplasmodial peptides and proteins

outside of the cell. In bacteria, many proteins reach the extracellular milieu via different one- or two-step secretion pathways (Christie, 2019). The two-step pathways rely on N-terminal signal peptides that direct proteins to the general secretory (Sec) or twin-arginine translocation (TAT) export apparatus in the inner membrane (IM) (Christie, 2019). Importantly, signal peptides can be predicted from a bacterial genome sequence based on their conserved features such as length, hydropathy profiles, and cleavage sites (Nielsen et al., 1997; Bagos et al., 2010; Petersen et al., 2011). We reasoned that increasing the level of antiplasmodial peptides released would lead to more efficient antiplasmodial bacterial strains. We report here the evaluation of twenty different *Asaia* Sec or TAT signal peptides for their ability to mediate what is likely to be non-specific, heterologous release from the periplasm and improve the ability of *Asaia* to act as a paratransgenesis platform. Increasing the amount of antiplasmodials released from the cell likely leads to greater suppression of parasites, but can also compromise the fitness of the resultant bacterial strains; therefore, careful strain construction should be considered.

MATERIALS AND METHODS

Media and Antibiotics

For plasmid cloning, *E. coli* Top10F' cells were cultured using standard Luria Bertani (LB) broth [1% tryptone, 0.5% NaCl, 0.5% yeast extract (w/v)] and LB agar (LB broth with 15 g/L agar). Media was supplemented with 30 μg/mL kanamycin. All *Asaia* strains were cultured in mannitol broth [0.5% yeast extract, 0.3% peptone, 2.5% mannitol (w/v)] or mannitol agar (mannitol broth with 15 g/L agar), both adjusted to a pH of 6.5 before sterilization. Davis minimal media broth [0.7% dipotassium phosphate, 0.2% monopotassium phosphate, 0.05% sodium citrate, 0.01% magnesium sulfate, 0.1% ammonium sulfate (w/v)] was used for cell collection and Davis minimal media agar (minimal broth with 15 g/L agar) supplemented with 0.5% (w/v) arabinose solution was used for colonization assessments. Media was supplemented with 120 μg/mL kanamycin for plasmid selection and also 100 μg/mL ampicillin for colonization assessments. Both liquid and solid media cultures for all strains were grown at 30°C, with agitation for liquid cultures. All bacterial strains and plasmids used in this study are described in **Table 1**.

Mosquito and Parasite Maintenance

Anopheles stephensi (a gift from the Johns Hopkins Malaria Research Institute) were maintained on 10% (w/v) sucrose solution at 29°C and 70% humidity with a 12 h day:12 h night light cycle. Larvae were reared at 29°C in pans and fed on crushed Tetramin Tropical Tablets for Bottom Feeders. Pupae were collected by hand and allowed to emerge as adults in 0.03 m^3 screened cages. *Plasmodium berghei* strain ANKA2.34 was maintained by passage through 7- to 8-week-old outbred female ND4 Swiss Webster mice (Charles River Laboratory) using standard procedures (Sinden et al., 1996). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and Duquesne University IACUC protocol #1810-09. All surgery was performed using anesthesia as outlined below, and all efforts were made to minimize suffering.

Genome Prediction of *Asaia* Extra-Cytoplasmic Proteins

An *Asaia bogorensis* SF2.1 genome sequence was annotated by the NCBI Annotation Pipeline version 2.0[1] and a total of 3,005 protein-coding genes were identified (Shane et al., 2014). SignalP4.1 signal peptide prediction software was used to analyze the identified protein coding sequences (Nielsen et al., 1997). The top 20 predicted proteins are listed in **Supplementary Table 1** (Bongio, 2015).

Plasmid Construction

All plasmid construction and propagation was performed in *E. coli* Top10F' (Invitrogen). The pNB92 plasmid was used for construction of all *Asaia* sp. signal sequence vectors (**Figure 1A**) (Bongio and Lampe, 2015). This plasmid uses the pBBR broad-host range origin of replication, a constitutively active neomycin phosphotransferase promoter (P_{nptII}), and the neomycin phosphotransferase II gene (nptII) conferring kanamycin resistance. A multiple cloning site after the P_{nptII} promoter allowed for in frame gene fusions with the *E. coli phoA* gene (without its native secretion signal = '*phoA*) to function as a reporter for protein localization (Manoil et al., 1990). **Supplementary Table 2** lists the top 20 predicted exported proteins and their signal peptides that were identified from the *Asaia* SF2.1 genome (Bongio, 2015). Gblock (IDT) synthetic dsDNA fragments were designed conservatively, using the first 150 nucleotides of each protein and included *Nde*I and *Pac*I restriction digestion sites before and after these sequences, respectively (**Supplementary Table 1**). The pNB92 vector and each gblock were fully digested with *Nde*I and *Pac*I restriction enzymes and recovered from gel electrophoresis after size confirmation with the Gel/PCR Fragment Extraction Kit (IBI Scientific). Each gblock was individually assembled into the digested pNB92 vector using an optimized temperature-cycle ligation procedure and electroporated into *E. coli* Top10F' electrocompetent cells (Lund et al., 1996). Clones were verified by PCR using the primer set in **Supplementary Table 1**, visualized through gel electrophoresis, and sequence verified. Plasmids were electroporated into *Asaia* SF2.1 electrocompetent cells, plated on selective media, and verified through PCR using the same primer set.

Plasmids expressing the antiplasmodial effector scorpine were constructed by digesting the plasmid pNB97 (Siders) and the constructs created above with the *Pac*I and *Sbf*I restriction enzymes (Conde et al., 2000; Bongio and Lampe, 2015). The scorpine gene fragment from pNB97 and the vector backbone from the signal sequence constructs were purified and assembled together using standard ligation procedures. A (Gly-Gly-Gly-Gly-Ser)$_3$ flexible linker was also inserted between the scorpine and *'phoA* gene fragments to promote proper folding of the fusion protein (**Figure 1B**). This double-stranded

[1]https://www.ncbi.nlm.nih.gov/genome/annotation_prok/

DNA fragment was achieved by annealing single-stranded complementary oligonucleotides together following the IDT standard protocol, which were designed with *Sbf*I restriction sites on both ends for ligation insertion (**Supplementary Table 1**). Briefly, the 500 μm oligonucleotide stock solutions were diluted to a final concentration of 100 μm in deionized water. The oligonucleotides were mixed in equal molar amounts, along with 2 μl of 10× Duplex Buffer [0.98% potassium acetate, 0.72% HEPES (w/v), pH 7.5] and deionized water to bring the final volume to 20 μl. The mix was heated to 94°C for 2 min and allowed to gradually cool. The mix was diluted 1:1000 in deionized water and 3 μl was used for ligation reactions. The products were transformed via electroporation into *E. coli* Top10F' cells, PCR and sequence verified, and electroporated into *Asaia* SF2.1 and plated on selective media.

Alkaline Phosphatase Reporter Screen

Passage of alkaline phosphatase (PhoA) across the inner membrane (IM) in *Asaia* was detected using 5-bromo-4-chloro-3′-indolyl phosphate (BCIP) supplemented media as previously described (Bongio and Lampe, 2015). Briefly, clones expressing the PhoA reporter constructs were plated on selective mannitol agar supplemented with 25 μg/mL BCIP and 30 μg/mL Na_2HPO_4. Since wild-type *Asaia* demonstrates a low-level of natural phosphatase activity, sodium phosphate was included as an inhibitor to reduce the number of false positives. Colonies were assessed for any color change after 72 h of growth at 30°C.

ELISA

For the detection of PhoA in the cell culture fractions, the *Asaia* strains expressing the PhoA constructs were grown to an OD_{600}

TABLE 1 | Strains and plasmids used in this study.

Strains	Characteristics		References
E. coli Top10F'	F′{lacIq, Tn10(TetR)} mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara leu) 7697 galU galK rpsL (StrR) endA1 nupG		Durfee et al. (2008)
Asaia SF2.1	Wild type strain isolated from *Anopheles* mosquitoes		Favia et al. (2007)
Plasmids	**Characteristics**	**Stable in *Asaia*?**	**References**
pNB92	Kan^R, pBBR origin, P_{nptII} promoter, '*phoA* insert and MCS for secretion signal fusion construction	Yes	Bongio and Lampe (2015)
pNB95 (Sider)	pNB92 with siderophore receptor gene cloned	Yes	Bongio and Lampe (2015)
pNB97 (Siders)	pNB95 with siderophore receptor-scorpine-PhoA effector construct	Yes	Bongio and Lampe (2015)
pNB141	pNB92 with MYC tag-PhoA construct	Yes	Bongio (2015)
pDCP[1]	pNB92 with dipeptidyl carboxypeptidase II signal	Yes	This study
pGGTP	pNB92 with gamma-glutamyltranspeptidase signal	Yes	This study
pHyp1	pNB92 with hypothetical protein 1 signal	Yes	This study
pHyp2	pNB92 with hypothetical protein 2 signal	Yes	This study
pCG6 (TonB)	pNB92 with TonB dependent receptor protein 1 signal	Yes	Shane et al. (2018)
pTonB2	pNB92 with TonB dependent receptor protein 2 signal	No	This study
pHyp3	pNB92 with hypothetical protein 3 signal	No	This study
pAChan	pNB92 with ammonium channel/transporter	Yes	This study
pPIsom	pNB92 with peptidyl-prolyl *cis–trans* isomerase signal	Yes	This study
pHyp4	pNB92 with hypothetical protein 4 signal	Yes	This study
pHyp5	pNB92 with hypothetical protein 5 signal	No	This study
pALys	pNB92 with alginate lyase signal	Yes	This study
pHyp6	pNB92 with hypothetical protein 6 signal	Yes	This study
pTonB3	pNB92 with TonB dependent receptor protein 3 signal	Yes	This study
pHyp8	pNB92 with hypothetical protein 8 signal	Yes	This study
pPerox	pNB92 with peroxiredoxin signal	No	This study
pHyp9	pNB92 with hypothetical protein 9 signal	No	This study
pMXKDX	pNB92 with pentapeptide MXKDX repeat protein signal	No	This study
pABCTrans	pNB92 with ABC-type phosphate transport signal	Yes	This study
pCopB	pNB92 with copper resistance protein CopB signal	No	This study
pHyp1s	Hypothetical protein 1 signal-scorpine-$(GGGGS)_3$- PhoA effector construct	Yes	This study
pTonBs	TonB dependent receptor 1 signal-scorpine-$(GGGGS)_3$- PhoA effector construct	Yes	This study
pHyp4s	Hypothetical protein 4 signal-scorpine-$(GGGGS)_3$- PhoA effector construct	Yes	This study

Transgenic Asaia strains are referred to as the plasmid names.

[1] *Plasmid sequences for plasmids constructed for this study were deposited into GenBank under accession numbers MW132102-MW132123.*

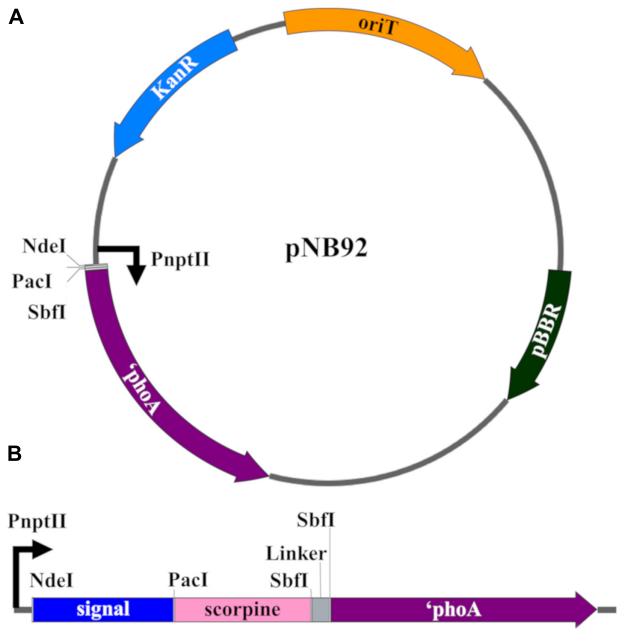


FIGURE 1 | Genetic constructs for the creation of transgenic *Asaia* strains. **(A)** pNB92 vector backbone for secretion signal insertion (Bongio and Lampe, 2015). The first 150 nucleotides of predicted signal sequence were inserted between the *Nde*I and *Pac*I restriction sites for fusion with the reporter *E. coli* PhoA protein lacking its native signal sequence (phoA). pBBR, origin of replication; KanR, kanamycin resistance; oriT, origin of transfer; PnptII, promoter. **(B)** Construct for antiplasmodial effector strains. The ORF encoding the antimicrobial scorpine effector as well as a $(GGGGS)_3$ flexible linker were inserted between the *Pac*I and *Sbf*I restrictions sites for fusion between the signal sequences and '*phoA* coding sequence.

of 1.0. One mL of each culture was centrifuged at 2000 × *g* (5000 RPM) for 5 min. The supernatant was removed and saved while the cell pellet was washed three times and resuspended in 1 mL Tris-buffered saline (TBS) [0.605% Tris-Cl, 0.876% NaCl, pH 7.5 (w/v)]. A second cell pellet from the same culture and isolated at the same time was resuspended in 1 mL of a 20% B-Per-TBS (v/v) solution (Thermo-Fisher Scientific, #78243) and vortexed for 2 min to lyse the cells. Two hundred microliters of the supernatant, whole cell, and cell lysate fractions were bound in wells of a NUNC-Immuno Maxisorp 96-well plate (VWR, cat. # 62409-024) overnight at 4°C. Plates were washed three times with TBS, and then blocked by adding 200 µl of 2% BSA-TBS (TBS with 2% (w/v) fraction V BSA) and incubating for 2 h at room temperature. The plate was again washed three times with TBS. Then, 100 µl of a 1:3000 dilution of rabbit polyclonal anti-PhoA-HRP antibody (GeneTex, #GTX27319) in 2% BSA-TBS was added to each well and incubated for 1 h at room temperature. The plate was washed eight times with 0.1% Tween20 (v/v)-TBS (TBS-T) for 2 min per wash. To visualize the protein, 50 µl of 1-Step Ultra TMB-ELISA (Thermo-Fisher Scientific, #34028) was added and the reaction allowed to proceed for 10–20 min at room temperature. To stop the reaction, 50 µl of 2 M H_2SO_4 was added, and the absorption at 450 nm was measured using a SpectraMax i3x plate reader (Molecular Devices), using the absorption at 655 nm for reference. These assays were performed five separate times and the results analyzed by one-way ANOVA with Dunnett's correction.

Western Analysis

Asaia strains expressing the PhoA constructs were grown overnight. Cultures were streaked on mannitol plates and grown at 30°C for 48 h. Colonies were collected by flooding the plates with 1 mL of minimal media, gently scraping the cells from the plate, and collecting the liquid into centrifuge tubes. These samples were then centrifuged at 13,800 × *g* (12,000 RPM) for 5 min. The supernatants were collected and placed on ice while the cell pellet was resuspended in 1 mL of 20% B-Per-TBS. The total protein concentration for each of the pelleted samples was analyzed through a Bradford assay (Thermo-Fisher Scientific, #23236) after accounting for any excess protein concentration from the mannitol medium. A dilution factor was established for each pellet sample to achieve a concentration of 1200 µg/ml for the pellet. The same dilution factor was then applied to the supernatant. In this way, the supernatant fractions were scaled relative to each other based on the total protein content of the cell pellet from which they were derived. Seventy-five microliters of each adjusted supernatant was added to 25 µl of 3× Laemmli buffer and the samples were boiled for 8 min.

Fifteen microliters of each sample and 8 µl of Precision Plus Protein Kaleidoscope ladder (Bio-Rad cat. #161-0375) were loaded onto on a 10% Mini-PROTEAN TGX Precast gel (Bio-Rad) and separated at 200 V for 35 min. Proteins were then transferred onto a PVDF membrane in a Bio-Rad transfer apparatus using Tris-glycine transfer buffer [0.303% (w/v) Tris, 1.127% (w/v) glycine, 10% methanol (v/v)] at 100 V for 1 h. The membrane was dried overnight, then was stained for total protein as a loading control using Revert 700 total protein stain (LI-COR cat. #926-11010) following the manufacturer instructions and imaged using an Odyssey FC dual mode imaging system (LI-COR) using the 700 nm infrared fluorescent detection channel for 2 min. The membrane was then rinsed in water and then blocked with 50% v/v Odyssey blocking buffer in TBS [50% (v/v) fraction V LI-COR Odyssey Buffer with TBS] for 1 h at room temperature with agitation and rinsed three times in deionized water for 5 min. The membrane was incubated in the primary antibody solution containing a 1:5,000 mouse monoclonal anti-PhoA antibody (Millipore, Temecula, CA, United States, MAB1012) diluted in 50% v/v Odyssey blocking buffer in TBS-T overnight at 4°C with agitation. The next day, the membrane was washed three times for 10 min with TBS-T. It was incubated for 1 h at room temperature with agitation in the secondary antibody solution containing a 1:20,000 IRDye® 800CW goat monoclonal anti-mouse antibody (LI-COR, cat. # 925-32210) diluted in 50% v/v Odyssey blocking buffer in TBS-T with 0.01% w/v SDS. Two washes for 10 min with TBS-T was followed by one wash for 10 min with TBS. The membrane was visualized on an Odyssey FC dual mode imaging system (LI-COR) using the 800 nm infrared fluorescent detection channel for 2 min. Image Studio Software 5.0 (LI-COR) was used for blot visualization and band quantification. In order to normalize the amount of protein in each lane of the western blot, the amount of protein in each entire

lane was quantified, the lane with the most protein determined, and each lane of the blot scaled to the highest amount. This scaling factor was then applied to each of the bands quantified using the anti-PhoA antibody.

Fitness Assessments of *Asaia* Strains

Two methods were used to assess the fitness of *Asaia* strains. First, the maximum growth rate of each strain was measured following the procedure outlined in Shane et al. (2018). Each *Asaia* strain was inoculated at 0.1 OD_{600} in 200 μl of a 96 well plate. The OD_{600} was analyzed over 24 h at 15 min intervals using a SpectraMax i3x (Molecular Devices). SoftMax Pro 7 software (Molecular Devices) was used to create growth curves of collated replicates of each strain until they reached stationary phase. Growth curves were further analyzed using the package growthrates59 (Petzoldt, 2017) in RStudio to find the maximum growth rate of each strain of *Asaia*. Data was visualized in RStudio using boxplot.

A mosquito colonization experiment was also used to assess the fitness of the strains. Each *Asaia* strain was fed to female *An. stephensi* mosquitoes at a 0.1 OD_{600} dilution in a sugar meal. After 36 h, mosquito midguts were dissected and homogenized using a tissue grinder. Fifteen midguts for each strain were pooled and diluted in 1000 μl of TBS. These samples were again diluted 10-fold in TBS and 100 μl of each dilution was plated on kanamycin and ampicillin supplemented minimal media with arabinose. CFUs for each strain were counted and compared to the total number of CFUs collected across test groups. Data was visualized in RStudio using boxplot.

Plasmodium berghei Parasite Inhibition

The ability of the antiplasmodial *Asaia* strains to inhibit *Plasmodium berghei* development in mosquitoes was evaluated according to Shane et al. (2018). Adult female ND4 Swiss Webster mice were infected with *P. berghei* ANKA2.34 and parasites were allowed to develop in the mice until parasitemia level reached 4–10%. At this point the mice were sacrificed and blood was collected via cardiac puncture. The infected blood was diluted with RPMI media (Gibco) to 2% parasitemia, then 200 μl (5×10^7 parasites) was injected intraperitoneally into an uninfected mouse. At the time of this transfer, each *Asaia* scorpine strain to be tested was diluted to 0.1 OD_{600} in the sugar meal and fed to 20–25 female *An. stephensi* mosquitoes in individual cups with screen lids. Thirty-six h post-infection each test group of mosquitoes was blood-fed on the infected mouse for 6 min each. The ability of the parasite to undergo exflagellation was also tested at this time using 6 μl ookinete media [1 L RPMI media supplemented with 0.2% sodium bicarbonate, 0.005% hypoxanthine, 0.00025% xanthurenic acid (w/v)] mixed with 10% (v/v) fetal bovine serum, 2 μl of 1 mg ml^{-1} of heparin in sterile phosphate buffer (PBS) [0.8% NaCl, 0.02% KCl, 0.144% Na_2HPO_4, 0.024% KH_2PO_4, pH 7.2 (w/v)], and 2 μl of blood collected from a tail prick of the mouse. At least two exflagellation events occurred for each malarial trial. Exflagellation occurs when microgametes exit red blood cells after a female mosquito takes a *Plasmodium*-infected blood meal, and can be monitored by microscopy. The number of these events in the blood meal is a measure of how infectious it is to the mosquito.

Mosquitoes that did not take in a blood meal were removed, and parasites were allowed to develop in the rest of the mosquitoes for 14 days at 19°C in order to form oocysts. After 14 days, the mosquito midguts were dissected and stained with a 10-fold dilution of 1% (v/v/) mercurochrome stain (Sigma Aldrich Product# M7011) in PBS for 30 min. They were then left to destain for 5 min in sterile PBS. The midguts were analyzed at 200× magnification and the number of oocysts per midgut were counted for each test group. All steps in this process were performed blindly and ordered randomly. Data was visualized in RStudio using Bee Swarm.

Statistics and Reproducibility

For all boxplots, the box bars are medians. The top and bottom of the boxes represent the first and third quartile of the data spread. The lower and upper bounds of the whiskers are the lowest datum still within 1.5× interquartile range (IQR) of the lower quartile, and the highest datum still within 1.5× IQR of the upper quartile, respectively. Significance for all tests was set to $P < 0.05$. Variance was estimated using standard error of the mean and is appropriately similar between test groups of each experiment. Significance of the mean was calculated using one-way ANOVA with Dunnett's correction in RStudio appropriate for multiple comparisons to a single control with normal distribution unless otherwise noted.

In **Figure 7**, Suppression of *P. berghei* development by paratransgenic *Asaia* strains, the data are pooled from three individual experiments. The median value of oocysts per midgut for the pooled data from the three experiments was calculated by and compared between treatments using quantile regression in RStudio (Cade and Noon, 2003). Quantile regression is a non-parametric test that compares subsets of a data set individually and is useful for data showing unequal variation (Cade and Noon, 2003). The significance of the difference in *P. berghei* oocyst prevalence was evaluated using binomial χ^2 tests with 1 degree of freedom. All colony and oocyst counts were done blindly regarding which strain was evaluated, and the strains were ordered randomly.

RESULTS

Asaia Genome Prediction of Exported Proteins

Annotation of the *Asaia* SF2.1 genome identified 3,005 total protein-coding genes (Shane et al., 2014). SignalP4.1 signal peptide prediction software further identified 228 proteins which were predicted to be exported to the periplasm via the Sec or the Tat system (Bongio, 2015). Among the top 20 predictions were peptidyl-dipeptidase DCP and gamma-glutamyltranspeptidase secreted proteases, multiple TonB dependent receptor proteins and an ammonium transporter protein, a peptidyl-prolyl *cis–trans* isomerase folding chaperone, and the digestive enzyme alginate lyase (**Supplementary Table 2**). A siderophore receptor protein (Sider) that had been isolated previously in an *Asaia*

genomic library screen using the reporter protein alkaline phosphatase (PhoA) was not identified from this genomic analysis (Bongio and Lampe, 2015). While these proteins are predicted to be exported past the inner membrane, some may only be localized to the periplasm or the outer membrane. We decided to focus our attention on the top 20 proteins with the highest scores.

Alkaline Phosphatase Protein Localization Using *Asaia* Signal Sequences

The vector pNB92 was used for construction of reporter constructs using the signals from the top 20 putative secreted protein genes (Bongio and Lampe, 2015). This vector contains the gene coding the *E. coli* PhoA protein lacking its native signal peptide (**Figure 1A**). The first 150 nucleotides of each signal were inserted in front of the *'phoA* gene, allowing for fusion of the predicted signal peptides to the PhoA reporter protein. These constructs were transformed into *Asaia* SF2.1 cells to create new strains that are referred to following the names of the plasmids they are carrying (**Table 1**). Thirteen of the *Asaia* transformants grew while seven did not, even after repeated attempts at transformation, indicating that the latter might be toxic when overexpressed in *Asaia* in these configurations (**Table 1**).

The successful clones were first screened for alkaline phosphatase activity by plating them on 5-bromo-4-chloro-3′-indolyl phosphate (BCIP) supplemented agar. BCIP can detect PhoA exported across the IM of Gram-negative cells, thus indicating that PhoA could be actively cleaving BCIP as a secreted protein, a membrane bound protein, or from within the periplasm (Brockman and Heppel, 1968). From this screen, nine strains showed a deep blue color change after 3 days of growth (Hyp1, Hyp2, TonB, AChan, PIsom, Hyp4, Hyp6, TonB3, and Hyp8), indicating cleavage of BCIP in the medium. The wild type *Asaia* SF2.1 colonies had no color change.

For the clones that showed a color change, an ELISA was performed to determine if PhoA was being released beyond the outer membrane (**Figure 2A**). Cultures were separated into the supernatant, whole cell, and cell lysate fractions, and the protein of interest was detected using an anti-PhoA-HRP conjugated antibody. Of the nine clones identified in the BCIP screen, six strains (Hyp 1, Hyp2, TonB, PIsom, Hyp4, and Hy8) showed PhoA protein in the supernatant and/or the whole cell fractions. Four of these (Hyp2, TonB, PIsom, and Hyp4) showed significantly more PhoA protein in the supernatant when compared to the previously identified siderophore receptor (Bongio and Lampe, 2015) Sider signal peptide (**Figure 2B**; $P \leq 0.0209$). The other three strains AChan, TonB3, and Hyp6 exhibited no detectable PhoA protein in any fraction.

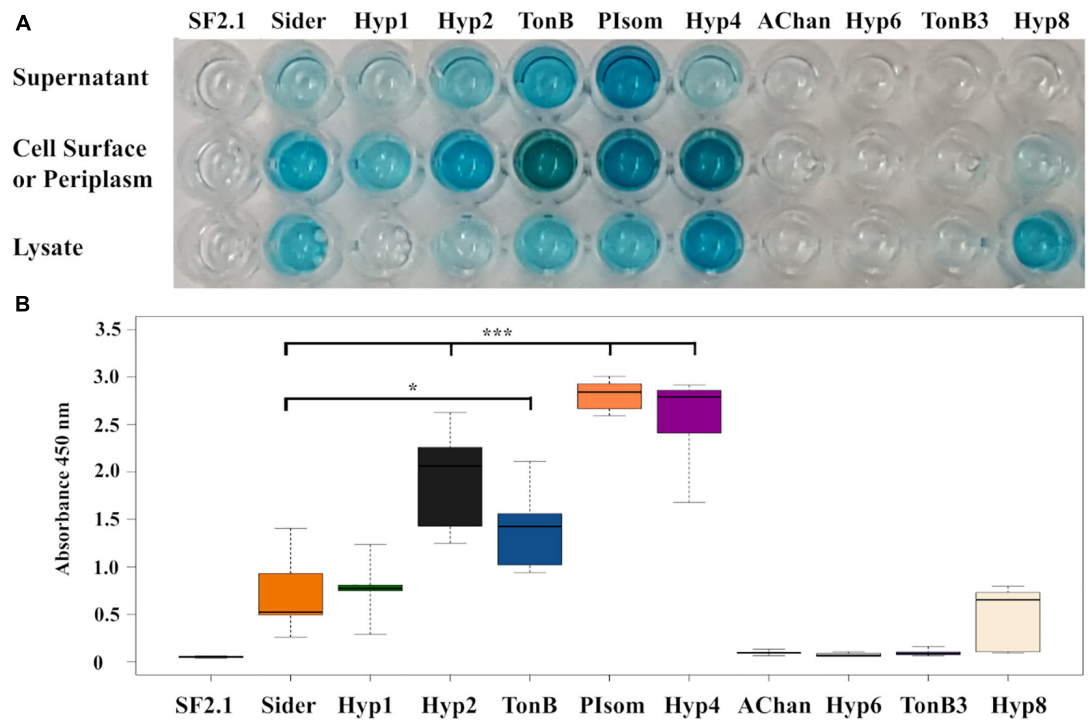


FIGURE 2 | Alkaline phosphatase localization in transgenic *Asaia* strains using ELISA. **(A)** A representative ELISA that utilized an anti-PhoA-HRP antibody to detect the presence of the alkaline phosphatase protein in the supernatant, cell surface/periplasm, and the cell lysate fractions of PhoA-only *Asaia* strains. SF2.1, wild-type *Asaia*; Sider, previously identified siderophore receptor signal (Bongio and Lampe, 2015). **(B)** Quantification of supernatant fractions of the ELISA analysis. Relative levels (expressed as absorbance values measured at 450 nm) of substrate cleaved by the HRP-conjugate anti-PhoA antibody in the supernatant fraction across five separate trials. Statistical significance was determined using one-way ANOVA with Dunnett's correction where significance is represented by $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ with experimental replicates. SF2.1, wild-type *Asaia*; Sider, previously identified siderophore receptor signal (Bongio and Lampe, 2015).

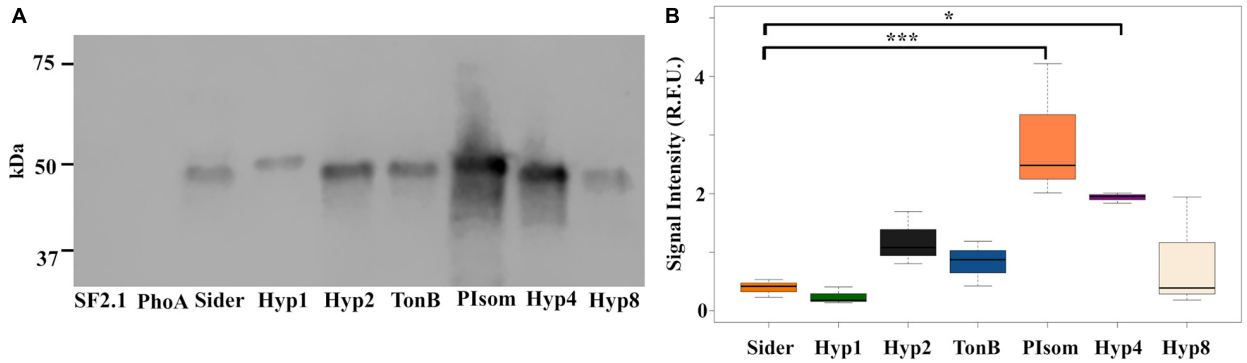


FIGURE 3 | Abundance of alkaline phosphatase protein in the supernatants from *Asaia* using predicted N-terminal signal peptides. **(A)** Representative western blot of supernatants from transgenic *Asaia* strains that showed localization of the PhoA reporter protein to the supernatant in the ELISA. The PhoA protein was detected using a monoclonal anti-PhoA antibody. **(B)** PhoA protein abundance in the supernatant. Western blots for all strains were repeated three times, and the signal intensities for the PhoA band were quantified without using a correction for lane to lane variation in total protein as an internal control. Statistical significance was determined using one-way ANOVA with Dunnett's correction where significance is represented by $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ with experimental replicates. No protein was detected in the SF2.1 or the PhoA (with no signal sequence) lanes. For the other strains, only significant comparisons are shown. SF2.1, wild-type *Asaia*; PhoA, alkaline phosphatase with no signal sequence; Sider, previously identified siderophore receptor signal. The same analysis correcting for variations in total protein between lanes as an internal control is shown in **Supplementary Figure 2**.

In order to further quantify the amount of protein in the supernatant relative to that of Sider, a western blot analysis was carried out on the supernatant of the six strains that were positive for PhoA in the ELISA using the anti-PhoA antibody. No signal was seen in the lanes with the SF2.1 strain and the PhoA strain with no signal sequence (=PhoA), while the rest of the supernatants had a small protein band around 49.9 kDa, which is the predicted size of the full PhoA protein after signal cleavage (**Figure 3A**).

Quantification of this analysis introduces a methodological problem. Typical western analysis uses internal controls of some housekeeping protein(s) whose amounts do not vary under different conditions (Yosef et al., 2010; Wu et al., 2012). No such control is available for the supernatant fraction that we are analyzing here. Protein mass normalization between lanes can be achieved by using total protein stains as an alternative (e.g., Revert 700 Total Protein Stain, LI-COR). We performed that control, but in this analysis we do expect variations in the amount of protein in the supernatant, so the suitability of this particular control is suspect. In our analysis, supernatant amounts varied such that they reflected the total protein content of the cell pellets from which they were derived. We therefore quantified the amount of PhoA in the supernatant produced by each strain with and without total protein normalization (**Figure 3B** and **Supplementary Figure 2**). The overall pattern of the relative protein amounts without total protein stain normalization essentially reproduced the results of the ELISA assay albeit with some differences in significances. Quantification of the PhoA-reactive bands without normalization showed that both the PIsom and Hyp4 strains had significantly increased amount of PhoA protein in the supernatant (one-way ANOVA with Dunnett's correction, $P \leq 0.0387$) when compared to the Sider signal peptide, while Hyp2 and TonB were not significantly different although they were significantly different in the ELISA ($P \geq 0.4474$) (**Figure 3B**). Quantification of the western analysis with total protein normalization did not reproduce the results of the ELISA and showed only Hyp4 as releasing significantly more protein than did Sider (one-way ANOVA with Dunnett's correction, $P = 0.00443$) (**Supplementary Figure 2**).

Fitness Assessments of Alkaline Phosphatase *Asaia* Strains

Two fitness assessments were carried out on the PhoA strains to determine if any were at a disadvantage when compared to the *Asaia* SF2.1 strain. First, the assessment of the maximum growth rates (μmax) of the strains were compared (**Figure 4A**). None of the transgenic strains grew as well as the *Asaia* SF2.1 wild-type strain (one-way ANOVA with Dunnett's correction, $P \leq 0.001$). When the newly constructed strains were compared to the Sider strain, only Hyp4 showed a significantly lower μmax (one-way ANOVA with Dunnett's correction, $P = 0.0423$).

The relative ability of the strains to colonize the mosquito midgut was also assessed (**Figure 4B**). The transgenic strains were fed to mosquitoes, midguts were dissected and plated under conditions that selected for *Asaia* growth, and CFUs were counted across all strains. None of the new transgenic strains were able to colonize the midgut as well as the Sider strain (one-way ANOVA with Dunnett's correction, $P < 0.001$). The Hyp2 strain colonized the midgut significantly better than four of the other new transgenic strains (one-way ANOVA with Dunnett's correction, $P \leq 0.0469$).

Antiplasmodial Effector Release Using *Asaia* Signal Sequences

An ORF encoding the antimicrobial peptide scorpine (Conde et al., 2000; Carballar-Lejarazú et al., 2008) was introduced into the six vectors that showed PhoA localization in the supernatant in the ELISA and western blot assays. A flexible linker was inserted between the scorpine and *'phoA* gene

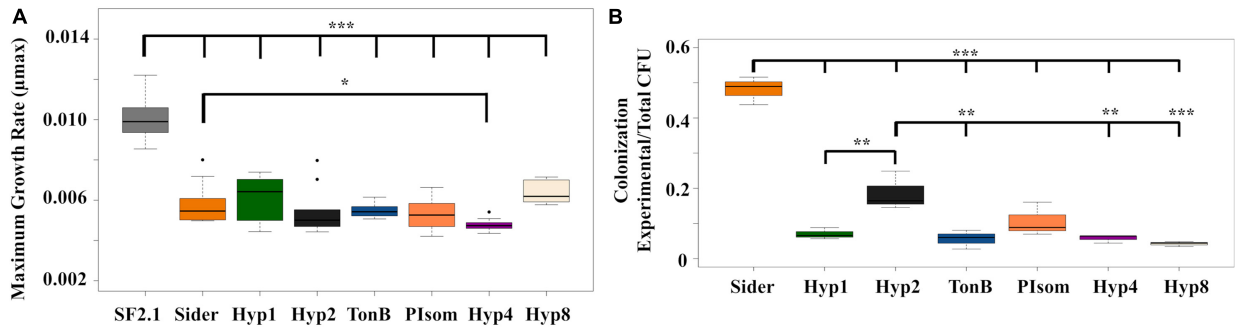


FIGURE 4 | Fitness assessments of alkaline phosphatase *Asaia* strains. **(A)** Growth curves and maximum growth rates (μmax) were calculated from 10 individual isolates of each strain grown over log phase of the bacteria using the package growthrates59 (Petzoldt, 2017) in RStudio. Only Hyp4 showed a significantly lower μmax than the Sider strain. **(B)** Relative colonization of mosquito midguts by *Asaia* strains. *Asaia* strains were fed to mosquitoes and 15 midguts from each sample were pooled and plated on selective media. Transgenic CFUS were counted for each isolate and taken as a ratio of the total across all strains. The Sider strain had the highest rate of colonization, followed by the Hyp2 strain. Statistical significance for each experiment was determined using one-way ANOVA with Dunnett's correction where significance is represented by $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ with experimental replicates (**A**. $n = 10$, **B**. $n = 3$). Only significant comparisons are shown.

fragments to promote proper folding and the independent function of each part of the fusion protein (**Figure 1B**). Attempts to create antiplasmodial *Asaia* strains with the Hyp2, PIsom, and Hyp8 signals proved problematic, resulting in no colonies on the selective plates after multiple transformation attempts suggesting that these configurations were toxic to *Asaia*. Western blot analysis was carried out on the supernatant of the new antiplasmodial strains Hyp1s, TonBs, and Hyp4s as well as Siders, the antiplasmodial strain using the siderophore receptor signal (**Table 1**). The supernatants for Hyp1s, TonBs, and Hyp4s showed two or three prominent bands, the largest one corresponding to the predicted size of the intact scorpine-PhoA protein at 57 kDa (**Figure 5A**). The other two bands were at approximate sizes of 51 kDa and 47 kDa and are most likely the product of additional cleavage within the protein either within the periplasm or as the protein exits the cell (**Figure 5A**).

Quantification of the protein released into the supernatant was carried out for all strains. The protein normalization problem discussed earlier for the strains expressing only PhoA is relevant here as well. In addition, the fact that the scorpine-PhoA fusion appears to be proteolytically cleaved means that accurately assessing the amount of scorpine reaching the supernatant is problematic since our reporter tag becomes separated from scorpine.

In order to estimate the amount of scorpine leaving the cell, we performed two different kinds of measurements. In the first, we quantified all of the protein fragments that reacted to the anti-PhoA antibody and reported that as a single value for the antiplasmodial strains (**Figure 5B** and **Supplementary Figure 4**). This measurement assumes that the scorpine that was cleaved also left the cell and so the smaller forms of reactive PhoA are a proxy for the presence of scorpine. We also quantified only the largest band which corresponds to the scorpine-PhoA fusion and thus is a direct measure of scorpine presence outside the cell in that form (**Supplementary Figures 5, 6**). There are, thus, four separate ways to measure the amount of scorpine that has left the antiplasmodial *Asaia* strains depending on the assumptions used. **Figure 5B** shows data for one of these sets of assumptions, namely allowing the amount of protein in the supernatant to vary (but normalized to the amount of protein in the cell pellet) and quantifying all of the antibody reactive PhoA protein bands assuming that all of the scorpine originally translated as a fusion protein left the cell. Under these conditions, Hyp4s showed significantly more protein than Siders (one-way ANOVA with Dunnett's correction, $P < 0.001$). Hyp1s also exhibited significantly more protein than the Siders strain (one-way ANOVA with Dunnett's correction, $P < 0.001$), in contrast to the Hyp1 PhoA only strain result (**Figure 3B**). The TonBs strain also showed full protein in the supernatant fraction, but not to a significantly greater degree than Siders (one-way ANOVA with Dunnett's correction, $P = 0.465$).

Analysis of the western results using the other three sets of assumptions is shown in **Supplementary Figures 4–6**. Under all sets of assumptions, Hyp1s released more scorpine than did Siders (one-way ANOVA with Dunnett's correction, $P \leq 0.00681$). TonB does as well when measured without total protein normalization quantifying only the largest reactive band (one-way ANOVA with Dunnett's correction, $P = 0.0456$).

Fitness Assessments of Paratransgenic *Asaia* Strains

Fitness assessments were carried out on the antiplasmodial strains to determine if any of the new strains showed a loss of fitness when compared to both Siders and the *Asaia* SF2.1 wild-type control. First, the maximum growth rates (μmax) of the strains were compared (**Figure 6A**). None of the antiplasmodial strains grew as well as the *Asaia* SF2.1 strain (one-way ANOVA with Dunnett's correction, $P \leq 0.001$). The Hyp1s strain showed a decrease in μmax compared to Siders while the other two antiplasmodial strains did not (one-way ANOVA with Dunnett's correction, $P < 0.0165$).

In addition, the relative ability of the antiplasmodial strains to colonize the mosquito midgut was assessed (**Figure 6B**). The

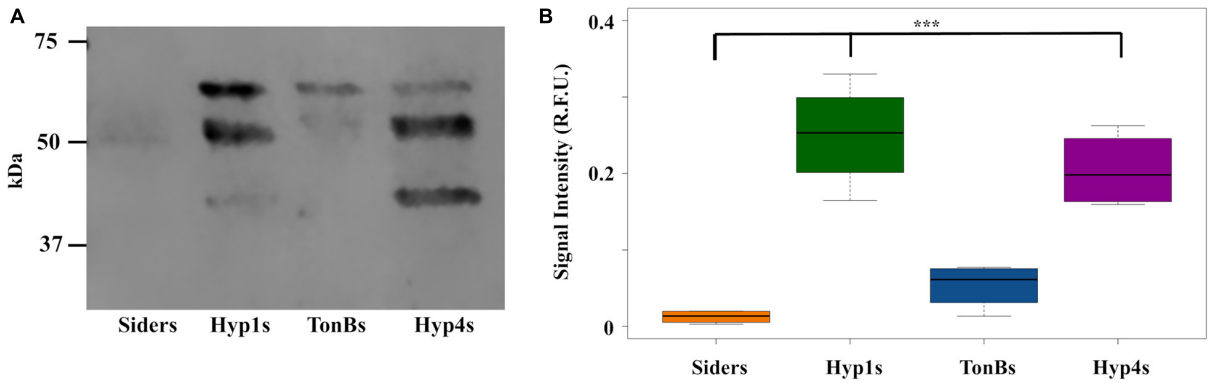


FIGURE 5 | Antiplasmodial effector abundance in the supernatants from *Asaia*. **(A)** Representative western blot of supernatants from antiplasmodial *Asaia* strains. The PhoA segment of the fusion protein was detected using a monoclonal anti-PhoA antibody. Three bands were detected with the antibody. The larger band (57 kDa) corresponds to the size of the scorpine-PhoA protein, while we interpret the other two bands (51 and 47 kDa) as most likely corresponding to cleavage products. **(B)** Fusion protein abundance in the supernatant. Western blots for all strains were repeated four times, and the signal intensities for the bands were quantified without using a correction for lane to lane variation in total protein as an internal control and reported as a single value. Statistical significance was determined using one-way ANOVA with Dunnett's correction where significance is represented by $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ with experimental replicates. Only significant comparisons are shown. SF2.1, wild-type *Asaia*; Siders, scorpine antiplasmodial strain using the previously identified siderophore receptor signal. Similar analyses to this figure using a correction for variations in total protein in each lane and quantifying only the largest reactive band in each lane are shown in **Supplementary Figures 4–6**.

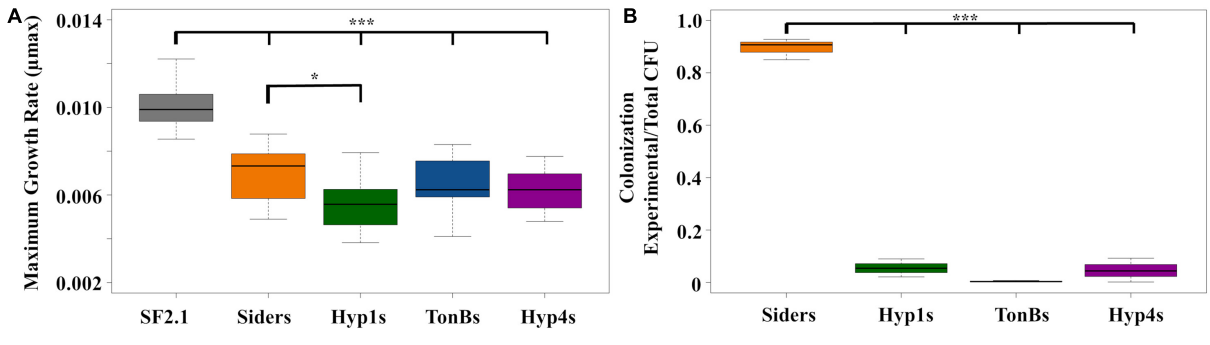
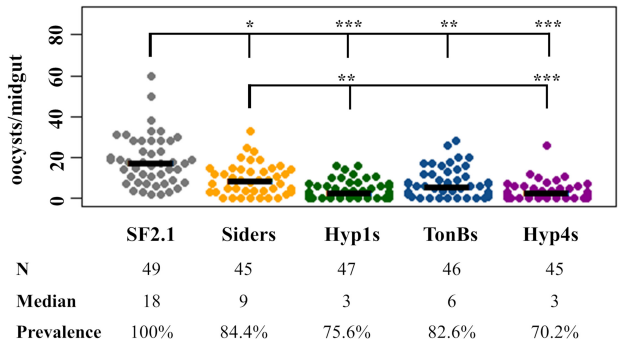


FIGURE 6 | Fitness assessments of antiplasmodial *Asaia* strains. **(A)** Maximum growth rates (μmax) were calculated from 10 individual isolates of each strain grown over log phase of the bacteria using RStudio. Only Hyp1s showed a significantly lower μmax than the Siders strain. **(B)** Relative colonization of mosquito midguts by antiplasmodial *Asaia* strains. Antiplasmodial *Asaia* strains were fed to mosquitoes and 15 midguts from each sample were pooled and plated on selective media. Transgenic CFUS were counted for each isolate and taken as a ratio of the total across all strains. Siders showed the highest rate of colonization. Statistical significance for each experiment was determined using one-way ANOVA with Dunnett's correction where significance is represented by $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ with experimental replicates (**A**. $n \geq 10$, **B**. $n = 3$). Only significant comparisons are shown.

antiplasmodial strains were fed to mosquitoes, midguts were dissected and plated, and CFUs were counted across all strains. Once again, the Siders had the highest rate of colonization in the midgut with the other three strains showing a substantially lower rate (one-way ANOVA with Dunnett's correction, $P < 0.001$).

Activity of Paratransgenic *Asaia* Strains Against *Plasmodium berghei*

The three antiplasmodial strains of *Asaia* developed here (Hyp1s, TonBs, and Hyp4s) were tested for their ability to prevent the development of *P. berghei* oocysts in *An. stephensi* female mosquito midguts, along with the Siders and SF2.1 strains as controls. The paratransgenic and wild-type strains were fed to female *An. stephensi* mosquitoes, which in turn fed on a *P. berghei* infected mouse. Mosquitoes that successfully blood-fed were dissected 14 days after the infective blood meal, and oocysts per midgut were counted (**Figure 7**). All of the paratransgenic strains significantly reduced the median number of oocysts when compared to the SF2.1 wild-type control *Asaia* strain (quantile regression, $P \leq 0.00029$). Hyp1s and Hyp4s had a significantly greater median oocyst reduction compared to the Siders strain (quantile regression, $P \leq 0.00181$), indicating that the increased levels of scorpine in the midgut had a stronger antiplasmodial effect in the mosquitoes. Another measure of antiplasmodial activity is prevalence. Prevalence is the fraction of mosquitoes of a population that have at least one oocyst, meaning that they may still be infective and can pass on the *Plasmodium* parasite. All of the antiplasmodial strains showed a significant reduction in prevalence of *P. berghei* infection when compared to SF2.1



	SF2.1	Siders	Hyp1s	TonBs	Hyp4s
N	49	45	47	46	45
Median	18	9	3	6	3
Prevalence	100%	84.4%	75.6%	82.6%	70.2%

FIGURE 7 | Suppression of *P. berghei* development by paratransgenic *Asaia* strains. In three separate trials, oocysts were counted in mosquitoes infected with *Asaia* strains that were fed on a *P. berghei* infected mouse. Each dot represents an individual midgut and the number of *P. berghei* oocysts it contained. Prevalence is the fraction of midguts with at least one oocyst. SF2.1 is the wild type *Asaia* strain (negative control) and Siders is the scorpine antiplasmodial strain using the previously identified siderophore receptor signal (positive control). All antiplasmodial strains significantly reduced the median number of oocysts (horizontal bars) compared to the wild-type strain (quantile regression, $P < 0.001$). The Hyp1s and the Hyp4s strains showed a significant reduction in the median number of oocysts (the bottom set of horizontal comparisons in the figure) compared to the Siders strain (quantile regression, $P \leq 0.00187$). The prevalence of infection (the top set of horizontal comparisons in the figure) was also significantly different between the wild-type and all antiplasmodial strains (χ^2, 1 df). There was no significant difference in prevalence of infection between the Siders strain and any of the new antiplasmodial strains (χ^2, 1 df). *P*-values: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

(15.6–29.8%). However, there was no significant difference in prevalence between the Siders strain and the Hyp1s, TonBs, and Hyp4s antiplasmodial strains (χ^2, $P \geq 0.1684$).

DISCUSSION

Malaria continues to plague humans in tropical areas across the globe (World Health Organization, 2020). Current preventative strategies have led to the evolution of mosquito and parasite resistance, and new strategies are desperately needed (Liu, 2015; Haldar et al., 2018). Two proposed strategies focus on reducing the vectoral capacity of mosquitoes through either genetically altering the mosquito genome itself or through *paratransgenesis* whereby symbiotic microorganisms are genetically modified to affect the mosquito's phenotype (Kitzmiller, 1972; Alphey, 2014; Adelman and Tu, 2016). Many barriers exist for the use of transgenic mosquitoes given that several species of mosquitoes vector malaria parasites, population genetic considerations like reproductive isolation, and allelic variation within gene drive target sites that may render them ineffective (The malERA Consultative Group on Vector Control, 2011; Wang and Jacobs-Lorena, 2013; Carballar-Lejarazú and James, 2017). Paratransgenesis may be more advantageous due to the ease of engineering the symbionts, ease of dispersal to natural populations, and lower fitness costs to the mosquito host itself (Wang et al., 2017; Bilgo et al., 2018). Many paratransgenesis candidates have been proposed, including bacterial symbionts such as *Pantoea agglomerans* and *Serratia marcescens AS1*; when engineered to express antiplasmodial effectors, these bacteria were able to significantly inhibit *Plasmodium* parasite infection while having little to no impact on the host mosquito mortality and fecundity compared to wild-type strains (Wang et al., 2012, 2017). Here, we looked to improve paratransgenesis using *Asaia bogorensis* SF2.1 by isolating novel signal peptides and examining their ability to deliver the antiplasmodial peptide, scorpine, outside the cell. *Asaia* is not naturally antiplasmodial so it must be genetically engineered to produce antiplasmodials.

Previous work investigated the use of signal peptides in *Asaia* SF2.1 to deliver antiplasmodial effector molecules within mosquito vectors. Common bacterial signal sequences such as *E. coli* OmpA and those from closely related species *Gluconobacter oxydans* and *Gluconacetobacter diazotrophicus* were originally tested in *Asaia*, but none mediated export of a reporter protein (Bisi unpublished; Bongio, 2015). A genetic library screen from *Asaia* SF2.1 yielded only one signal, a siderophore receptor signal (=Sider), that was successful in the release of antiplasmodial effectors (Bongio and Lampe, 2015). Driving the export of the antimicrobial scorpine with the identified siderophore signal led to significant *Plasmodium* oocyst inhibition, but only reduced the prevalence, or the fraction of mosquitoes of a population that have at least one oocyst, by 20% (Bongio and Lampe, 2015). Prevalence is important since a mosquito with even one oocyst may still be infective and can pass on the *Plasmodium* parasite. The *Asaia* genomic fusion containing the signal sequence is also very long, at over 500 amino acids in length, which could have an increased fitness cost to the bacterial strains that carry it. Therefore, we hypothesized that increasing the amount of toxin released from the cell using different N-terminal signal peptides would lead to significantly improved levels of paratransgenesis.

In this study, we isolated six new signals that mediated measurable release of the reporter protein, PhoA. When used to mediate the release of an antiplasmodial scorpine-PhoA fusion protein, the behavior of the new signals was unpredictable. For example, no transformation could be obtained with scorpine-PhoA fusions fused to either the Hyp2, PIsom, or Hyp8 signals, even though these constructs were stable in *E. coli*. Another signal, Hyp1, performed poorly when fused only with PhoA, while it was one of the better performers when fused to scorpine-PhoA. Based on these results, further use of the signals in *Asaia* to drive the release of other antiplasmodials will most likely have to be evaluated empirically.

Scorpine is a strong antimicrobial molecule that seems to share properties of both cecropin and defensin (Conde et al., 2000). Though the exact mechanism by which it interrupts *Plasmodium* and other microbial cells is unknown, its cationic properties are thought to contribute to binding to the negatively charged lipids on membranes to cause disruption and eventually cell lysis (Carballar-Lejarazú et al., 2008). It has also been shown to affect the fitness of *Asaia*, so constitutive expression using these constructs might be toxic for the cells and may need to be regulated (Shane et al., 2018). Other antiplasmodials that are specific to *Plasmodium* can also be considered for use to

limit fitness costs to the bacterium, such as SM1 and EPIP, a *Plasmodium* enolase–plasminogen interaction peptide (Ghosh et al., 2001, 2011).

We relied on western analysis to determine the amount of scorpine released by strains constructed with the new signal peptides. Proteolytic cleavage, however, appeared to occur which separated the reporter PhoA from the antiplasmodial scorpine making relative concentrations of scorpine released difficult to quantify precisely. The known antiplasmodial behavior of scorpine and the mosquito midgut experiment performed here offer some insight on how to interpret these data, however. Purified scorpine is known to be highly active against *Plasmodium berghei* mosquito life stages (gametocytes and ookinetes) in a dose-dependent manner (Conde et al., 2000). Indeed, the ED_{50} of scorpine was 0.7 μM against ookinetes, the stage that actively invades midgut cells and leads to the formation of oocysts (Conde et al., 2000). Thus, more scorpine in the midgut is expected to correlate to greater parasite killing power. The mosquito experiments performed here identified two strains of *Asaia* (Hyp1s and Hyp4s) that significantly decreased the median number of oocysts per midgut beyond that produced by our previous strain using the signal from the siderophore receptor (**Figure 7**). These two strains also released significantly more scorpine when measured by the western analysis that assumed that all of forms of PhoA could be used as a proxy for the amount of scorpine released from the cell (**Figure 5B**). These data are not conclusive since they depend upon particular assumptions regarding western quantification, but they do suggest that increasing the amount of scorpine released from *Asaia* strains improves paratransgenesis.

Paratransgenesis in *Asaia* would likely be dramatically improved if we could achieve *bona fide* secretion in this species. Although signal peptides are relatively easy to predict from primary sequence data due to their N-terminal position and structure (Nielsen et al., 1997; Bagos et al., 2010; Petersen et al., 2011), identifying proteins that are actually secreted is much more difficult because there are no universal markers for secretion in mature bacterial proteins, although verified substrates secreted by particular bacterial secretion systems are known (Eichinger et al., 2016; An et al., 2017; Zeng and Zou, 2019). Newer *in silico* tools like Bastionhub are becoming available to predict secreted proteins (Wang et al., 2021) and these can be expected to aid the development of native secretion systems in the various bacterial species that are being developed for paratransgenesis, including *Asaia*.

In conclusion, we successfully identified and tested six new *Asaia* signal sequences that delivered heterologous protein outside the cell. These should prove useful to create new paratransgenic strains in the future, especially since paratransgenic strains for field release will necessarily need to produce more than one effector protein to decrease the chances of parasites evolving resistance to any single effector. Our simple hypothesis that increasing the amount of antiplasmodial proteins released from *Asaia* strains would lead to improved paratransgenesis was difficult to prove conclusively given that the scorpine-PhoA fusion protein underwent proteolytic cleavage separating the reporter from the antiplasmodial peptide. In addition, expression of heterologous proteins using these new signals generally decreased the fitness of the *Asaia* strains. Even so, this research brings *Asaia* one step closer for field-readiness and shows the attractiveness of paratransgenesis and the ease of its implementation, which can easily be combined with the other measures for both vector and parasite control.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, MW132102–MW132123 and https://datadryad.org/stash, doi: 10.5061/dryad.tx95x69wj.

ETHICS STATEMENT

The animal study was reviewed and approved by Duquesne University Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

CG designed the experiments, built the *Asaia* strains, tested them for protein localization and antiplasmodial effects, and wrote the manuscript. MB and SM performed the fitness experiments. DL designed the experiments and co-wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.633667/full#supplementary-material

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Identification of a Novel *Brevibacillus laterosporus* Strain With Insecticidal Activity Against *Aedes albopictus* Larvae

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Bacterial species able to produce proteins that are toxic against insects have been discovered at the beginning of the last century. However, up to date only two of them have been used as pesticides in mosquito control strategies targeting larval breeding sites: *Bacillus thuringensis* var. *israelensis* and *Lysinibacillus sphaericus*. Aiming to expand the arsenal of biopesticides, bacterial cultures from 44 soil samples were assayed for their ability to kill larvae of *Aedes albopictus*. A method to select, grow and test the larvicidal capability of spore-forming bacteria from each soil sample was developed. This allowed identifying 13 soil samples containing strains capable of killing *Ae. albopictus* larvae. Among the active isolates, one strain with high toxicity was identified as *Brevibacillus laterosporus* by 16S rRNA gene sequencing and by morphological characterization using transmission electron microscopy. The new isolate showed a larvicidal activity significantly higher than the *B. laterosporus* LMG 15441 reference strain. Its genome was phylogenomically characterized and compared to the available *Brevibacillus* genomes. Thus, the new isolate can be considered as a candidate adjuvant to biopesticides formulations that would help preventing the insurgence of resistance.

Keywords: *Aedes albopictus*, *Brevibacillus laterosporus*, biopesticides, genome sequencing, soil microbiota community

INTRODUCTION

The vector control market, which includes chemical, biological, and physical (e.g., UV lights and traps) strategies, was valued at USD 15.12 billion in 2017 and is projected to reach 20.37 billion by 2023. The segment likely to grow at the highest rate is the one of biological control: globally the biopesticide market has an estimated value of USD 4.3 billion in 2020, and it is expected to reach USD 8.5 billion in the next five years (Markets and markets, 2018). This dramatic increase can be attributed to the growing concerns on the environmental impact of chemical insecticides and the continuous increase of insecticide-resistance populations in many vector species. Moreover, unlike conventional insecticides which often target a broad spectrum of insects, including beneficial

species such as pollinators, bioinsecticides provide a more targeted activity toward selected species. Furthermore, these products are quickly biodegraded, leaving virtually no harmful residues and having limited long-term impact on the environment.

The biocontrol of mosquitoes began with the identification of bacteria active against Diptera, specifically *Bacillus thuringiensis* var. *israelensis* in 1977 (Goldberg and Margalit, 1977) and *Lysinibacillus sphaericus* strain 1593 (Singer, 1973). These two bacterial species achieved commercial success and are now broadly used.

The toxic activity of *B. thuringiensis* var. *israelensis* is due to four major crystal proteins, Cry4Aa, Cry4Ba, Cry11Aa, and Cyt1Aa, whose genes reside on a 128 kb plasmid (pBtoxis) (Berry et al., 2002; Ben-Dov, 2014). Other proteins, such as Cry10Aa, Cyt2Ba, Cyt1Ca, P19, and P20, contribute to the toxicity of *B. thuringiensis* var. *israelensis* (Palma et al., 2014). The larvicidal activity of the Cry and Cyt toxins is due to their ability to perforate midgut epithelial cells membranes, with the Crys binding to membrane receptors (Feldmann et al., 1995) and Cyt1Aa binding unsaturated phospholipids (Thomas and Ellar, 1983; Du et al., 1999). The major pitfalls of *B. thuringiensis* var. *israelensis* is its sensitivity to UV-damage (Myasnik et al., 2001), which required the development of stabilizing formulations (Lacey, 2007) and biotechnological approaches, including transgenic crops or recombinant bacteria expressing *B. thuringiensis* toxins (Federici et al., 2003; Sanahuja et al., 2011; Ursino et al., 2020).

One case of mosquito population resistant to *B. thuringiensis* var. *israelensis* has been described (Paul et al., 2005); however, most targeted studies have reported no resistance after long periods of treatment (Goldman et al., 1986; Becker and Ludwig, 1993). One of the obstacles to the development of resistance to *B. thuringiensis* var. *israelensis* is the synergism between Cry toxins and Cyt1Aa, with the latter functioning as a surrogate receptor able to promote toxin binding to host target membranes (Pérez et al., 2005). Nevertheless, moderate resistance may occur (Boyer et al., 2007; Paris et al., 2010), underlying the possibility that, in some instances, *B. thuringiensis* var. *israelensis* might persist in the environment for a long time, enhancing the likelihood of evolving resistant mosquito populations. Indeed, *B. thuringiensis* var. *israelensis* has low survivability in the environment but, in specific conditions, it persists and proliferates (Tilquin et al., 2008; Melo-Santos et al., 2010).

Lysinibacillus sphaericus is a second, potent mosquitocidal bioinsecticide. During sporulation it produces two separate proteins, BinA (42 kDa) and BinB (51 kDa), that form a binary toxin which accumulates as parasporal crystalline inclusions (Alexander and Priest, 1990). Some strains also produce non-crystal mosquitocidal toxins (Mtx1, Mtx2, and Mtx3) during vegetative growth. Upon binding to brush border membranes of midgut cells, *L. sphaericus* toxins are internalized and induce cell death either via ADP-ribosylation, as is the case of Mtx1 toxin (Thanabalu et al., 1993), or activation of apoptosis (Tangsongcharoen et al., 2015). As binding of Bin toxin to midgut cells is mediated by a single receptor (maltase), resistance is easily acquired as a consequence of mutations in this toxin binding protein (Darboux et al., 2002). The insurgence of *L. sphaericus* resistant populations has been described since 1997 (Nielsen-Leroux et al., 1997) and resistance in field populations of various countries has been reported (Su et al., 2018, 2019).

The highly effective results obtained with *B. thuringiensis* var. *israelensis* and *L. sphaericus* led to several screening campaigns allowing the identification of other useful bacterial biopesticide species, such as *Chromobacterium subtsugae* (Martin et al., 2007), *Yersinia entomophaga* (Hurst et al., 2011), and *Brevibacillus laterosporus* (de Oliveira et al., 2004; Ruiu, 2013).

Many strains of *B. laterosporus* have been isolated and the list of insects susceptible to their entomopathogenic activity includes Coleoptera, Lepidoptera (de Oliveira et al., 2004), mosquitoes, black flies (Favret and Yousten, 1985; Rivers et al., 1991), and house flies (Ruiu et al., 2007). Its pathogenicity against Diptera has been associated to the characteristic canoe-shaped parasporal body (CSPB) which consists of four major proteins: CpbA, CpbB, CHRD, and ExsC (Marche et al., 2017). Ingestion of lysates of recombinant *Escherichia coli* strains expressing these proteins results in the death of house flies, implying their role as insecticidal toxins (Marche et al., 2017).

Here we report the isolation of a novel *B. laterosporus* strain with high toxicity against mosquito larvae. This strain was isolated in a screening campaign of bacterial isolates active against *Aedes albopictus*, a globally distributed invasive species vector of many mosquito-borne diseases (Paupy et al., 2009). We carried out morphological, genomic, and insecticidal characterization of the new isolate, and determined that its larvicidal activity is significantly higher than that of the LMG 15441 reference strain. Thus, the new isolate characterized in this paper can be considered as a candidate for the development of novel biocontrol formulations, alone or in combination with *B. thuringiensis* var. *israelensis* and *L. sphaericus* preparations to enhance their efficacy and avert the insurgence of resistance.

MATERIALS AND METHODS

Bacterial Strains and Growth Media

Lysinibacillus sphaericus strain 1593 (*Bacillus* Genetic Stock Center ID 13A1; Priest et al., 1997), *B. laterosporus* strains LMG 15441 (*Bacillus* Genetic Stock Center ID 40A1; Rivers et al., 1991) and DSM25 (Shida et al., 1996) were used as positive controls. Strains 1593 and LMG 15441 were purchased from the *Bacillus* Genetic Stock Center (Columbus, OH, United States); strain DSM25 was purchased from DSMZ – German Collection of Microorganisms and Cell Cultures GmbH (Braunschweig, Germany). Growth media employed in this work include Luria–Bertani medium (LB), T3 medium (3 g/L tryptone, 2 g/L tryptose, 1.5 g/L yeast extract, 0.05 M sodium phosphate (pH 6.8), 0.005 g/L $MnCl_2$) (Martin and Travers, 1989) and BP medium (7 g/L Bactopeptone, 6.8 g/L KH_2PO_4, 0.12 g/L $MgSO_4{\cdot}7H_2O$, pH 7.4; BP medium is completed by addition of 10 μM $MnSO_4{\cdot}4H_2O$, 50 μM $ZnSO_4{\cdot}7H_2O$, 50 μM $FeSO_4$, 100 μM $CaCl_2{\cdot}4H_2O$, 0.3% glucose just before use] (Lecadet et al., 1980).

Samples Collection, Screening, and Isolation of Bacteria With Larvicidal Activity Toward *Ae. albopictus*

Fourty-four soil samples were collected from different geographical areas [**Supplementary Table 1**: Italy (23), Cameroon (4), Zimbabwe (8), Philippines (3), United Kingdom (1), Cuba (1), Myanmar (1), Kenya (1), Pakistan (1), and Tajikistan (1)] for the isolation of bacteria with insecticidal activity toward larvae of *Ae. albopictus*. Soil samples (about 100 g each) were collected from 2 to 5 cm below the surface using a sterile spatula and transferred in sterile 50 mL Falcon tubes. Collected samples were transported to the laboratory and stored at 4°C.

A protocol for selecting and growing the sporulating and cultivable bacteria of each soil samples was developed. In order to enrich in entomopathogenic species, methods previously described for the isolation of *B. thuringiensis* from soil were combined and adapted (Travers et al., 1987; Santana et al., 2008; Patel et al., 2013). One gram of each sample was incubated at 80°C for 5 h before being inoculated in 20 mL LB medium supplemented with 0.25 M sodium-acetate. After 4 h of incubation at 30°C (200 rpm), 1 mL of culture was centrifuged at 1,000 rpm for 1 min to settle down soil particles. The supernatant was transferred into a sterile test tube and treated at 80°C for 10 min. Serial dilutions of each sample were spread on T3-agar and 0.25 mL of supernatant were used to inoculate 25 mL of T3 medium which is optimal for *B. thuringiensis* sporulation. After 48 h of growth at 30°C (200 rpm), each culture was diluted 50-fold in 25 mL of BP medium and incubated at 30°C, 200 rpm, for 72 h. BP is a complete medium for *B. thuringiensis* growth and sporulation. Appropriate dilutions (10^{-4}, 10^{-6}, 10^{-7}, and 10^{-8}) of each culture were spread onto T3-agar medium plates and the rest of the cultures were collected by centrifugation at 10,000 rpm for 10 min at 4°C. Pellets were washed three times with NaCl 1 M, EDTA 10 mM and twice with dH_2O before being stored at −80°C or immediately used in larvicidal assays.

Cultures showing larvicidal activity were selected for further experiments. Colonies grown on T3-agar plates on which dilutions of active cultures had been spread were isolated to single colonies. Single clones were grown in 20 mL of BP for 72 h (30°C, 200 rpm) and pellets were collected, washed, and stored as described above before being assayed for their larvicidal activity.

Larvicidal Assays and Determination of LC_{50}

All experiments were performed using *Ae. albopictus* Rimini strain which was reared at 28°C, in a 12 h-light/12 h-dark photoperiod, with 70% of humidity.

The mixtures of cells and spores obtained after culture of soil inocula were screened for their larvicidal activity using 10 second-instar larvae of *Ae. albopictus* Rimini strain placed in 10 mL of dH_2O without any nutritional supplement. Pellets were resuspended in dH_2O and added at the final concentration of 2 g/L (expressed as biomass wet weight/litre). The same conditions were used in bioassays performed to screen individual bacteria isolates for potential larvicidal activity. In these screening assays, the concentration of 2 g/L was chosen to be able to detect activity even in soil samples with a low concentration of larvicidal bacteria and in bacterial isolates with low activity. A negative control group only exposed to distilled water was included in each experiment. Suspensions of *L. sphaericus* strain 1593 and *B. laterosporus* strain LMG 15441 collected after 72 h of growth in BP medium were used as positive controls. Mortality was recorded at 24 h intervals until 72 h from the beginning of the assay. The experiments were carried out at room temperature (22–25°C) in a laboratory location exposed to natural sunlight. Each test was performed in duplicate. Survival curves of each isolate were compared to those of the positive controls using a Long-Rank (Mantel–Cox) test in Prism GraphPad; *P*-values were corrected using False Discovery Rate.

Dose-response curves for strains SAM19, LMG 15441, and DSM25 were performed using 25 second-instar larvae of *Ae. albopictus* Rimini strain placed in 100 mL of dH_2O. Larvae were exposed to serial dilutions (range from 320 to 5 mg/L) of cells-spores suspensions in distilled water, without adding food. For each strain, five to seven concentrations were tested alongside a negative control group. Each test was performed in duplicate (technical replicate) and the entire experiment was repeated three times. The number of cfu and spores per mL used in the assay was determined by plating appropriate dilutions of each suspension before and after heat treatment at 80°C for 10 min. The experiments were carried out at room temperature and mortality was recorded at 24 and 48 h. Mortality was calculated according to the following formula:

$$mortality(\%) = \frac{X - Y}{X} 100$$

where X = percentage survival in the untreated control and Y = percentage survival in the treated sample.

Based on the obtained data, we performed a probit analysis using R studio (library ecotox and SciViews) and the results were visualized using ggplot2 (R Core Team, 2020).

Identification of Isolates by PCR and 16S rRNA Sequencing

Selected bacterial isolates showing larvicidal activity against *Ae. albopictus* were initially identified by performing colony PCRs targeting conserved regions of genes encoding known entomopathogenic toxins of *L. sphaericus*, *B. thuringiensis* var. *israelensis* and *B. laterosporus*. Primers employed in this work are reported in **Table 1**. Genes encoding the mosquito larvicidal toxins BinA, BinB, Mtx1, Mtx2, and Mtx3 of *L. sphaericus* were amplified by colony multiplex PCR (Jagtap et al., 2009). The same approach was used for the detection of *B. thuringiensis* var. *israelensis* toxin genes *cry4*, *cry10,* and *cry11* (Vidal-Quist et al., 2009). For the identification of *B. laterosporus* strains, a set of primers targeting the gene encoding the conserved 28 kDa spore surface protein was employed (Marche et al., 2019). The taxonomic classification of SAM19 was determined by sequencing the V3–V4 hypervariable regions of the 16S rRNA gene. The fragment of interest was amplified using

TABLE 1 | Primers used for the detection of mosquitocidal toxin genes in the isolates with larvicidal activity against *Ae. albopictus*.

Organism	Target gene(s)	Primer	Sequence (5′–3′)	Positive isolates	References
Bacillus thuringiensis var *israelensis*	*cry4*	cry4f cry4r	GCATATGATGTAGCGAAACAAGCC GCGTGACATACCCATTTCCAGGTCC	–	Vidal-Quist et al., 2009
	cry10	cry10f cry10r	TATTGTTGGAGTTAGTGCAGGTATTATTGTAG TATTCCATGTTGCGTTAGTATTAGTTC	–	Alberola et al., 1999
	cry11	cry11f cry11r	TTAGAAGATACGCCAGATCAAGC CATTTGTACTTGAAGTTGTAATCCC	–	Vidal-Quist et al., 2009
Lysinibacillus sphaericus	*binA*	binAf binAr	CCAGAAAACGAGCAATACCC GACCACATGCTTTGCCAATA	–	Jagtap et al., 2009
	binB	binBf binBr	CCCCAAACATCCTTACTTGAGA GCGCACTTCCTTTAACTGCT	–	
	mtx1	mtx1f mtx1r	ATTCCCTCTTTTGCTTCTCCA AGCACTATGAGGTGTCCAAGG	CB35, CB50, TJ9	
	mtx2	mtx2f mtx2r	TGATTGCAAGTTTTTTGTTTG CAGATGCTTCCCCAGATGTTA	–	
	mtx3	mtx3f mtx3r	TAGCTTTCCAGATGCAGCAA CGAAGTCTCATTTGCCTGACT	–	
Brevibacillus laterosporus	*cpbA*	cpbAf cpbAr	CTGCTACTAGTT GATCTAAG CTGATTGGTAGCT TAGGTA	CR1 CR4 LC1 LC2 NA2 NA6 SAM1 SAM2 SAM3 SAM14 SAM15 SAM19	Marche et al., 2019

primers 16Sf (5′-CCTACGGGNGGCWGCAG) and 16Sr (5′-GACTACHVGGGTATCTAATCC) (Klindworth et al., 2013).

Transmission Electron Microscopy

Samples were collected at different time points during growth in BP medium at 30°C and pellets were prefixed in Karnowsky's fixative in cacodylate buffer (pH 7.2). After post-fixation in 2% OsO_4 in 0.1 M cacodylate buffer for 1.5 h at 4°C, samples were washed, dehydrated through a progressive ethanol gradient, transferred to propylene oxide and embedded in Epon 812. Thin sections (80 nm) were stained with saturated uranyl acetate, followed by Reynolds lead citrate and examined with Zeiss EM900 transmission electron microscope at 80 kV.

Genomic Analysis of SAM19

Genomic DNA was extracted from SAM19 using the DNeasy Blood and Tissue Kit (QIAGEN). DNA sequencing was performed by Illumina MiSeq with a Nextera-XT pair-end library. 1,308,373 read pairs were produced. After a preliminary quality check with FastQC (Wingett and Andrews, 2018), reads were assembled with SPAdes 3.10 (Bankevich et al., 2012). The genome was annotated with Prokka (Seemann, 2014). Accordingly, the genome assembly was polished by removing short (<500 bp) contigs bearing no annotated gene. The draft genome sequences of *B. laterosporus* SAM19 has been deposited in GenBank under accession number JADGMT000000000.

For phylogenomic analysis, the predicted protein sequences of a representative set of 12 *B. laterosporus* genomes, plus *Bacillus agri* as outgroup, were downloaded from NCBI. Maximum likelihood phylogenomic analyses were performed on a concatenated set of single copy orthogroups, as previously described (Floriano et al., 2018).

In order to perform comparative analyses on genome structure and content, the genome sequences of two closely related strains from phylogenomics (DSM25 and BGSP7) were downloaded from NCBI. After reciprocal reorientation of contigs in case of draft assemblies, synteny analyses were performed with MUMmer (Delcher et al., 2002), using the NUCmer aligner and filtering out alignments <1,000 bp and <80% identity. Average nucleotide identity (ANI) values were calculated with the Enveomics online suite (Rodriguez-R and Konstantinidis, 2016). Annotation of possible antimicrobial molecules was performed using the on-line software AntiSMASH (Antibiotics and Secondary Metabolite Analysis Shell; Medema et al., 2011) and BAGEL4 (de Jong et al., 2006).

Clusters of Orthology Groups (COGs) in the predicted proteins of SAM19 and the two references were predicted with the NCBI pipeline (Galperin et al., 2015), and the respective COG repertoires directly compared.

RESULTS

Isolation of Bacterial Strains With Larvicidal Activity Against *Ae. albopictus*

The sporulating and cultivable bacterial communities of 44 soil samples were grown and screened for their larvicidal activity against second instar larvae of *Ae. albopictus*. To this purpose, based on methods previously described for the isolation of *B. thuringiensis* from soil samples (Travers et al., 1987; Santana et al., 2008; Patel et al., 2013), a new strategy for the selection and cultivation of sporulating bacteria enriched in entomopathogenic strains was developed (described in section "Materials and Methods" and **Figure 1**). The mixtures of cells and spores obtained from each soil sample were resuspended in dH_2O and used in larvicidal assays at the final concentration of 2 g/L (**Figure 1**). After 24 h of treatment, eight mixtures of soil bacteria (YA, CB, AR, LC, TC, VV, NA, and TJ) caused more than 30% larval mortality. Larvicidal activity increased over time and, 48 h after the beginning of the assay, five additional suspensions of soil bacteria (CR, LB, SAM, ZBC, and ZBD) killed at least 30% of *Ae. albopictus* larvae (**Figure 2A**). For each of the final 13 active samples, purified clones were grown in BP medium and the mixtures of cells and spores were harvested after 72 h of growth. Pellets were used in larvicidal assays at the final concentration of 2 g/L. After 24 h from the beginning of the assay, 10 bacterial clones isolated from three different soil samples induced more than 30% mortality against second instar larvae of *Ae. albopictus*. Five additional clones isolated from Cuba, Kenya, and Tajikistan also displayed larvicidal activity, but the percentage of larval mortality induced at 24 h was below 30% (**Figure 2B**). Larval mortality induced by each strain increased over time, reaching 100% in the case of clones isolated from soil collected in the Sampaloc lake area (Philippines) (**Figure 2B**). Furthermore, statistical analysis of the survival curves indicated that the Philippine isolates, with the exception of SAM3, were the only strains not being significantly different ($p > 0.05$) from the two positive controls (*L. sphaericus* and *B. laterosporus*), suggesting a similar larvicidal activity.

Identification of the Mosquitocidal Isolates

In order to assess whether the isolated clones belonged to known entomopathogenic bacterial species, their genomic DNA was used as a template in PCR reactions targeting known entomopathogenic toxin-encoding genes of the species *B. thuringiensis* var. *israelensis*, *L. sphaericus,* and *B. laterosporus* (**Table 1**). While no amplification products were obtained using primers pairs annealing to conserved regions of the *B. thuringiensis* var. *israelensis* toxin genes *cry4*, *cry10,* and *cry11*, an amplicon corresponding to the *L. sphaericus mtx1* toxin gene was obtained in three (CB35, CB50, and TJ9) of the five clones with lower larvicidal activity (**Figure 2B** and **Table 1**). More interestingly, the use of primers targeting a gene encoding a highly conserved 28 kDa spore surface protein of *B. laterosporus* yielded an amplification product in all other active clones (**Table 1**; Marche et al., 2019).

Due to their low level of activity, the *L. sphaericus* isolates CB35, CB50, and TJ9 (**Figure 2B**) were not studied any further. Among the *B. laterosporus* clones, those isolated from soil collected in the Sampaloc lake area (Philippines, SAM) induced higher mortality; clone SAM19, displaying the highest larvicidal activity after 24 h of treatment, was selected for further characterization.

BLAST analysis of its 16S rRNA gene sequence confirmed its classification as *B. laterosporus*. Cell and spore morphology were examined by transmission electron microscopy (TEM). To this purpose, SAM19 and the wild type *B. laterosporus* reference strain LMG 15441 were grown in synchronized cultures in BP medium and cultures were collected at different time points. As shown in **Figure 3**, during late stationary phase (24 h), an electrodense structure, probably related to the nascent parasporal body, could be observed at one pole of SAM19 spores, which were still contained within their mother cells. At 48 h, when sporulation was complete, a lamellar CSPB, firmly anchored to one side of the spore coat, was observed. The morphology of the mature spore appears similar to that of the reference strain LMG 15441 and to other *B. laterosporus* strains with entomopathogenic activity against Diptera (Marche et al., 2017).

Genome Sequence and Phylogenomic Analysis of *B. laterosporus* SAM19

The final draft genome assembly suggests that the chromosome of SAM19 is 5,550,463 bp-long (174 contigs; N50 = 90,219 bp; L50 = 20, largest contig = 316,450 bp; GC = 40.1%): 5,252 protein coding genes and 108 ncRNA genes were annotated.

Phylogenomic analyses confirmed the assignment of SAM19 to the *B. laterosporus* species (**Figure 4**). In particular, this new isolate forms a monophyletic lineage (100% support) with strains BGSP7 and DSM25, being more closely related with BGSP7 (93% support). These two strains were thus selected for more detailed comparative analyses.

Larvicidal Activity of *B. laterosporus* SAM19

The larvicidal activity of SAM19 was determined and compared with that of the phylogenetically proximal strain DSM25. Strain LMG 15441 was included in the analysis: despite being on a different phylogenetic branch with respect to SAM19, it shows phylogenetic proximity with UNISS18, a *B. laterosporus* strain with entomopathogenic activity (Ruiu et al., 2007; **Figure 4**). As reported in **Figure 5**, the lethal effects were concentration dependent and at 24 h post infection SAM19 displayed the highest larvicidal activity, with a LC_{50} of $10^{1.233}$ mg/L (confidential limits $10^{1.154}$–$10^{1.307}$), corresponding to $10^{5.287}$ cfu/mL ($10^{5.192}$–$10^{5.381}$) and $10^{5.153}$ spores/mL ($10^{5.077}$–$10^{5.229}$) (**Supplementary Table 2**). SAM19 LC_{50} values did not change significantly after 48 h (**Supplementary Figure 1** and **Supplementary Table 2**), confirming that the effect on larval viability is exerted within 24 h since exposure to the strain suspension. Interestingly, for

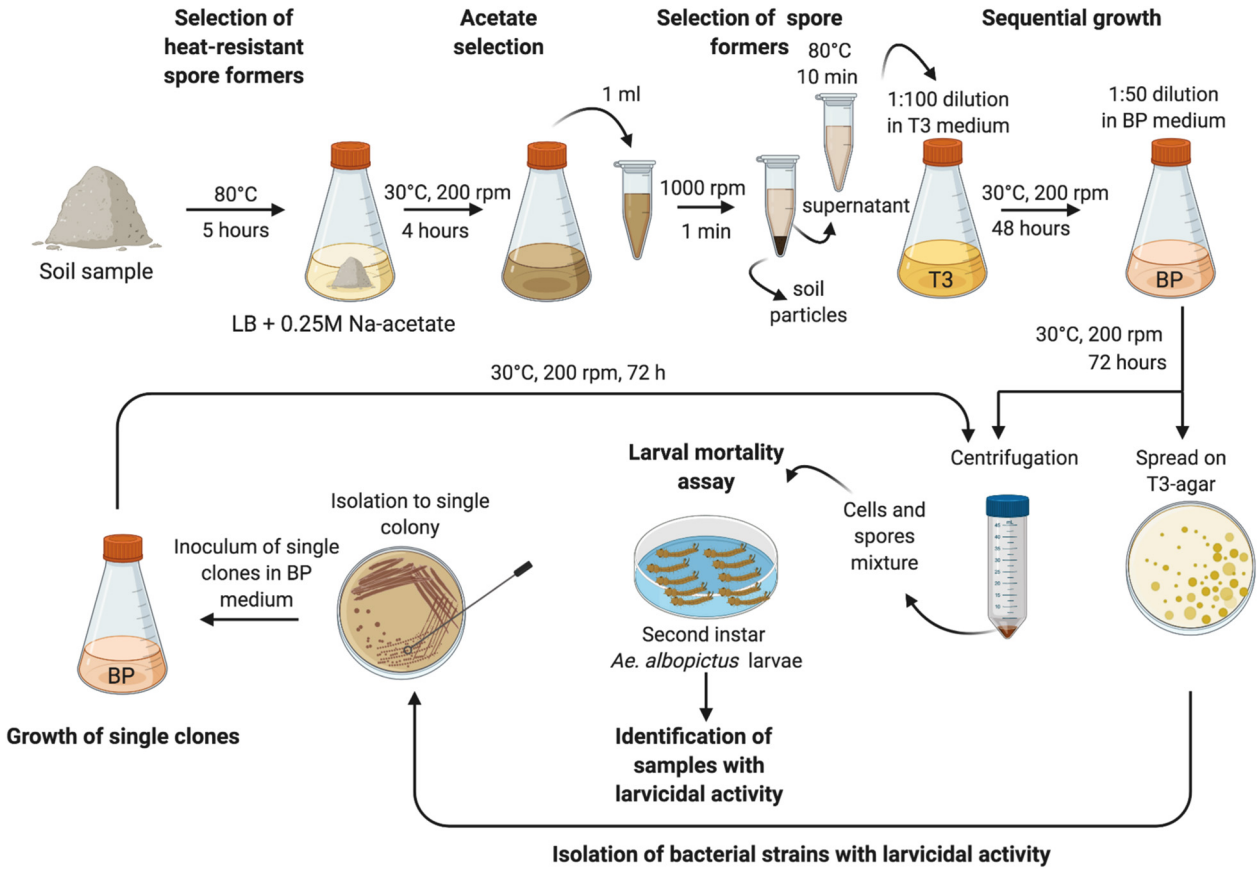

FIGURE 1 | Schematic representation of the protocol used for the identification of soil samples enriched with bacteria with insecticidal activity and for the isolation of bacterial strains with larvicidal activity against *Ae. albopictus*. Created with BioRender.com.

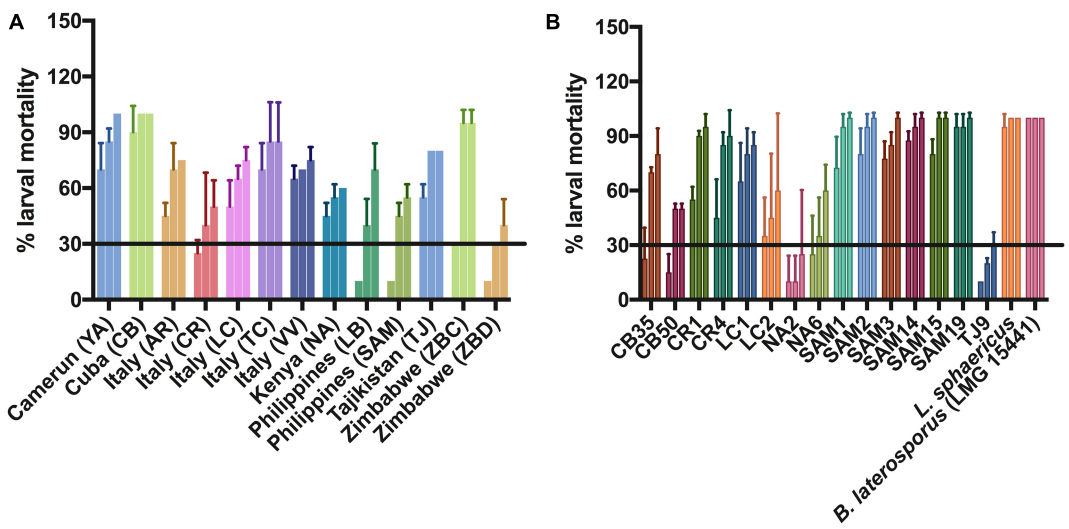

FIGURE 2 | Percentage of *Ae. albopictus* larval mortality at 24, 48, and 72 h after treatment with mixtures of cells and spores of cultures originating from the inoculum of 13 soil samples **(A)** or of single bacterial isolates **(B)**. Suspensions were added at the final concentration of 2 g/L. For each sample, the first, second and third bar represent larval mortality at 24, 48, and 72 h, respectively. Under the employed conditions, treatment with *B. laterosporus* strain LMG 15441 induced 100% of larval mortality in all experiments [Standard deviation (SD) = 0]. Data are the average ± SD of two independent experiments, each performed in duplicate.

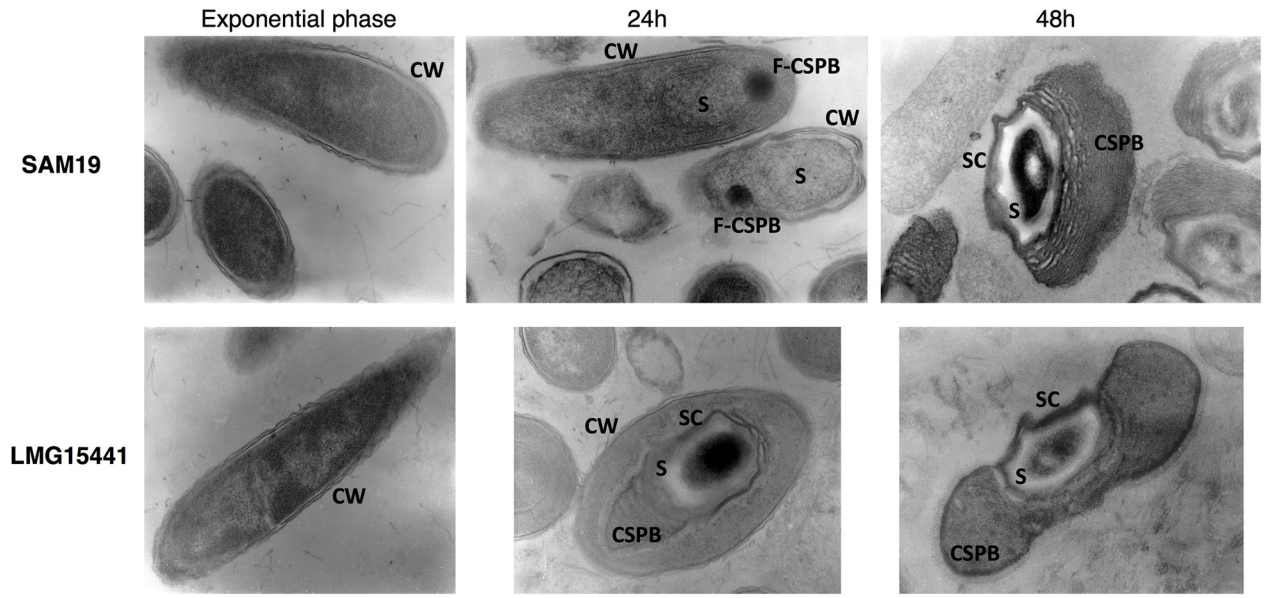

FIGURE 3 | Transmission electron microscopy (TEM) images of *Brevibacillus laterosporus* strains SAM19 and LMG 15441 at different growth stages: Vegetative cell during exponential phase; Sporangium including forming spore at 24 h after inoculum; Free spore. CW, cell wall; S, spore; SC, spore coat; F-CSPB, forming canoe-shaped parasporal body; CSPB, canoe-shaped parasporal body.

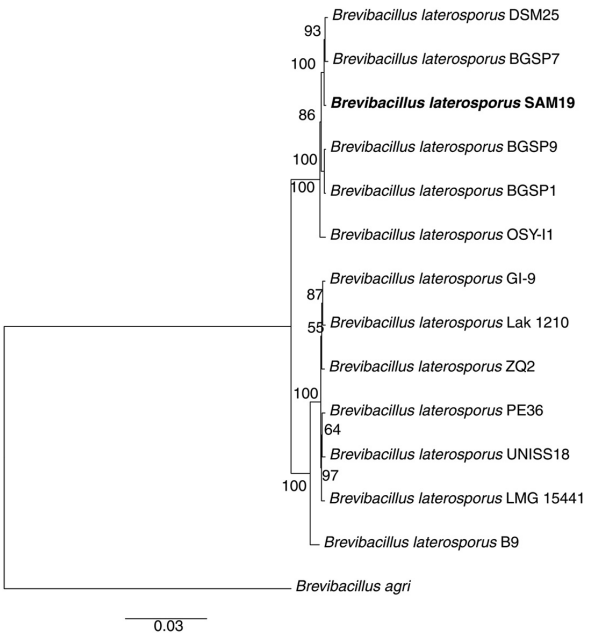

FIGURE 4 | Maximum likelihood phylogenomic tree obtained with RAxML (Stamatakis, 2015) showing the relationships of SAM19 with representative *B. laterosporus* strains. Numbers of branches stand for bootstrap supports with 100 pseudo-replicates, scale bar for estimated proportional sequence divergence.

all three strains LC_{50} values expressed as cfu/mL and spores/mL are similar to each other (**Supplementary Table 2**), consistent with the fact that larvicidal activity is associated with the spore.

Gene Content Analysis

In order to investigate whether the lower LC_{50} of SAM19 could be associated to unique functions encoded by the new isolate, its genome was compared with those of DSM25 and BGSP7. Coherently with phylogenetic proximity, SAM19 showed a very high level of synteny (14 and 8 inversions) and an ANI of 99.11 and 99.13% with DSM 25 and BGSP7, respectively (**Supplementary Figure 2**).

93.3% of the 1,910 COGs present on the genome of SAM19 were shared with the other two strains; only 75 were unique to this new isolate. BLAST analysis of the corresponding proteins revealed that homologues with >95% sequence identity were present in other *B. laterosporus* strains. No clear candidates with a potential involvement in virulence could be identified (**Supplementary Datasheet 1**).

As SAM19 is phylogenetically close to the antimicrobials producer strain BGSP7, its genome was also searched for the presence of genes coding for secondary-metabolites biosynthesis and bacteriocins using the online bioinformatic tools AntiSMASH (Antibiotics and Secondary Metabolite Analysis Shell; Medema et al., 2011) and BAGEL4 (de Jong et al., 2006). Non-ribosomal peptide synthetase clusters encoding bogorol A and brevicidine were identified by AntiSMASH analysis. Moreover, SAM19 genome displayed the potential to produce numerous bacteriocins, including laterosporulin, sactipeptides, UviB, and lanthipeptide class I (**Supplementary Table 3**).

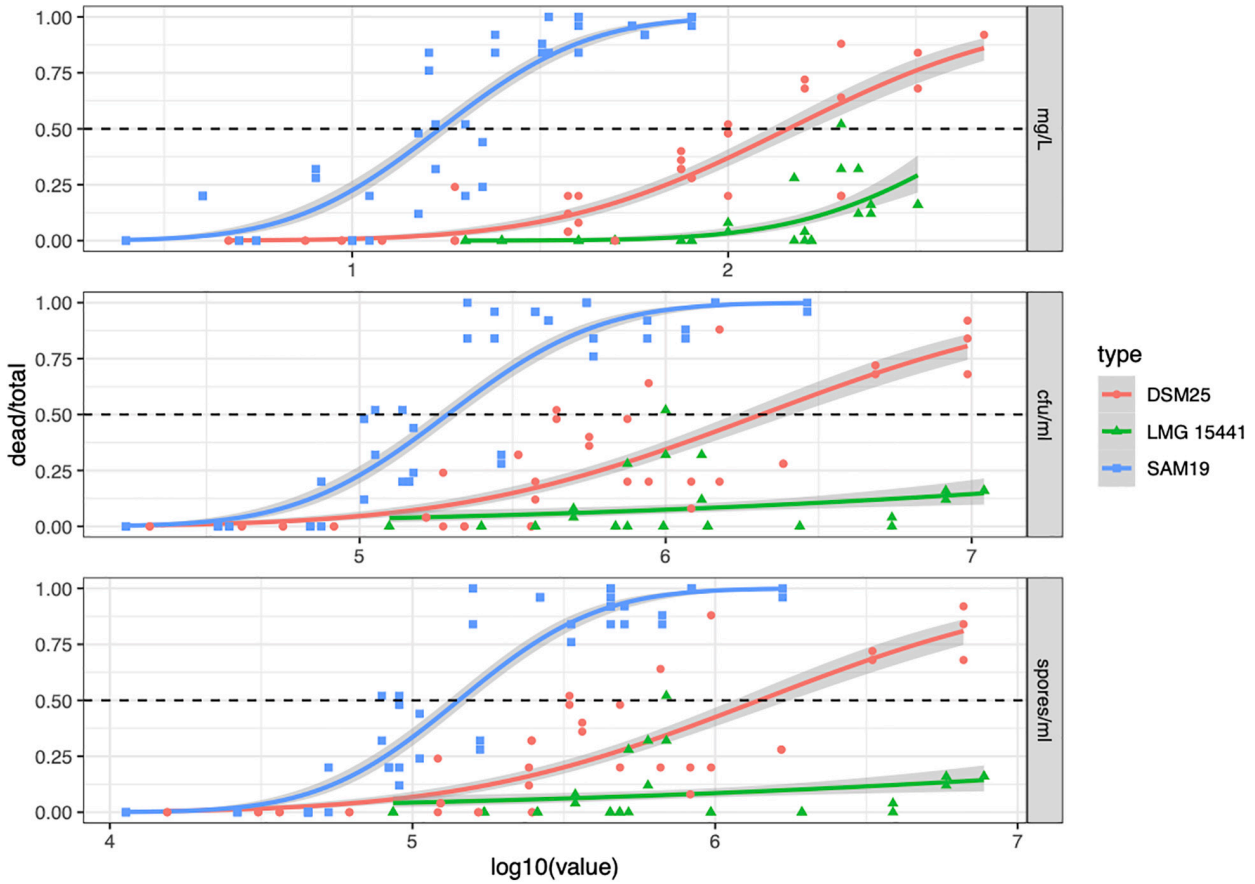

FIGURE 5 | Probit analysis of mortality data of *Aedes albopictus* larvae treated with *Brevibacillus laterosporus* strains SAM19, DSM25, and LMG 15441. The analysis was performed on data collected at 24 h after exposure to bacteria. The dead/total ratio is reported as a function of the concentration (log10) of each suspension expressed as mg/L **(upper panel)**, cfu/mL **(middle panel)** or spores/mL **(lower panel)**.

DISCUSSION

Bacillus thuringensis is the most widely employed biopesticide. Its high insecticidal activity, the specificity of each subspecies for a limited clade of insects (Ehling-Schulz et al., 2019), its low environmental impact and the possibility of using genes encoding toxic proteins to generate transgenic organisms make the use of *B. thuringiensis* a successful strategy to control insect pests. Currently, there are more than 98 formulated bacterial pesticides commercially available (Lacey et al., 2015).

The two main pitfalls of the large use of *B. thuringiensis* are its short lifetime and concerns about public health. As the stability of insecticidal crystalline proteins is hampered by solar radiation, effective control of insect pests requires continuous spraying of *B. thuringensis* formulations. Furthermore, *B. thuringensis* belongs to the *Bacillus cereus* group together with *Bacillus cereus sensu stricto*, *Bacillus anthracis, Bacillus weihenstephanensis, Bacillus mycoides, Bacillus pseudomycoides, Bacillus cytotoxicus*, and *Bacillus toyonensis*. *B. cereus* is recognized to secrete the emetic toxin cereulide, coded by the *ces* operon (Agata et al., 1995). Although there is no evidence that *B. thuringiensis* secretes it, concerns were raised by the European Food Safety Authority on the possibility that some *B. thuringensis* strains might contain genes for its synthesis, or that some formulations might contain other *B. cereus* species, requiring the full sequencing of the *B. thuringensis* strains used (EFSA Panel on Biological Hazards (Biohaz), 2016). Notably, *ces* genes are located in a transposable element in *B. weihenstephanensis* (Mei et al., 2014), raising questions about the possible horizontal transfer between species.

In view of these premises, the search for possible alternative biopesticides is increasing, particularly to control vector-borne diseases. It is worth noting that vector control is still the most effective strategy for the prevention of many insect-borne diseases, particularly for mosquito-borne diseases, including malaria, dengue and Zika. Their endemic area are the tropical and subtropical regions, but climate change and intense human commercial activities are expanding the geographical distribution of many mosquito species, allowing the transmission of mosquito-borne diseases in new countries. This is the case of *Ae. albopictus*, which spread from South-East Asia to all over the world and which caused in the last decade several outbreaks of chikungunya and dengue in Europe (Benedict et al., 2007; Gossner et al., 2018).

In this context, this work was aimed at isolating novel cultivable bacterial strains with insecticidal activity toward larvae of *Ae. albopictus*. To this purpose, a novel method to rapidly identify soil samples colonized by bacteria with larvicidal activity was developed. In order to have a collection as diversified as possible, samples were collected from different areas of Italy, from African (Kenya, Zimbabwe, and Camerun) and Asian countries (Pakistan, Tajikistan, Philippines, and Myanmar) as well as from Cuba and the United Kingdom. Different environments such as city, rural areas, agricultural lands, river basins, and lake areas were sampled (**Supplementary Table 1**). The classical protocol for the isolation of strains of *B. thuringiensis* var. *israelensis* is based on acetate selection followed by heat treatment: soil is inoculated in rich liquid medium supplemented with 0.25 M sodium acetate and, after 4 h of incubation at 30°C with aeration, culture supernatant is heated at 80°C. Since *B. thuringiensis* spores do not germinate in the presence of 0.25 M sodium acetate, germinated bacteria of species other than *B. thuringiensis* are heat killed (Travers et al., 1987). However, since *B. thuringiensis* concentration in soil can be very low, this strategy was reported to have low efficiency and alternative methodologies, including dry-heat pre-treatment of soil samples and enrichment method, were developed (Santana et al., 2008; Patel et al., 2013). All these methods require microscopic examinations of single clones for their ability to produce spores and crystals after at least 72 h of growth on sporulation agar-medium. Isolating, cultivating, and assaying the larvicidal activity of a high number of single clones for each soil sample is a time-consuming procedure. To overcome this limitation, we developed a new two steps procedure. In the first step, a mixed population of sporulating cultivable bacteria was obtained and tested for its larvicidal capacity. In the second step, single bacterial clones obtained from active soil samples were tested for their entomopathogenic activity. This method provides two major advantages: (i) multiple soil samples can be assayed in parallel and (ii) the bacterial population obtained from each soil contains a high percentage of active bacteria. We cannot exclude that entomopathogenic bacteria could be present at low density even in soil samples that displayed low or null larvicidal activity under employed conditions. Surprisingly, the majority of active clones were not *B. thuringiensis* var. *israelensis* strains but were identified as *L. sphaericus* and *B. laterosporus*, two known entomopathogenic species. While *L. sphaericus* isolates showed lower activities compared to the control reference strain 1593, isolated *B. laterosporus* strains displayed high toxicity.

Brevibacillus laterosporus has attracted increasing attention as a producer of antimicrobial compounds and secondary metabolites and is used as probiotic for humans (Ruiu, 2013). Importantly, strains with insecticidal activities against Diptera, Lepidoptera, and Coleoptera have been reported, making it an important candidate for the biocontrol of different pests with very limited risks for public health (de Oliveira et al., 2004; Ruiu, 2013).

The genome of the newly isolated strain was sequenced and the phylogenomic analysis revealed its close proximity to strains DSM25 and BGSP7. DSM25 is a known entomopathogen active against *Culex quinquefasciatus* and *Aedes aegypti* (Favret and Yousten, 1985; Rivers et al., 1991). BGSP7 was recently isolated from silage and selected for its ability to produce antimicrobial molecules active against Gram-negative (*Klebsiella pneumoniae* Ni9 and *Pseudomonas aeruginosa* MMA83) and Gram-positive (*Staphylococcus aureus* ATCC25923 and *Listeria monocytogenes* ATCC19111) multi-drug resistant pathogens (Miljkovic et al., 2019). Moreover, antifungal activity and toxicity toward larvae and adults of the potato beetle *Leptinotarsa decemlineata* were reported for BGSP7 (Miljkovic et al., 2019).

Even though the possible antimicrobial activity of SAM19 was not investigated in this work, bioinformatic analysis of its genome provided evidence of the presence of genes encoding antimicrobials.

The genome of SAM19 revealed the presence of 75 COGs not shared with its closest relatives DSM25 and BGSP7. However, analysis of the corresponding proteins did not highlight any specific feature suggesting higher toxicity of SAM19 compared to DSM25. Interestingly, SAM19 unique COGs included different phage related proteins, suggesting the possibility that phage functions and lysogenic conversion could play a role in the increased entomopathogenic activity.

The insecticidal activity of some strains of *B. laterosporus* has been associated to the presence of cytoplasmic inclusions containing insecticidal crystal proteins (Orlova et al., 1998). As revealed by TEM observations, SAM19 does not display intracellular crystalline inclusions. Entomopathogenic *B. laterosporus* strains lacking parasporal crystals have been previously described. Among these, the strain UNISS18 is characterized by insecticidal activity against *Musca domestica* and *Ae. aegypti* (Ruiu et al., 2007). This strain, however, is phylogenomically distant from SAM19. Its pathogenicity has been correlated to different virulence factors, including two surface proteins, CbpA and CbpB, associated to the CSPB. While a gene (LICBGMMG_04116) encoding a protein with 91.89% identity to UNISS18 CpbA was identified in SAM19, the new isolate did not display any CpbB homologue. A BLAST search analysis revealed that SAM19 encodes proteins previously identified as putative virulence factors expressed by UNISS18 during growth in the insect body (Marche et al., 2018). These proteins, with a high level of sequence identity (81.41–100%) with their UNISS18 homologues (**Supplementary Table 4**) include chitinases ChiA and ChiB (LICBGMMG_02893 Chitinase A1; LICBGMMG_01497 Chitodextrinase), a collagenase-like protease PrtC (LICBGMMG_01889), GlcNAc-binding protein (LICBGMMG_01411), protective antigen proteins (LICBGMMG_05212; LICBGMMG_04772; LICBGMMG_05296), bacillolysin (LICBGMMG_04689), thermophilic serine proteinase (LICBGMMG_02557), and the insecticidal toxin Mtx (LICBGMMG_04980 Epsilon-toxin type B). The toxicity of the new isolate against *Ae. albopictus* could therefore result from the concerted activity of a variety of virulence factors.

Further studies are needed to better characterize the toxicity of the newly identified SAM19 *B. laterosporus* strain against other mosquito and, more generally, Diptera species and to test the strain stability in field settings, using formulated or unformulated preparations. The higher toxicity of SAM19, compared with other *B. laterosporus* strains used as reference, supports the

possibility of adding this strain to the arsenal of eco-compatible tools that can be used to control mosquitoes and to limit the spread of invasive species and their associated diseases. Noteworthy, since *B. laterosporus* strains have been previously described for their toxicity to insects (Coleoptera, Lepidoptera, and Diptera), nematodes and mollusks (Ruiu, 2013), it will be important to assess possible off-target effects of SAM 19 to other species and organisms. Furthermore, the identification of genes encoding antimicrobial molecules suggests its potential use for antimicrobial production.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, JADGMT000000000; https://www.ncbi.nlm.nih.gov/biosample/, SAMN16515084.

AUTHOR CONTRIBUTIONS

GB, PG, EF, GG, and AA contributed to the conception and design of the study. GB, SM, PG, VS, and EU screened the collection of soil samples, isolated bacterial strains with larvicidal activity against *Ae. albopictus,* and identified and characterized SAM19. CF, MC, and DS performed SAM19 genome assembly, annotation, and phylogenomic analysis. GR, EC, and LS performed TEM analysis of SAM19. GB, PG, MC, and DS drafted the manuscript. GB, PG, and EF finalized the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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frontiers in Microbiology

REVIEW
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Mosquito Trilogy: Microbiota, Immunity and Pathogens, and Their Implications for the Control of Disease Transmission

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In mosquitoes, the interaction between the gut microbiota, the immune system, and the pathogens that these insects transmit to humans and animals is regarded as a key component toward the development of control strategies, aimed at reducing the burden of severe diseases, such as malaria and dengue fever. Indeed, different microorganisms from the mosquito microbiota have been investigated for their ability to affect important traits of the biology of the host insect, related with its survival, development and reproduction. Furthermore, some microorganisms have been shown to modulate the immune response of mosquito females, significantly shaping their vector competence. Here, we will review current knowledge in this field, focusing on i) the complex interaction between the intestinal microbiota and mosquito females defenses, both in the gut and at humoral level; ii) how knowledge on these issues contributes to the development of novel and targeted strategies for the control of mosquito-borne diseases such as the use of paratransgenesis or taking advantage of the relationship between *Wolbachia* and mosquito hosts. We conclude by providing a brief overview of available knowledge on microbiota-immune system interplay in major insect vectors.

Keywords: *Wolbachia*, vector-borne diseases, control strategies, pathogens, insects

GENERAL INTRODUCTION

Bloodsucking insects are important vectors of pathogens that cause a variety of severe diseases worldwide, with a strong impact on human and animal health (Lee et al., 2018; Boulanger et al., 2019). Concern about vector-borne diseases has increased in the last decade, also because of the geographical spread of several insect vectors, caused by intense trade and climate changes (de La Rocque et al., 2011; Caminade et al., 2019).

In particular, mosquitoes are major vectors of pathogens, including protozoa (e.g., *Plasmodium* spp. which causes malaria), nematodes (e.g., filariae), and viruses (e.g., dengue, chikungunya, West Nile, and Zika). Over 3,500 species of mosquitoes have been described, but only a limited number of them can function as disease vectors, and varying levels of specificity are observed for different types

of pathogens. Overall, mosquito-borne pathogens are estimated to cause around 500,000 deaths each year, with billions of people exposed to the risk of contracting these infectious agents[1].

So far, the most effective preventive strategies to limit the impact of mosquito-borne diseases have focused on controlling mosquito vector populations heavily relying on the use of insecticides and personal preventive measures, such as insecticide-treated nets (ITN) (Wangdi et al., 2018; Carnevale and Gay, 2019). For example, massive use of LLINs (long-lasting insecticidal nets, ITN with longer duration of effectiveness due to the incorporation of the insecticide into fibers during the manufacturing process) has greatly contributed to combat malaria (Carnevale and Gay, 2019). However, the efficacy of these control measures is hampered by the selection and spread of resistance (Hemingway, 2018), which is a complex phenomenon that accounts for modifications of multiple biochemical processes in mosquitoes (Hemingway, 2018; Ingham et al., 2020) or, also, for alterations of the mosquito biting behavior (e.g., shifts from an indoor- to an out-door host-seeking behavior) (Moiroux et al., 2012; Kreppel et al., 2020; Perugini et al., 2020). The massive use of insecticides raises also concerns, in relation to the impact on non-target species and the environment (Mansouri et al., 2017). Furthermore, the spread of invasive mosquito species to new areas requires constant monitoring and availability of new and alternative control strategies, considering that the control methodologies applied in the area of origin of a given species are not always suitable to be used in different countries and environmental conditions (Bellini et al., 2020).

The improvement of integrated vector control strategies, and in particular the development of novel environment-friendly insecticides and control approaches, is therefore urgent. In this context, insect microbiota already inspired the development of innovative control tools, such as the use of "symbiotic control" to target insect pests and vectors.

In this review we will focus our attention on the interactions between the microbiota and the vector host, with particular emphasis on the immune response. We will describe how this interaction shapes, at least partially, the vectorial capacity of mosquitoes; we will then describe the microbiota- and symbiont-based strategies that are used to control mosquitoes and mosquito-borne diseases, or that have been proposed but not yet applied. Finally, we will provide an overview of the current knowledge about the interaction between microorganisms and the immune system in other bloodsucking insect vectors.

THE INTERPLAY BETWEEN FEMALE MOSQUITO IMMUNE SYSTEM, GUT MICROBIOTA AND VECTOR COMPETENCE

The vector competence of mosquitoes is a biological trait that is influenced by multiple factors (Azar and Weaver, 2019). It is shaped, in the first instance, by the genetic variability of the immune effectors of the mosquito; for example, *thioester-containing protein 1* gene have multiple alleles that determine differences in susceptibility of *Anopheles* mosquitoes to the malaria infection (Le et al., 2012). The genomic variants of vectored pathogens or parasites can also play a major role, such as the case of the E1-226V variant of chikungunya virus that is preferentially transmitted by *Aedes albopictus* (Schuffenecker et al., 2006). Lastly, vector competence in mosquitoes can be also affected by the composition of the microbiota (Boissière et al., 2012).

Microorganisms, indeed, colonize different organs and tissues in mosquitoes, including gut, salivary glands and reproductive tissues (Segata et al., 2016; Scolari et al., 2019; Gao H. et al., 2020). They influence many aspects of the mosquito biology, including reproduction, development, adult survival and, overall, immunity (Coon et al., 2014). The main sites where cellular and humoral components of adult mosquito immunity exert their functions against invaders are the hemocoel with the circulating hemolymph, that contains the immune cells called hemocytes (Hillyer, 2010, 2016; Raddi et al., 2020), and the gut, which receives the sugar and blood meals and that hosts a major component of the insect microbiota (gut-associated microbiota).

For the purpose of this review, we will focus our attention on how bacteria interact with the gut of adult female mosquitoes and shape the immune responses after a blood meal (summarized in **Figure 1**). Blood meal, indeed, causes a proliferation of midgut microbiota (Gusmão et al., 2010; Kumar et al., 2010; Oliveira et al., 2011; Barletta et al., 2017) that, for instance, peaks at around 30 h after meal in *Anopheles gambiae* (Kumar et al., 2010).

Female mosquitoes acquire pathogens together with the blood meal and the microbes residing in the gut have a profound effect on the outcome of the infection (Cirimotich et al., 2011b; Dennison et al., 2014; Jupatanakul et al., 2014; Scolari et al., 2019).

For example, axenic *An. gambiae* mosquitoes are more susceptible to *Plasmodium* infection; conversely the co-feeding of a mixture of *Escherichia coli*, *Staphylococcus aureus* bacteria, and *Plasmodium falciparum* gametocytes decreases infection levels (Dong et al., 2009). Similarly, axenic *Ae. aegypti* have higher midgut dengue virus titers compared to normal septic mosquitoes (Xi et al., 2008) and some field-derived bacterial isolates affect dengue virus infection when introduced in axenic mosquitoes (Ramirez et al., 2012). Notably, the effect of microbiota on viral infection is specific and varies with the insect host and the virus: for example, it has been shown that axenic *An. gambiae* mosquitoes are less susceptible to o'nyong'nyong virus infection (Carissimo et al., 2015).

The protective role of the microbiota can be exerted by a specific class of microorganisms. It is the case of *Enterobacteriaceae* in *Anopheles mosquitoes*, which have a protective effect on *Plasmodium* infection (Cirimotich et al., 2011a; Boissière et al., 2012). In *Ae. aegypti*, different strains with different susceptibility to dengue infection harbor specific bacterial species that might be related to their vectorial capacity, with *Pedobacter* sp. and *Janthinobacterium* sp. identified only in resistant strains, while *Bacillus* sp. only in susceptible strains (Charan et al., 2013).

[1]https://www.who.int/news-room/fact-sheets/detail/vector-borne-diseases

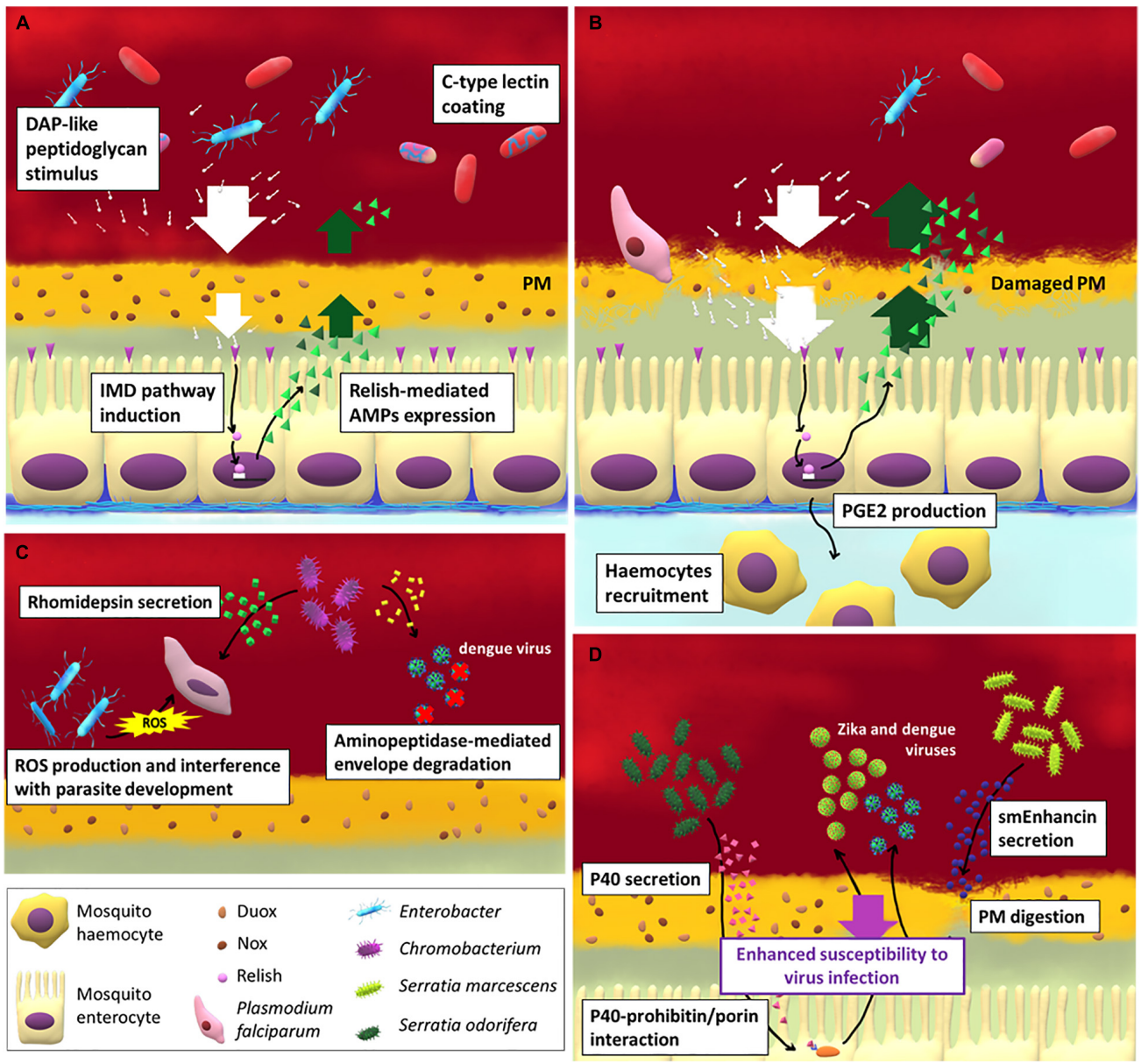

FIGURE 1 | The interaction between gut immune response, microbiota and pathogens in mosquito females. **(A)** The strong increase of gut-associated microbial load after blood meal induces the activation of the IMD pathway in midgut epithelial cells and the release of antimicrobial peptides (AMPs). The contact between bacterial cells or bacterial-associated molecules (such as peptidoglycans) and epithelium is partially prevented by the peritrophic matrix (PM) which forms soon after a blood meal. Other mosquito-secreted molecules can be exploited by the bacteria as protection from AMPs, such as C-type lectins. When the PM integrity is impaired **(B)** by the action, for example, of the *Plasmodium* ookinetes, the IMD pathway is activated and hemocytes are recruited to the infection site at the base of the epithelium thanks to the release of prostaglandin E2 by midgut cells. Apart from activating vector immune response some bacterial species are able to directly limit **(C)** or to favor **(D)** pathogen and virus infection in mosquitoes.

Physiological features and/or the genome variability of the mosquito vector can modulate vector competence in reason of their effect on the composition of gut bacteria community. The regulation of specific metabolic processes, as the branched chain amino acid degradation pathway, plays a role in the modulation of the microbial load of different *Aedes aegypti* strains (Short et al., 2017) that may in turn affect vector competence. Furthermore, genetic variation in immune genes encoding proteins with type III fibronectin domains (FN3D) in the gut correlates with interspecific variation of the load of *Serratia marcescens*, a common component of *Anopheles* gut *Enterobacteriaceae* (Stathopoulos et al., 2014). Indeed, silencing of three *FN3D* genes modulates *S. marcescens* load and alters the gut bacteria population favoring *Enterobacteriaceae* in *Anopheles* mosquitoes (Stathopoulos et al., 2014). This interaction, in turn, influences vector competence, since the abundance of

Enterobacteriaceae in the mosquito midgut affect *Plasmodium* infection (Boissière et al., 2012).

Humoral Immune Responses Mediated by the Gut and Interactions With the Associated Microbiota

The mosquito immune responses against infectious agents involves multiple pathways and effector molecules, which are summarized in **Table 1**.

The gut of mosquito females houses a wide spectrum of bacterial species, the most common of which are Gram-negative (Gendrin and Christophides, 2013; Scolari et al., 2019; Gao H. et al., 2020). Humoral responses against microbial pathogens have been deeply characterized in *Drosophila* and involve different pathways (Buchon et al., 2014; Mussabekova et al., 2017). Among them, the IMD pathway is conserved in mosquitoes (Christophides et al., 2002) and it appears to be functionally involved in antibacterial defense against both Gram-positive and Gram-negative bacteria (Meister et al., 2005; Cooper et al., 2009; Magalhaes et al., 2010; Barletta et al., 2017). In mosquito females, IMD pathway is activated in response to the proliferation of midgut microbiota that is triggered by the blood meal (Kumar et al., 2010; Barletta et al., 2017). The microbe-associated molecular pattern (MAMP) that triggers the activation of this pathway in Gram-negative bacteria is the diaminopimelic acid (DAP)-type peptidoglycan of the cell wall. In *Drosophila*, this molecule is recognized by two peptidoglycan recognition proteins (PGRP), i.e., the membrane-bound PGRP-LC in the anterior midgut and the intracellular PGRP-LE in the middle and posterior midgut (Kaneko et al., 2006; Buchon et al., 2014). Other pattern recognition proteins (PRRs) participate in the regulation of IMD pathway in a tissue specific manner: in the gut, it is positively regulated by PGRP-LA, while the amidases PGRP-LB and PGRP-SC, which cleave peptidoglycan into non-immunogenic fragments, negatively regulate the pathway (Zaidman-Rémy et al., 2006; Paredes et al., 2011; Gendrin et al., 2017). In mosquitoes, PGRP-LC is the main receptor that mediates immune response against Gram-positive and Gram-negative infections, with the isoform PGRP-LC3 recognized as key modulator of these responses at early stages of hemolymph colonization (Meister et al., 2009; Stathopoulos et al., 2014) and the isoform PGRP-LC1 having a main role in the midgut response (Rodgers et al., 2020). Similarly to *Drosophila*, PGRP-LC interacts with polymeric DAP-type peptidoglycan, while PGRP-LA and PGRP-LB positively and negatively regulate the pathway in *Anopheles* mosquitoes (Gendrin et al., 2017; Gao L. et al., 2020).

In *Drosophila*, the binding of the peptidoglycan ligand causes the dimerization of the receptor, activating an intracellular signaling cascade: the adaptor protein IMD is cleaved by the protease Dredd (Kim et al., 2014) and is rapidly ubiquitinated. This modification leads ultimately to the activation of the NF-κB transcription factor Relish, through the activity of Dredd and of the transforming growth factor β activated kinase-1 and the I-kappa B kinase complex (Paquette et al., 2010). Notably, the *An. gambiae* genome encodes two isoforms of the Relish homolog (i.e., REL-2); the short isoform, REL-2S, is involved in the response against Gram-negative bacteria, while the long isoform, REL-2F, against Gram-positives (Meister et al., 2005). It has been demonstrated that in *Anopheles dirus* REL-2F is involved in protection against both Gram-positive (with Lys-type peptidoglycan) and Gram-negative bacteria (with DAP-type peptidoglycan) (Khan et al., 2016). Relish, in turn, induces the expression of antimicrobial peptides (AMPs). These peptides have a highly conserved structure and they might exert their antimicrobial activity through peptide-lipid interaction or receptor-mediated recognition processes (Bulet et al., 1999). In mosquitoes, there are two classes of AMPs (defensins and cecropins) that have been found in many other insects, and one class, gambicins, that seems to be mosquito specific (Levashina, 2004).

Interestingly, it has been reported a direct interaction between PGRP-LD and gut-associated microbiota in *Anopheles*. Silencing of *PGRP-LD*, led to an over-activation of the immune response, leading to an over-expression of multiple AMP in *An. stephensi* prior blood feeding that causes a reduction of the bacterial load in the mosquito gut (Song et al., 2018).

A role of an immunomodulatory peroxidase (IMPer) and a dual oxidase (Duox) secreted by midgut cells in modulating gut-associated microbiota in *Anopheles* has also been described (Kajla et al., 2016) (see also section "The Interplay Between Physical Barriers Defenses in the Gut, Immune Responses, Microbiota and Implications for Vector Competence"). Indeed, when the peroxidase is silenced in *Anopheles stephensi* midgut, bacterial growth is significantly reduced by the overexpression of nitric oxide (NO) synthase gene (*NOS*), a final effector of the JAK/STAT pathway, while no significant recruitment of the classical immune pathways was observed (Kajla et al., 2016). Since NOS is a negative regulator of *Plasmodium* development (Oliveira et al., 2011), the authors suggested that the induction of the JAK/STAT pathway might be a strategy to modulate the vectorial capacity of *Anopheles* mosquitoes.

The expression of *Duox* is also regulated by a gut-membrane-associated protein, named Mesh, and the reduction of Duox activity lead to the increase of the microbiota load, suggesting that reactive oxygen species (ROS) might participate in controlling gut microbial homeostasis (Xiao et al., 2017). Notably, it has been also shown that blood meal-derived heme can decrease ROS levels in the mosquito midgut, allowing proliferation of bacteria (Oliveira et al., 2011).

The homeostatic balance governed by a tight control of both AMP transcripts and *Duox* expression is further confirmed by the effect of the mechanism exerted by the pathogenic fungus *Beauveria bassiana*: this fungus induces dysbiosis in the mosquito midgut by altering the expression of AMP transcripts and *Duox* with the secretion of the toxin oosporein, inducing bacterial growth, promoting the overgrowth of the opportunistic bacteria *S. marcescens*, which, once in the hemocoel, favors septicemia and thus the killing of mosquitoes (Wei et al., 2017).

On the other hand, the antimicrobial effect of AMPs produced by the mosquito against gut-associated microbiota is counteracted by multiple mechanisms: it has been demonstrated, for example, that the coating of bacteria with C-type lectins expressed in the mosquito midgut counteracts AMPs activity

TABLE 1 | Major humoral immune pathways in mosquitoes.

Immune pathway	Pathogen/parasite	Trigger	Intracellular actors	Effectors
Toll	• Gram positive bacteria • Fungi	Binding of pathogen-derived ligands to PRRs that triggers proteolytic cleavage of the cytokine Späetzle which binds to the membrane receptor Toll	MyD88, Tube, Pelle, Relish 1, Cactus	AMPs
	• Viruses	Interaction of the virus with Späetzle or with the membrane receptor Toll		AMPs
IMD	• Gram negative bacteria	Binding of pathogen-derived ligands to PGRP membrane receptors (mainly PGRP-LC)	PGRP-LE, IMD, FADD, Dredd, Caspar, Relish 2	AMPs
	• Viruses	Binding of the virus to an unknown membrane receptor		AMPs Vago (JAK-STAT activator)
JAK-STAT	• Viruses • Parasites	Binding of Upd ligand to Domeless membrane receptor or of Vago to an unknown membrane receptor	Hop (JAK), SOCS, STAT, PIAS	AMPs Antiparasitic factors (e.g., TEP1 opsonization factor, NOS)

The table summarizes the main features of the innate immune pathways characterized in mosquitoes (Cirimotich et al., 2009; Sim et al., 2014; Kumar et al., 2018; Mukherjee et al., 2019; Tikhe and Dimopoulos, 2021). AMP, antimicrobial peptide; PGRP, peptidoglycan recognition proteins (e.g., PGRP-LC); PRR, pattern recognition receptors (e.g., PGRP-SA, -SD); Upd, unpaired.

and favors gut microbiota homeostasis (Pang et al., 2016; Li et al., 2020).

The priming of the mosquito innate immune response by gut-associated microbiota can partially explain the effect of microbiota on pathogen virulence (Dong et al., 2009). In particular, some bacteria species are able to promote AMP genes expression in the gut, thus exerting a protective role against pathogens: this is the case of *Proteus* sp. in *Ae. aegypti* against dengue (Ramirez et al., 2012) and *S. marcescens* in *An. stephensi* against *Plasmodium berghei* (Bai et al., 2019).

The Interplay Between Physical Barriers Defenses in the Gut, Immune Responses, Microbiota and Implications for Vector Competence

An important immune role in the midgut of many insects is exerted by the peritrophic matrix (PM), a gel-like structure produced by midgut (Type I PM) or cardia region (Type II PM) cells (Hegedus et al., 2009). The PM is a non-cellular, selectively permeable layer composed by a scaffold of chitin fibrils associated with glycoproteins and proteoglycans that, among other functions, represents the first line of defense providing a physical barrier between the gut flora and the epithelium (Hegedus et al., 2009). In adult mosquitoes the PM is absent but in females the distension of the midgut induced by blood ingestion triggers the formation of a thick layer of Type I PM (around 20 μm) that surrounds the blood bolus (Shao et al., 2001).

As already mentioned, during blood meal, the load of gut-associated microbiota strongly increases and, interestingly, in *Anopheles* the synthesis and the integrity of PM appears to be microbiota dependent (Rodgers et al., 2017; Song et al., 2018) as already observed for other arthropod vectors (Weiss et al., 2013; Narasimhan et al., 2014). It is unclear which signaling pathway is responsible for this phenomenon, even though a potential role for the JAK/STAT pathway, which in mosquitoes has been implicated in antiviral response (Souza-Neto et al., 2009; Jupatanakul et al., 2017), has been suggested (Rodgers et al., 2017).

The structural integrity of PM is necessary for a proper response against pathogens: for example silencing of *PGRP-LD* in *An. stephensi* causes a dysbiosis, as a consequence of the altered expression of genes that codify for structural components of the PM and thus for its integrity (Song et al., 2018). Noteworthy, the fragmentation of the PM consequent to silencing increases the vectorial potential of the mosquito thanks to the enhanced susceptibility to *P. berghei* infections (Song et al., 2018).

In *An. gambiae* mosquitoes in addition to PM, the formation of a mucin-barrier lining the epithelium has been proposed (Kumar et al., 2010). In particular, upon the increase of microbiota load induced by blood meal, IMPer and Duox enzymes are secreted and their role in a process of crosslinking between mucins that may be secreted on cell surface is proposed. Although the presence of this mucin coat has to be demonstrated yet and the mechanism by which this coat should not interfere with physiological absorption/secretion processes at microvillar surface is still unknown, this mucin-barrier may regulate the access of immune elicitors secreted by bacteria to the epithelium and, vice versa, the access of immune effectors secreted by midgut cells into the endoperitrophic space where bacteria proliferate.

When PM integrity is disrupted by ookinete invasion in malaria-vectors, the direct contact between bacteria and midgut epithelial cells primes the immune cellular response in the hemocoel (Barletta et al., 2019). Hemocytes are recruited at the midgut basal surface by the prostaglandin E2 (PGE2) that is produced and secreted by the midgut cells. Hemocytes secrete an alpha macroglobulin with a structure similar to complement C3 protein in vertebrates, named thioester-containing protein 1 (TEP1) (Blandin et al., 2004; Baxter et al., 2007), which is involved in the lysis of pathogens, mainly *Plasmodium* ookinetes. In particular, TEP1 is a complement-like opsonin that upon binding to pathogens and parasites promote their recognition

by hemocytes and thus promote their phagocytosis or lysis. The link between microbiota-induced immune priming and *TEP1* expression has been further demonstrated in *An. dirus* (Wang Y. et al., 2013), showing that the microbiota participates in orchestrating the epithelial and complement-like immune responses. Hemocytes, in particular granulocytes, also participate in the phagocytosis of circulating microbes, while oenocytes are major players in the melanization response (Hillyer and Strand, 2014). The activation of this system heavily affect *Plasmodium* infection: the recruitment of hemocytes in proximity of the midgut basal surface (Barletta et al., 2019) and the production of NO (Kajla et al., 2016) leads to nitration of epithelial cells, which is required for a proper immune response against these parasites (Oliveira et al., 2012).

Direct Effect of Gut-Associated Microbiota on Pathogen Transmission

Some gut bacterial species can affect pathogen transmission directly, without influencing the mosquito immune response. *Pseudomonas rhodesiae*, *Enterobacter ludwigii*, and *Vagococcus salmoninarium*, isolated from the *Ae. albopictus* midgut, directly inhibit La Crosse virus infection, suggesting that they may produce anti-viral molecules (Joyce et al., 2011). *Chromobacterium* sp. *Panama* strain produces an aminopeptidase that degrades the dengue virus envelope protein, reducing dengue virus infection in *Ae. aegypti* (Ramirez et al., 2014; Saraiva et al., 2018a). The same species also produces an antiparasitic protein, named rhomidepsin, which restricts *P. falciparium* infection in *An. gambiae* (Saraiva et al., 2018b). An *Enterobacter*, isolated from wild *Anopheles arabiensis* mosquito populations in Zambia, has been demonstrated to generate ROS and to interfere with *P. falciparum* development before invasion of the midgut epithelium (Cirimotich et al., 2011a).

Bacteria may also enhance the infection of vectored pathogens. *Serratia odorifera* suppresses the immune response of the host by secreting a polypeptide, P40, that interacts with the mosquito prohibitin, similar to a cysteine rich protein present in some venoms, required for virus infection in mosquitoes (Londono-Renteria et al., 2015). As a result, susceptibility of *Ae. aegypti* to both dengue and chikungunya viruses infection is enhanced (Apte-Deshpande et al., 2012, 2014). Similarly, *S. marcescens* secretes smEnhancin, a protein that digests mucins associated with the PM, making mosquitoes more susceptible to virus infection (Wu et al., 2019).

The relationship between gut-microbiota and pathogens transmitted by mosquitoes is not only one way, but it is more and more clear that pathogens can shape the microbial load in the mosquito midgut and/or the composition of the bacterial population. For example, during the pre-invasive phase, *Plasmodium vivax* significantly decrease microbial load and *16S rRNA* gene expression was not detectable before 36 h post meal, the time frame when ookinetes/early oocysts invaded the gut (Sharma et al., 2020). This suggests that *Plasmodium* can restrict bacterial growth minimizing the impact of microbiota on the mosquito immune response by out-competing the bacteria before ookinete invasion.

Finally, viral infection can shape the composition of the gut microbial community: Zika virus alters the microbiota profile in *Ae. aegypti* (Villegas et al., 2018), and chikungunya virus increases the abundance of *Enterobacteriaceae* in *Ae. albopictus* (Zouache et al., 2012).

MICROBIOTA-MEDIATED CONTROL OF VECTOR-BORNE DISEASES

The knowledge accumulated on the interaction between insects and resident microbiota inspired the development of new strategies for the control of vector-borne diseases, since the modulation or manipulation of microbiota may have a strong impact on the host fitness and its resistance to pathogens and parasites (Gendrin et al., 2013; Gupta and Nair, 2020). The main microbiota-mediated interventions for the control of vector-borne diseases include: i) the manipulation of the symbionts for the expression of effector molecules (i.e., paratransgenesis, Wang and Jacobs-Lorena, 2017), summarized in **Figure 2**; ii) the introduction of microorganisms (bacteria or fungi) into the insect in order to reduce vector competence (van Tol and Dimopoulos, 2016), also outlined in **Figure 2**.

Paratransgenesis for the Control of Vector-Borne Diseases

In arthropods, paratransgenesis is based on the genetic manipulation of symbionts for the production of effector molecules (e.g., antipathogens or immunomodulatory), followed by the re-introduction of the modified symbiont into the arthropod host, to reduce its vector competence (Ogaugwu and Durvasula, 2017; Wang and Jacobs-Lorena, 2017; Gao H. et al., 2020; **Figure 2**). The choice of a good candidate symbiont is crucial (Hoy, 2013). First, the symbiont should be stably associated with the insect vector, efficiently transmitted vertically and/or horizontally, and persist long enough to produce the effector molecules (Wilke and Marrelli, 2015). Second, the symbiont should be easily culturable and should be genetically manipulable (Wang and Jacobs-Lorena, 2017). Third, the engineered microorganism should have the same fitness of the wild type strain and should not affect the fitness of the host (van Tol and Dimopoulos, 2016). Finally, to better enhance the effect, the symbiont should secrete the antagonistic molecule to guarantee its interaction with the target pathogen (Wang and Jacobs-Lorena, 2017). Paratransgenesis was initially applied for the control of Chagas disease by exploiting the symbiont *Rhodococcus rhodnii*, engineered for the production of the AMP cecropin A in the host, the triatomine bug *Rhodnius prolixus* (Durvasula et al., 1997). Since then, several projects have explored paratrangenesis as a strategy to control malaria. In 2007, Riehle et al. (2007) engineered the bacterium *Escherichia coli* for the expression of the two anti-plasmodial molecules (i.e., salivary gland and midgut peptide 1 and the phospholipase-A2 PLA2). Although a significant inhibition of the parasite *P. berghei* development was detected, the persistence of the bacterium in the gut was very low and the expression of functional PLA2 was toxic to the bacterium (Riehle et al., 2007). The mosquito

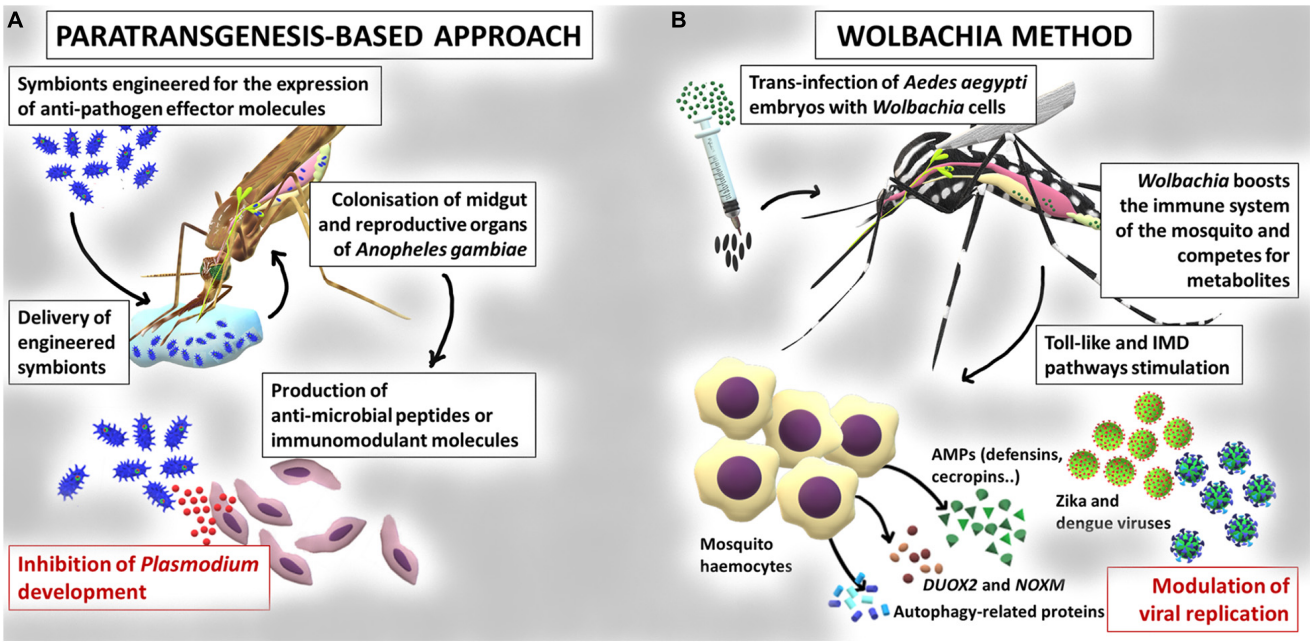

FIGURE 2 | Paratransgenesis as a tool for the control of mosquito-borne diseases. **(A)** Example of application of a paratransgenesis based-approach for the control of mosquito vector competence. Engineered symbionts colonize midgut and reproductive organs of *Anopheles gambiae* mosquitoes and express anti-pathogen effector molecules, leading to the inhibition of *Plasmodium* parasite development. **(B)** Example of application of the *Wolbachia* based-approach. *Wolbachia* is artificially introduced into the *Aedes aegypti* mosquitoes; these bacteria can block development of viruses such as dengue and Zika, through priming the response of the insect immune system or competing for nutrients.

symbiotic bacteria belonging to the genera *Pantoea*, *Serratia*, and *Asaia*, have been regarded as very promising for paratransgenesis purposes. *Pantoea agglomerans* is a non-pathogenic bacterium, widespread in different mosquitoes belonging to the genus *Anopheles* and, differently from *E. coli*, can efficiently persist in the insect gut (Riehle et al., 2007). This bacterium has been engineered for the expression of five anti-*Plasmodium* factors which have determined a strong inhibition of the development of the parasite (Wang et al., 2012). *Serratia* colonizes male and female of *An. stephensi* mosquitoes with a very low fitness cost for the insect (Chiamaka et al., 2020). The release of five single effector molecules by this modified bacterium or their simultaneous expression efficiently inhibited *P. falciparum* infection in mosquitoes (Wang et al., 2017). Finally, the bacterium *Asaia*, commonly found in *Anopheles* and *Aedes* mosquitoes (Favia et al., 2007; Crotti et al., 2009) has been successfully engineered for the secretion of different effector proteins resulting in a significant inhibition of *P. berghei* development (Bongio and Lampe, 2015; Shane et al., 2018). In addition, more recently, a modified strain of the bacterium *Asaia*, able to stimulate the immune system of mosquitoes, has been proposed for the control of the heartworm *Dirofilaria immitis* (Epis et al., 2020). Examples of paratransgenic control approaches come also from the study of leishmaniases and trypanosomiasis. Engineered bacteria of the genus *Bacillus*, among others, are under study for their potential to reduce the capability of sand flies to transmit *Leishmania* (Wijerathna et al., 2020). In African trypanosomiasis, the symbiont of the genus *Sodalis* has been studied as a candidate vector to be exploited to block trypanosome transmission in the tsetse flies. Especially, attacin is a well characterized inducible immune peptide studied as an effector molecule for the engineering of *Sodalis* with specificity against some Gram-negative bacteria and protozoa (Aksoy et al., 2008).

In addition to bacteria, other microorganisms have been investigated for their potential to be exploited in paratransgenesis, in particular fungi and viruses. *Metarhizium robertsii* (previously named *M. anisopliae*), a fungus that infects several insects and proliferates in the hemolymph, was engineered to produce antimalaria effector proteins with encouraging results (Fang et al., 2011). As for viruses, densonucleosis viruses have been proposed as attractive agents for viral paratransgenesis in *Aedes* and *Anopheles* mosquitoes; Ren et al. (2008) described an efficient *An. gambiae* densovirus (*Ag*DNV) which can be potentially used for the control of malaria by transduction of anti-*Plasmodium* peptides or insect-specific toxins. The same densovirus was proposed by Suzuki et al. (2014) as over-expression system for the malaria vector *An. gambiae*. Moreover, the pathogenic *Aedes* DNV (*Ae*DNV) was manipulated to express the green fluorescent protein (Afanasiev et al., 1999) and the microRNAs that target host genes (Liu et al., 2016).

An important key point in the paratransgenic approach is the choice of the molecules with antagonistic activity against pathogens or parasites (Wang et al., 2017). While in the case of malaria parasites there are different effector molecules

successfully studied and tested (Bisi and Lampe, 2011; Fang et al., 2011; Dehghan et al., 2017), in the case of viral infections the research is much more limited (Gao H. et al., 2020). **Supplementary Table 1** highlights several effector molecules, including AMPs and specific single chain antibodies, currently investigated for their anti-parasite activities.

Before paratransgenesis is applied in large-scale in the field, an intermediate step is required to validate laboratory-based findings; recently, a semi-field study provided evidence for the potential capability of engineered *Asaia* bacteria to invade mosquito populations (Mancini et al., 2016). Many questions are still open about the introduction and maintenance of the engineered bacteria in mosquito populations; exploiting a bacterium that is naturally vertically and/or horizontally transmitted offers the possibility of a stably spreading the symbiont among target mosquito populations (van Tol and Dimopoulos, 2016). To date, one of the most important tools for the dissemination of engineered bacteria to mosquitoes is based on sugar baits (Lindh et al., 2006; Wang et al., 2012; Wang and Jacobs-Lorena, 2017). Furthermore, Bilgo et al. (2018) tested in a field study the attractivity and effectiveness of sugar baits as a delivery method for modified bacteria (Bilgo et al., 2018); in brief, they highlighted that Window entry trap (WET) attractive sugar bait stations are the most promising tool to introduce and spread engineered bacteria through the mosquito population. Despite these promising results and applications in semi-field condition or in the field, a real application of paratransgenesis has not yet been realized and possible disadvantages of this strategy are still to be investigated. Safety and risk assessments on humans and on non-target organisms, horizontal gene transfer, stability of the engineered symbionts in a natural habitat are some of the issues that will have to be addressed before the application (Coutinho-Abreu et al., 2010).

Colonization of Mosquitoes With Microorganisms

The second microbiota-mediated intervention exploits the introduction of non-modified microorganisms into the insects able to impair vector competence. The impairment may occur by different mechanisms such as resource competition with the vectored pathogen or parasite, stimulation of the host immune response, reduction of host lifespan (Cirimotich et al., 2011b; Dennison et al., 2014). Different bacteria isolated from the insect gut have been studied for their capability to affect pathogen transmission. Interestingly, a recent study showed that the bacterium *S. marcescens*, isolated from the midguts of field-collected mosquitoes, could negatively affect *Plasmodium* development in *An. stephensi* mosquitoes by activating immune response and in particular modulating effector genes such as *TEP1* and *fibrinogen immunolectin 9* (Bai et al., 2019). Moreover, Cappelli et al. (2019) described the interactions between the bacteria *Asaia* and the immune system of the mosquitoes *An. stephensi*; in particular, the introduction of *Asaia* triggers mosquito immune responses, eliciting an anti-*Plasmodium* response.

To date, the most promising microbiota-mediated intervention is based on the release of *Ae. aegypti* mosquitoes infected with a *Wolbachia* strain isolated from *Drosophila melanogaster* for the control of dengue virus (Hoffmann et al., 2011; Walker et al., 2011; O'Neill, 2018; see dedicated section).

Wolbachia and the Immune System of Mosquitoes

Wolbachia is one of the most fascinating microorganisms associated with arthropods, due to its ability to influence the reproductive biology of the hosts, their metabolism, and immunity (Werren et al., 2008). The *Wolbachia* encompasses obligate intracellular bacteria, members of the order Rickettsiales, first observed in the mosquito *Culex pipiens* by Hertig and Wolbach (1924). *Wolbachia* is widespread in insect species and populations, but patchily distributed among them. In a seminal study, insects from 65% of the examined species tested positive for *Wolbachia*, with different prevalence rates within infected species, in some cases reaching fixation (Hilgenboecker et al., 2008). Among mosquitoes, *Wolbachia* has consistently been detected in species from the genera *Culex*, *Aedes*, *Coquillettidia*, *Mansonia*, and *Uranotaenia* (Huicong et al., 2020), where it is found both in reproductive organs and somatic tissues. These localizations are coherent with the effects that *Wolbachia* has on the hosts, i.e., with its capability to influence the mosquito survival and fertility. In general, the presence of these bacteria in insects determines reproductive alterations, such as feminization of genetic males, parthenogenesis and the killing of male embryos (sex-ratio distortions) and cytoplasmic incompatibility (CI). CI provides a reproductive advantage to *Wolbachia* infected females over uninfected ones, resulting in a rapid spread of *Wolbachia* into the host population (Jiggins, 2017). CI is caused by the sperm from infected males, which is capable of reducing the fertility of uninfected females. Briefly, the molecular mechanism at the basis of CI has been recently elucidated: CI displays as embryonic death when a male expressing prophage WO genes *cifA* and *cifB* mate with an uninfected female or a female infected by an incompatible *Wolbachia* strain. In mosquito females harboring a compatible *cifA*-expressing strain rescue the embryonic development (LePage et al., 2017; Shropshire et al., 2021). *Wolbachia* has recently been detected in *Ae. aegypti* and in some species of *Anopheles* mosquitoes, although its presence is in general variable, in terms of prevalence and abundance, from species to species (Baldini et al., 2014; Balaji et al., 2019). As for the presence of *Wolbachia* in *Anopheles*, a negative correlation between *Wolbachia* infection and *Plasmodium* was observed in *An. gambiae*, in which the presence of *Wolbachia* reduces malaria transmission with effects on sporozoites (Shaw et al., 2016; Gomes et al., 2017). More recently, the description of novel *Wolbachia* strains in *Anopheles* mosquitoes was reported on two large studies in Africa (Jeffries et al., 2018; Ayala et al., 2019); in these researches the authors proved that the *Wolbachia* prevalence varied among *Anopheles* species, suggesting that the sample size can be a key factor to detect the infection. Moreover,

recent papers emphasized that the evidence for the infection of *Wolbachia* in *Anopheles* mosquitoes is largely molecular, which implies that active *Wolbachia* infections had not always been discriminated from the simple presence of "traces" of *Wolbachia* or its DNA (Chrostek and Gerth, 2019; Ross et al., 2020). However, another possible explanation for the limited presence of *Wolbachia* in several *Anopheles* mosquitoes can be the preponderant role of *Asaia* bacteria in these mosquitoes (Favia et al., 2007; Chouaia et al., 2012). In fact, *Asaia* symbionts had been shown to interfere with the vertical transmission of *Wolbachia* and to negatively correlate with *Wolbachia* in mosquito reproductive tissues (Hughes et al., 2014; Rossi et al., 2015).

Prior to the observation of naturally infected individuals of *Ae. aegypti*, stable and heritable *Wolbachia* infections had been generated in laboratory colonies of this species, by embryonic microinjection of *Wolbachia* from donor species (Xi et al., 2005; **Figure 2**). After the release of infected mosquitoes, *Wolbachia* was then able to spread into wild *Ae. aegypti* populations, by means of the CI mechanism (Xi et al., 2005; Hoffmann et al., 2011; Nazni et al., 2019). *Wolbachia* was also stably introduced into a colony of *An. stephensi*, where the bacteria increased host resistance to *P. falciparum* (Bian et al., 2013). A similar phenomenon was observed in *Ae. aegypti* where different *Wolbachia* strains have been shown to inhibit the infection by viruses of medical relevance, such as dengue (Moreira et al., 2009; Bian et al., 2010), chikungunya (Moreira et al., 2009), West Nile (Hussain et al., 2013), Zika (Aliota et al., 2016), and filarial worms (Kambris et al., 2009).

A stable infection of *Wolbachia* into a novel mosquito host implies that this symbiont must be able to cope with the host immune system. Thus, has *Wolbachia* evolved mechanisms to suppress or stimulate the immune system of the hosts?

Actually, when *Wolbachia* bacteria infect a new host, they are able to stimulate the mosquito immune system, including the Toll and IMD pathways. In detail, Pan et al. (2018), reported that the suppression of either the IMD pathway alone or both the Toll and IMD pathways reduced *Wolbachia* load in *Ae. aegypti*; on the other hand, the activation of these pathways increased *Wolbachia* load, suggesting that host innate immunity is utilized to establish and promote this new host-microbial symbiosis. Various studies indicated that *Wolbachia*-mediated interference with pathogens is associated with a boosted immunity in mosquitoes (Kambris et al., 2009, 2010; Moreira et al., 2009; Bian et al., 2010; Hughes et al., 2011). Overexpression of AMPs, such as *defensins* and *cecropins*, and of several Toll pathway genes, is induced by *Wolbachia* in *Ae. aegypti*, providing evidence that immune activation is crucial in the inhibition of dengue infection in these mosquitoes. Comparing the transcripts of *Wolbachia*-infected *Ae. aegypti* mosquitoes with wild type mosquitoes, Pan et al. (2012) described the up-regulation of genes in the midguts of *Wolbachia*-infected mosquitoes: *defensin C*, *attacin*, *cecropin D*, *Copper superoxide dismutase*, 13 *cytochrome P450*, two putative *NADH dehydrogenase*, and three *heat-shock proteins*, *Gram-negative binding protein B1* (*GNBPB1*), *Relish-like protein 1A* (*REL1A*). Similarly, the components of the Toll pathway such as *GNBPB1*, *Spaetzle 3*, *myeloid differentiation primary response 88* and *REL1A* were also up-regulated. Moreover, they demonstrated that *Wolbachia* infection leads to an up-regulation of genes encoding a NADPH oxidase and a dual oxidase (DUOX2), which are involved in the generation of ROS. Specifically, this increased ROS level is correlated with the activation of the Toll pathway, which contributes to the production of antioxidants, defensins and cecropins (Bian et al., 2010; Luplertlop et al., 2011; Pan et al., 2012).

A recent study provided evidence for the effect of a protein of *Wolbachia* in the activation of the immune response of *Ae. aegypti* and *An. stephensi* mosquitoes, consisting in the expression of genes coding for cecropin, TEPs, leucine-rich repeat protein and CLIP-domain serine protease, plus NADPH-oxidases and NO synthase. This priming of the immune response of mosquitoes was associated with the inhibition of the development of the heartworm parasite *Dirofilaria immitis* (Epis et al., 2020; Varotto-Boccazzi et al., 2020).

Additionally, Zug and Hammerstein (2015) proposed the hypothesis that newly introduced *Wolbachia* triggers the immune response and causes oxidative stress by upregulating the expression of several immune effectors such as AMPs, autophagy-related proteins, and ROS. In *Drosophila*, a native *Wolbachia* infection increases ROS level, leading to oxidative stress, which is involved in the resistance of these flies against viral infection and replication (Wong et al., 2015). On the contrary, in *Ae. albopictus* mosquitoes, which are naturally infected by *Wolbachia*, the presence of the bacteria is not associated with oxidative stress, but with balanced redox homeostasis.

In summary, although *Wolbachia* often determines an up-regulation of mosquito immunity in newly infected hosts, immune priming is not regarded as the sole mechanism involved in the inhibition of pathogen transmission. For example, it has been proposed that competition between viruses and *Wolbachia* for intracellular cholesterol and amino acids can result in metabolite depletion and cellular stress, thus reducing viral replication (Caragata et al., 2014; Lindsey et al., 2018).

Normally, when *Wolbachia*-free insects are artificially infected with the symbionts, it is expected that an anti-microbial immune response could be triggered leading to the elimination of *Wolbachia* itself. However, *Wolbachia*, through the evasion of the AMP-based immune response or the suppression of the autophagy-associated immune defense, are able to prevent their elimination (Zug and Hammerstein, 2015). In parallel, natural selection could favor the presence of the endosymbiont *Wolbachia* improving the fitness of the insect host; indeed, other studies suggest that *Wolbachia* provides an advantage to the host in the form of metabolic provisioning (Brownlie et al., 2009; Gerth and Bleidorn, 2016). In the long term, natural selection is also expected to favor a reduction in the immune stimulating property of *Wolbachia*, with a stabilization of the association (Dedeine et al., 2003).

The artificially infection of *Aedes* mosquitoes by *Wolbachia* affects the relative abundance of resident bacteria, but not species diversity (Audsley et al., 2018), and this effect may be related to an activation of immune pathways such as Toll

and IMD (Rancès et al., 2012). Interestingly, in *Anopheles* mosquitoes, there are several bacterial species that negatively correlate with *Wolbachia*; for example, Hughes et al. (2014) demonstrated that native mosquito microbiota, in particular bacteria of the genus *Asaia*, is a major barrier for the transmission of *Wolbachia*. The same observation was reported in Rossi et al., 2015, in which, a mutual exclusion or a competition between *Asaia* and *Wolbachia* has been hypothesized in anophelines thus explaining the inability of *Wolbachia* to colonize the reproductive system.

Anyhow, due to the variable influence of *Wolbachia* on the composition of mosquito microbiota, e.g., in relation with the host species, developmental stage, sampling location (Muturi et al., 2016, 2017; Straub et al., 2020), an understanding of these factors is very important before *Wolbachia* is transinfected into a new mosquito species for the control of the pathogens.

Furthermore, another crucial aspect to be investigated is the long-term phenotypic stability of artificially infected *Ae. aegypti* mosquitoes in field conditions (O'Neill, 2018). As previously described, field application of *Wolbachia*-infected *Ae. aegypti* mosquitoes for the control of mosquito-borne viruses is relatively "new"; we can expect that this system (*Wolbachia-Ae. aegypti*) will evolve in the coming years (Dorigatti et al., 2018). Certainly, higher efficacy strains of *Wolbachia* must be investigated and the release of mosquitoes infected by two or more strains ("superinfected") might be proposed as an alternative strategy to manage potential reductions of the efficiency of single *Wolbachia* to interfere with pathogen transmission (Joubert et al., 2016).

THE INTERACTION BETWEEN MICROBIOTA AND IMMUNE SYSTEM IN OTHER INSECT VECTORS

The role of microbiota in the modulation of vector immune responses and in the regulation of vector competence, has been also studied in tsetse flies (Diptera: Glossinidae), sand flies (Diptera: Psychodidae) and triatoma bugs (Hemiptera: Triatominae), major vectors of African trypanosomiasis, leishmaniases and American trypanosomiasis respectively (Cirimotich et al., 2011b; Weiss and Aksoy, 2011; Wang J. et al., 2013; Telleria et al., 2018). Indeed, the comprehension of the intimate relationship between these insect vectors and resident microbiota may be pivotal for the development of new tools to counteract the transmission and spread of diseases, such as paratransgenesis (Weiss and Aksoy, 2011).

Due to their reproduction and feeding habits, the life of the immature stages of tsetse flies is characterized by a relative sterility (Wang J. et al., 2013), since the larva develops inside the female uterus where it is fed by the maternal accessory gland (i.e., the milk gland) that produces a highly nutrient secretion. Once deposited, the larva immediately pupate, and adults, that are exclusively hematophagous, feed on sterile blood of different mammalian hosts including humans (Wang J. et al., 2013). The microbiota associated with tsetse flies is thus relatively simple compared to other insects and essentially constituted by three bacterial symbionts and a salivary-gland associated Hytrosavirus (**Table 2**). Moreover, the environment may marginally contribute to the establishment of gut microbiota through the ingestion of bacteria present on host skin during blood meals (Geiger et al., 2014). The obligate association with *Wigglesworthia* during larval stage is responsible for proper development of an adult functional immune system, in particular of the pathways mediating cellular responses. *Wigglesworthia*-free larvae develop into adults unable to counteract the septicemia induced by normally non-pathogenic *E. coli* due to a decrease in sessile and circulating immune cells and failure in melanization reaction (Weiss et al., 2011). Although a similar effect was observed in laboratory colonies of flies depleted of *Sodalis* and *Wolbachia*, field-flies that do not harbor these symbionts possess a functional immune system (Weiss et al., 2012). Interestingly, *Wigglesworthia* is able to trigger tsetse flies antibacterial immune responses against trypanosome by inducing the production of a peptidoglycan recognition protein (i.e., PGRP-LB) and, by the recruitment of the IMD pathway, of anti-trypanosome effector molecules (Wang et al., 2009). In addition, the competence of tsetse flies for trypanosomes has been linked to the capacity of *Wigglesworthia* to produce folate (vitamin B9) *de novo*, which thus seems to be a key metabolite for these parasites (Rio et al., 2019).

The knowledge about the interplay between microbiota and immune system in sand flies and triatome bugs is quite fragmented, although a role of intestinal microbiota in the maintenance of gut homeostasis and immune activation in these vectors has been reported (Araújo et al., 2006; Ursic-Bedoya and Lowenberger, 2007; Waniek et al., 2011; Castro et al., 2012; Diaz-Albiter et al., 2012; Vieira et al., 2015; Telleria et al., 2018).

Sand flies larvae acquire their gut microbiota from food, which is represented by soil organic matter and sand flies adults from carbohydrate-rich fluids (plant sap and aphid secretions). In addition, adult females feed on blood, principally from birds and mammals. Gut microbiota presence and composition has an impact on insect reproductive fitness (Telleria et al., 2018) and allows the activation of important immune pathways for the production of humoral effectors that allow the coexistence of insect and resident microbiota (Telleria et al., 2018). Moreover, studies on the sand fly *Lutzomyia longipalpis* have highlighted a key role of gut microbiota on vector competence for *Leishmania* (Sant'Anna et al., 2014; Kelly et al., 2017) and even that *Leishmania* protects *L. longipalpis* against bacterial infection (Diaz-Albiter et al., 2012; Sant'Anna et al., 2014). Intriguingly, recent work has demonstrated a remarkable role of *Leishmania*-infected sand fly microbiota. When regurgitated on the skin of the secondary host during bite, sand fly microbes are able to initiate an immune reaction at the bite site that positively impacts on the progression of infection (Dey et al., 2018).

The triatomine gut is a complex environment where microorganisms and parasites coexist and challenge each other in different ways (Díaz et al., 2016; de Fuentes-Vicente et al., 2018). This association has been well studied in *R. prolixus*, one of the vectors of the protozoa *Trypanosoma cruzi* (Azambuja et al., 2017). *R. prolixus* acquires enteric microbiota

TABLE 2 | Tsetse fly symbionts, main features of the association, and symbiont role in the modulation of host biology.

Microorganism	Features of the acquisition and association with the flies	Present in all flies?	Role in host biology	Relevant bibliography about its role in the host
Wigglesworthia (Fam. Enterobacteriaceae)	• Maternally transmitted bacterial endosymbiont • Localized in the cytosol of bacteriocytes adjacent to anterior midgut and also contained in milk gland secretions • Obligate mutualist	Yes	• Nutritional function (these symbionts are equipped with the biosynthetic pathways to produce vitamins essential for the host requirements) • Immunological function	Rio et al. (2019) Wang J. et al. (2013) Weiss et al. (2011, 2013)
Sodalis (Fam. Enterobacteriaceae)	• Maternally transmitted bacterial symbiont • Located both intra- and extra-cellularly different tissues including midgut, fat body, milk gland and salivary glands • Commensal symbiont	No	• Unknown	Toh et al. (2006) Wang J. et al. (2013) Weiss et al. (2012, 2013)
Wolbachia (Fam. Rickettsiaceae)	• Bacterial endosymbiont transovarically transmitted via germ line cells • Exclusively localized in germ line tissues • Parasitic symbiont	No	• Manipulation of host reproduction by different mechanisms (e.g., cytoplasmic incompatibility)	Wang J. et al. (2013) Weiss et al. (2012, 2013) Doudoumis et al. (2013)
SGHV[1] (Fam. Hytrosaviridae)	• Horizontally transmitted during feeding • Located in salivary glands	No	• Replication causes the swelling of salivary glands (hypertrophy) • In the presence of the virus, tsetse flies may be symptomatic or asymptomatic	Wang J. et al. (2013) Kariithi et al. (2018)

[1]*Acronym for salivary gland hypertrophy virus.*

through horizontal transmission (i.e., by the consumption of feces of conspecifics or cannibalism, which allow the establishment of intestinal symbionts, such as *R. rhodnii* that provides vitamins to the bug) and through the skin of the animals during blood feeding, while infected blood is the source of *T. cruzi* (Azambuja et al., 2017). Although strain dependent, the capacity of the parasite to alter immune responses of the bug has been reported in different studies (Araújo et al., 2006; Ursic-Bedoya and Lowenberger, 2007; Waniek et al., 2011; Castro et al., 2012; Vieira et al., 2015). In particular, *T. cruzi* and *Trypanosoma rangeli* are able to trigger the production of immune effectors by the host (i.e., phenoloxidase and AMPs) that specifically reduce gut flora and, on the other hand, increase parasitemia (Araújo et al., 2006; Ursic-Bedoya and Lowenberger, 2007; Waniek et al., 2011; Castro et al., 2012; Vieira et al., 2015). In addition, the induction of a significant decrease of *R. rhodnii* load in the gut of *R. prolixus* infected with *T. rangeli* (but not with *T. cruzi*) has been observed (Eichler and Schaub, 2002).

CONCLUSION

The manipulation of the mosquito microbiota is an emerging strategy for the control of many deadly diseases, including malaria, dengue, chikungunya, and Zika. These strategies require a deep knowledge of the mosquito immunity and of the interactions occurring between the insect immune system and the microbiota. Three main applicative approaches are under study: i) development of microbial strains that express anti-parasitic or anti-viral effector molecules; ii) development of microbial strains expressing immune-priming molecules; iii) introduction of unmodified strains with immune-priming effects in mosquitoes and/or resource competitors that ultimately limit infections in the insects. The first two approaches require the release of genetically modified organisms in the field and, therefore, further studies are needed to understand the spread and the effect of these organisms in target and non-target species. The development of strategies for a safe removal of the organisms are necessary, in the case that adverse effects will be detected during releases in the field, as already suggested for transgenic mosquitoes (Zapletal et al., 2021). The development of these multiple tools in mosquito will foster the studies in other less-studied arthropod species, which anyhow can transmit a high number of human pathogens.

AUTHOR CONTRIBUTIONS

PG, SC, GB, and IA reviewed the mosquito immunity, the interaction with the mosquito gut microbiota, and the interactions of microbiota with other insect species. IV-B, FC, and SE reviewed the paratransgenesis and the applied application of the studies on microbiota interaction. All authors have made a

direct and intellectual contribution to the work and approved the manuscript for publication.

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SUPPLEMENTARY MATERIAL

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Trained Immunity in *Anopheles gambiae*: Antibacterial Immunity Is Enhanced by Priming via Sugar Meal Supplemented With a Single Gut Symbiotic Bacterial Strain

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Mosquitoes have evolved an effective innate immune system. The mosquito gut accommodates various microbes, which play a crucial role in shaping the mosquito immune system during evolution. The resident bacteria in the gut microbiota play an essential role in priming basal immunity. In this study, we show that antibacterial immunity in *Anopheles gambiae* can be enhanced by priming via a sugar meal supplemented with bacteria. *Serratia fonticola* S1 and *Enterobacter* sp. Ag1 are gut bacteria in mosquitoes. The intrathoracic injection of the two bacteria can result in an acute hemocoelic infection in the naïve mosquitoes with mortality of ∼40% at 24 h post-infection. However, the *Enterobacter* or *Serratia* primed mosquitoes showed a better 24 h survival upon the bacterial challenge. The priming confers the protection with a certain degree of specificity, the *Enterobacter* primed mosquitoes had a better survival upon the *Enterobacter* but not *Serratia* challenge, and the *Serratia* primed mosquitoes had a better survival upon the *Serratia* but not *Enterobacter* challenge. To understand the priming-mediated immune enhancement, the transcriptomes were characterized in the mosquitoes of priming as well as priming plus challenges. The RNA-seq was conducted to profile 10 transcriptomes including three samples of priming conditions (native microbiota, *Serratia* priming, and *Enterobacter* priming), six samples of priming plus challenges with the two bacteria, and one sample of injury control. The three priming regimes resulted in distinctive transcriptomic profiles with about 60% of genes affected by both bacteria. Upon challenges, different primed mosquitoes displayed different transcriptomic patterns in response to different bacteria. When a primed cohort was challenged with a heterogenous bacterium, more responsive genes were observed than when challenged with a homogenous bacterium. As expected, many canonical immune genes were responsive to the priming and challenge, but

much more non-immune genes with various functions were also responsive in the contexts, which implies that the prior priming triggers a delicately coordinated systemic regulation that results in an enhanced immunity against the subsequent challenge. Besides the participation of typical immune pathways, the transcriptome data suggest the involvement of lysosome and metabolism in the context. Overall, this study demonstrated a trained immunity via priming with bacteria in diet.

Keywords: mosquito, priming, trained immunity, RNA-seq, *Anopheles gambiae*, transcriptomic response

INTRODUCTION

Invertebrate organisms have evolved an effective innate immune system throughout evolution. The immune mechanisms effectively counteract various infectious agents such as viruses, bacteria, fungi, and parasites. The innate immune machinery is genetically encoded, consisting of pattern recognition receptors, immune pathways, and immune effectors. Upon recognition of pattern molecules from each type of invaders, respective immune pathways will be activated to produce relevant effectors to the pathogens (Baxter et al., 2017; Shaw et al., 2018). Although the innate immunity in invertebrates lacks the immune memory and specificity in a form defined as in the adaptive immunity in vertebrates, increasing evidence indicates that in invertebrates the immune efficacy can be enhanced by immune priming. In such cases, prior exposure to a pathogen can trigger better protection against a repeated challenge, i.e., "priming" followed by "challenge." Such phenomena have been defined as innate immune memory or trained immunity (Milutinovic and Kurtz, 2016; Netea and van der Meer, 2017; Gourbal et al., 2018; Melillo et al., 2018; Netea et al., 2020; Sharrock and Sun, 2020). In mosquitoes, priming effects have been demonstrated in several contexts. The presence of midgut microbiota is essential to prime and maintain a basal immunity against malaria parasite *Plasmodium falciparum* (Dong et al., 2009). There is a crosslinked physical barrier between gut microbes and gut epithelial cells, which limits the microbial immune elicitors to be sensed by epithelial cells and allows the immune permeability to the gut microbiota (Kumar et al., 2010). The *P. falciparum* invasions breach this barrier and trigger the immune response against bacteria, in turn, this heightened immunity indirectly enhances the resistance against *P. falciparum* (Rodrigues et al., 2010). *Asaia* is a gut bacterium present predominantly in the gut microbiota. The introduction of *Asaia* into midgut via diet modulated the transcription of certain immune genes in *Anopheles stephensi* and *Anopheles gambiae*, and this microbial manipulation elevated anti-*Plasmodium* immunity in *Anopheles stephensi* but not in *An. gambiae* (Cappelli et al., 2019). In addition, one of the two strains of *Serratia marcescens,* which were isolated from wild caught specimens of *Anopheles sinensis,* was able to inhibit the development of rodent malarial parasite *Plasmodium berghei* in *An. stephensi* (Bai et al., 2019). The colonization of the *Serratia* strain in the gut primed the antimalaria effect via modulating the immune genes including antimalaria effectors (Bai et al., 2019). The interactions with bacteria in the microbiota play a critical role in shaping mosquito immune system throughout the evolution. Priming effects have also been investigated in the immunity against bacteria. Brown et al. (2019) have shown that the treatment of *An. gambiae* larvae by injecting *Escherichia coli, Enterobacter* sp., or *Staphylococcus aureus* increased the number of circulating hemocytes and enhanced phagocytosis upon a challenge with *E. coli* in the eclosed adults. However, the prior infection with *E. coli* in larvae did not affect the survival upon the *E. coli* challenge in adults (Brown et al., 2019). In *An. gambiae* adults, a prior hemocoelic infection by injecting *E. coli* primed a stronger immunity against a second *E. coli* infection. The primed mosquitoes had more circulating hemocytes and elevated expression of *NOS* and *PPO6* genes upon the secondary infection (Powers et al., 2020). To further understand the innate immune system in mosquitoes, we examined the priming effect of gut bacteria on immune response in this study. *An. gambiae* mosquitoes were primed in sugar diet with native microbiota, and sugar meal supplemented with bacteria *Enterobacter* or *Serratia*, respectively. Post priming, we examined the effect on survival upon the challenge with the homogeneous or heterogeneous bacteria, respectively. Further, we analyzed the systemic transcriptomic response to the priming as well as the challenges using a homogeneous or heterogeneous bacterium in the primed mosquitoes.

MATERIALS AND METHODS

Mosquitoes

Anopheles gambiae Giles sensu stricto G3 strain was obtained from MR4 and was reared at 28°C with 80% humidity under a 10/14 h day–night light cycle. Larvae were fed on a diet of Brewer's yeast and cat food powder (1:2 ratio). Adults were maintained on 10% sucrose daily, and 5-day old females were fed on NIH Swiss outbred mice for blood meal to induce egg production. Eggs were collected on day 3 post blood feeding and placed in water pans.

Bacterial Feeding

Newly emerged adult mosquitoes excrete meconium (Moll et al., 2001) and initiate a new gut microbiota. Therefore, it is an appropriate time to establish a microbial community by introducing bacteria in the sugar meal. *Enterobacter* sp. Ag1 was originally isolated from the midgut of the G3 strain (Jiang et al., 2012). *Serratia fonticola* S1 was isolated from midgut of the wild-caught specimens of *Aedes albopictus* in Florida in July 2015. The *Enterobacter* strain is persistently present with G3 strain.

The *Serratia* strain is not a part of the gut community in the G3 mosquitoes. We used a PCR assay to examine the presence of *Serratia* and *Enterobacter* in the midgut DNA from the G3 mosquitoes. The primer sets targeting two *Serratia* genes, *DNA gyrase* and *glucose phosphatase*, and one *Enterobacter* gene, *DNA gyrase,* were used for PCR. Primer sequences were provided in **Supplementary Table 1**. The bacteria were tagged with GFP expressing plasmid using a method we described previously (Pei et al., 2015). The 10% sucrose sugar meal was supplemented with respective bacteria at OD_{600} of 1.0. The bacterial sugar meal was given to mosquitoes post eclosion for 3 days, and midgut at day 1 and day 3 post feeding was dissected to examine the presence of GFP tagged bacteria under a fluorescent microscope as described previously (Pei et al., 2015). Both *Enterobacter* and *Serratia* were observed in the gut. Three priming regimes were used, group I was given regular sugar meal without bacterial supplement, defined as native priming; group II was given sugar meal supplemented with *Enterobacter,* defined as *Enterobacter* priming; and group III was given sugar meal supplemented with *Serrati,* defined as *Serratia* priming.

Bacterial Injection

Bacteria *Enterobacter* and *Serratia* grow overnight in Luria Bertani broth containing ampicillin (100 μg/ml) at 28°C. Bacterial culture was normalized to OD_{600nm} = 1 and diluted with sterile H_2O to yielded approximately 1000 colony forming unit (CFU)/μl. On day 4 post the respective priming regimes described above, individual mosquitoes were injected with ∼100 nl of the bacterial solution, and approximately 100 bacterial cells were received per mosquito. Sterile water was injected as injury control. Survival at 24 h post infection was used to assess the antibacterial immunity. On 24 h post injection, mosquitoes were surface cleaned with dipping into two tubes of 70% ethanol sequentially, 15 s each. After cleaning, the thorax of an injected mosquito was homogenized in 50 μl sterile water, and 30 μl homogenates were spread to an LB plate with Ampicillin and cultured at 28°C overnight. The colonies on the plate were examined under UV light to visualize GFP tagged bacteria. In bacteria-injected mosquitoes, GFP-tagged bacteria were recovered, while in sterile water injected mosquitoes no GFP tagged bacteria were detected. The data were generated from three experimental replicates, each replicate had ∼40 females for injection. The survival rates between the cohorts were compared using Chi-square test.

Transcriptome Analysis

RNA-sequencing (RNA-seq) was used to compare transcriptomes. The samples of a total of 10 conditions were collected for RNA-seq, which included three priming regimes, each priming regime had two challenges with homogeneous and heterogeneous bacteria, and sterile water injection in mosquitoes with native community was used as injury control. Each condition had three replicates, therefore 30 samples were collected for RNA-seq. The scheme of bacterial priming, challenge, and RNA sampling was presented in **Figure 1**. The SRA biosample ID was listed in **Supplementary Table 6**. For each sample, RNA was isolated from 20 mosquitoes. The whole mosquitoes were used for RNA extraction using Trizol reagent (Invitrogen), and TURBO DNase I (Invitrogen) treatment was followed to remove genomic DNA contamination. The RNA samples were shipped to Genewiz for further processing to make cDNA libraries for sequencing using Illumina Hiseq, 2 × 150 bp paired-end chemistry. At least 25M clean reads were generated from each RNA sample, which provided a sequencing depth sufficient for transcriptome analysis. The reads were mapped against *An. gambiae* reference of transcripts (NCBI), which was implemented by using Array Star v.16 (DNAstar). Read counts were normalized using the median of ratios method (Li et al., 2020) using DESeq2 software (Love et al., 2014). In determining normalized read counts, this method accounts for sequencing depth and RNA composition by calculating normalization factors for each sample in comparison to a pseudo-reference sample. After determining normalized read counts, an independent filter was utilized which removed transcripts with normalized counts less than 5. This resulted in a dataset of 10,689 transcripts (**Supplementary Table 2**). The clustering of all samples revealed that replicate 2 of *Enterobacter* priming-*Serratia* infection was not consistent with the other two replicates, likely due to a quality issue, therefore, this replicate was removed from the analysis. Differentially expressed genes were identified using a negative binomial generalized linear model (GLM) available through DESeq2 (Love et al., 2014). Likelihood ratio tests were conducted to identify transcripts that exhibited differential expression between all groups. Pairwise differential expression comparisons were made, and statistical significance was determined by computing *q*-values that preserve the False Discovery Rate (FDR) (Storey, 2003; Storey and Tibshirani, 2003; Li et al., 2020). For example, concluding that a transcript was differentially expressed between two groups with a *q*-value of 0.05 would imply that there was a 5% chance (expected) that this conclusion was a false positive. To determine a lower dimensional representation of the transcriptomic data, principal components analysis (PCA) was conducted using regularized log-transformed (rlog) data. PCA seeks to find a small set of "principal components" that capture a large proportion of the variance in the original data (Johnson, 2019). The rlog data was determined using DESeq2, while the "prcomp" function in R (R Core Team, 2019) was utilized to determine the PCA. The proportion of the variance captured by each of the principal components was determined. To validate expression patterns revealed by RNA-seq, a selected set of genes was measured using quantitative RT- PCR. For each sample, RNA was extracted from 15 females using Trizol reagent. Genomic DNA contamination was removed by DNase I treatment as described above. cDNA synthesis was carried out using NEB ProtoScript II Reverse Transcriptase (NEB). The cDNA was used as a template for RT-PCR to determine the transcript abundance of target genes. Three cohorts of primed mosquitoes were challenged with homogeneous and heterogenous bacteria, respectively, and transcript abundance of five genes was examined by the qRT-PCR and compared with the expression level from RNA-seq. The primers used were present in **Supplementary Table 1**. No reverse transcriptase (NRT) and no template control (NTC) served as negative controls.

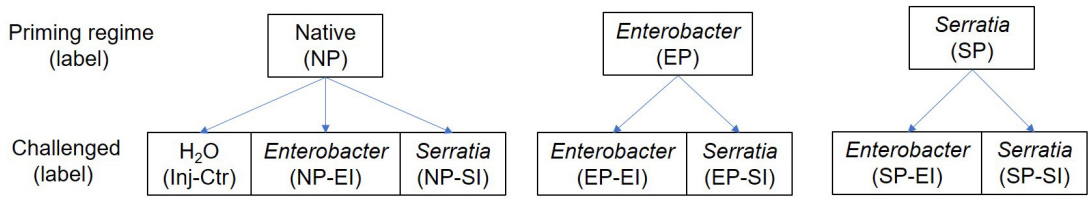

FIGURE 1 | Scheme of priming, challenge, and sampling for RNA-seq. Newly emerged *An. gambiae* G3 mosquitoes were set into three cohorts for priming treatment via sugar meal: native microbiota, supplemented with *Enterobacter* and *Serratia*. The primed cohorts were then subject to *Enterobacter* and *Serratia* challenge, respectively. The sterile H_2O injection in native primed cohort was used as injury control. RNA samples were extracted from each cohort for RNA-seq, a total of 30 samples were collected from 10 conditions with three replicates for each condition.

RESULTS

Gut Commensal Bacteria Caused an Acute and Virulent Infection in Hemocoel

The bacterium *Enterobacter* sp. Ag1 was isolated from the midgut of *An. gambiae* in the lab, and *Serratia fonticola* S1 was isolated from the midgut of wild-caught *Aedes albopictus*. Both bacteria are Gram-negative bacteria in order Enterobacterales. *Enterobacter* belongs to family Enterobacteriaceae, and *Serratia* belongs to family Yersiniaceae. The *Enterobacter* is persistently associated with G3 strain in our insectary (Jiang et al., 2012; Pei et al., 2015). We examined the presence of *Serratia* in the gut of the G3 mosquitoes using a PCR assay targeting two *Serratia* genes, *DNA gyrase subunit A*, and *glucose-1-phosphatase*. Metagenomic DNA from the whole body of larva and pupa, the midgut of sugar-fed mosquitoes (day 4 post eclosion) and day 4 post blood-feeding were subject to the PCR assay. Mosquito gene *rpS5* and *Enterobacter* gene *DNA gyrase subunit A* were used as a positive control. The mosquito *rpS5* amplicon was present in all four samples, and the *Enterobacter* gene was not detected in larva and pupa but was present in the adult midgut before and after blood feeding. However, none of the two *Serratia* genes was amplified in the four samples (**Supplementary Figure 1**). We concluded that the *Serratia* strain was not associated with the G3 mosquitoes in our insectary. Therefore, in this study, the *Enterobacter* strain represents a bacterium that has been associated with the G3 colony, and the *Serratia* strain represents a bacterium that has limited or no association with the G3 colony. Next, we tested the infection outcome of these two bacterial strains in the G3 mosquitoes. Injection of the bacteria, ∼100 CFU per mosquito, into the hemocoel caused an acute hemocoelic infection, almost all infected mosquitoes died in 3 days post injection, therefore survival at 24 h was the most informative data point to present infection outcomes. As shown in **Figure 2**, *Enterobacter* or *Serratia* injection resulted in an infection with a survival rate of 63.3% and 58.0% at 24 h post injection, respectively, while the injury control (injected with sterile water) had a survival rate of 84.2%. This shows that the two gut symbiotic bacteria can cause an acute virulent hemocoelic infection. *E. coli* as a representative of Gram-negative bacteria in family Enterobacteriaceae has been widely used in the studies of mosquito immunity. *An. gambiae* can tolerate *E. coli* in a large quantity in the hemocoel and survive up to 30 days until all die (Powers et al., 2020), which represents a chronic infection course. Therefore, the infection course and outcome are quite different between *E. coli* and the two gut bacteria, *Enterobacter* and *Serratia*. Therefore, we further studied the mosquito response to the acute and virulent infection model caused by the two bacteria.

Bacterial Feeding Primed Immunity Against Homogeneous Bacterial Infection

Mosquito midgut harbors a microbiota with various microbes. Previously, we have shown that feeding newly emerged mosquitoes with a sugar diet supplemented with a single bacterium can make the bacteria dominant in the gut microbial community (Pei et al., 2015). We tested the immune priming effect of single bacterial feeding on immunity against bacterial challenges. The scheme of priming and challenge was present in **Figure 1**. Newly emerged mosquitoes were fed with sugar meal supplemented with *Serratia* or *Enterobacter* (at a concentration of OD_{600} = 1.0) for 3 days. The mosquito cohort with the sugar meal without bacterial supplement was defined as priming with native microbiota. On day 4, the primed mosquitoes were challenged with *Enterobacter* or *Serratia*. Compared to the cohorts with native priming, the *Enterobacter* primed mosquitoes had a better survival upon the *Enterobacter* challenge (90.7 vs. 63.3%, **Figure 3A**), and the *Serratia* primed mosquitoes had a better survival upon the *Serratia* challenge (77.5 vs. 59.2%, **Figure 3B**). Then, we examined the specificity of priming effect against challenges using heterogeneous bacteria. The *Enterobacter* primed mosquitoes had a survival of 90.7% upon the *Enterobacter* challenge and 63.1% upon the *Serratia* challenge. Similarly, the *Serratia* primed mosquitoes exhibited a survival of 78.3% upon the *Serratia* challenges and 41.9% upon the *Enterobacter* challenge (**Figure 3C**). Overall, the bacterial priming via diet enhances antibacterial immunity, the trained immunity demonstrates a stronger protection against challenges with the homogeneous than the heterogeneous bacteria, suggesting that priming is specific at a certain level.

Transcriptomic Response to Bacterial Priming and Challenge

To identify systemic transcriptomic response to the priming and the bacterial challenge following priming, we conducted RNA-seq to compare transcriptomes in these 10 different conditions (**Figure 1**): priming without challenge (native microbiota,

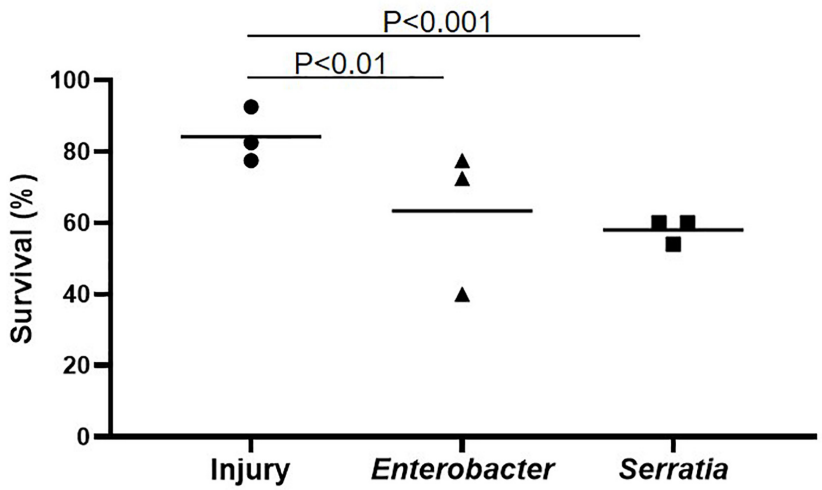

FIGURE 2 | Bacterial infection resulted in mortality. Naive mosquitoes were infected with *Serratia or Enterobacter* by intrathoracical injection. Sterile water injection was used as injury control. The average survival was generated from three replicates. Each replicate had 40 females. The survival was significantly reduced by *Serratia* and *Enterobacter* infection in comparison with injury, tested using Chi square.

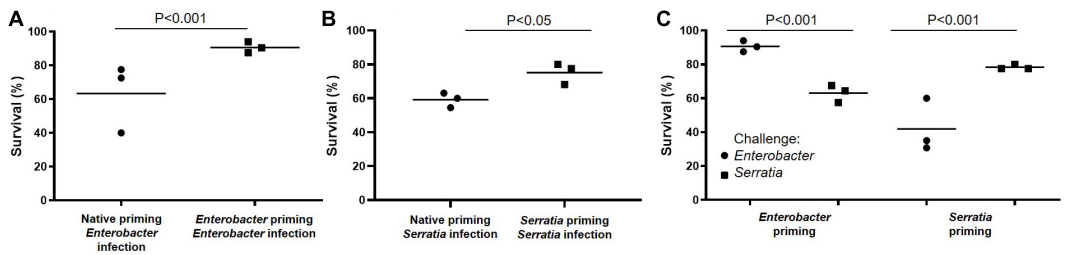

FIGURE 3 | Priming-enhanced antibacterial immunity was specific to the homogeneous bacteria. **(A)** Compared to native microbiota, the *Enterobacter* priming increased mosquito survival upon the *Enterobacter* challenge. **(B)** Compared to native microbiota, the *Serratia* priming increased survival upon the *Serratia* challenge. **(C)** *Enterobacter* priming increased the survival upon the *Enterobacter* challenge but not the *Serratia* challenge. The *Serratia* priming increased the survival upon the *Serratia* challenge but not the *Enterobacter* challenge. The survival data of each challenge were generated from three replicates, each had 40 mosquitoes. The survival difference was compared using Chi square test.

Enterobacter, and *Serratia* priming), priming plus challenge (native, *Enterobacter* and *Serratia* priming plus *Enterobacter* challenge; and native, *Enterobacter* and *Serratia* priming plus *Serratia* challenge) and injury control on mosquitoes with native microbiota.

Overview of Transcriptomic Responses to Priming and Bacterial Challenge

A principal component analysis was conducted to observe the transcriptomic response to the priming regimes and the impact of priming on transcriptomic response to the bacterial challenge (**Figure 4**). The transcriptome replicates from the primed cohorts without challenge were clustered closely with a distance from the other conditions (marked by a red circle). Intra-replicate variation of the cohorts with native microbiota appears to be higher than the other two primed cohorts, which may be related to the diverse microbial structure in the native microbiota. The injury controls (marked by a green circle) were separated from the cohorts with the bacterial challenge. The priming effect on the *Serratia* challenge was demonstrated by a clear separation of the three clusters (marked by three purple circles), suggesting the three priming regimes had distinctive effects on the *Serratia* challenge. The replicates of the *Enterobacter* challenge with respective priming regimes (marked by a single light blue circle) were clustered nearby with an interspersed pattern, suggesting that these priming regimes may have overlapping effects on the *Enterobacter* challenge. The transcriptomic patterns were corroborated by qRT-PCR data with five genes in six conditions. The folder changes in RNA-seq data and qRT-PCR were compared, the expression patterns of *DEF1, LYSC1,* and *CLIPA14* were consistent in all six conditions between the two types of data; and *PGRPLB* and *CLIPB12* were consistent in four of six conditions, respectively (**Supplementary Figure 2**).

Transcriptomic Responses to the Priming Without Challenge

The mosquitoes that were fed with regular sugar meals had native microbiota, which was defined as a native primed cohort.

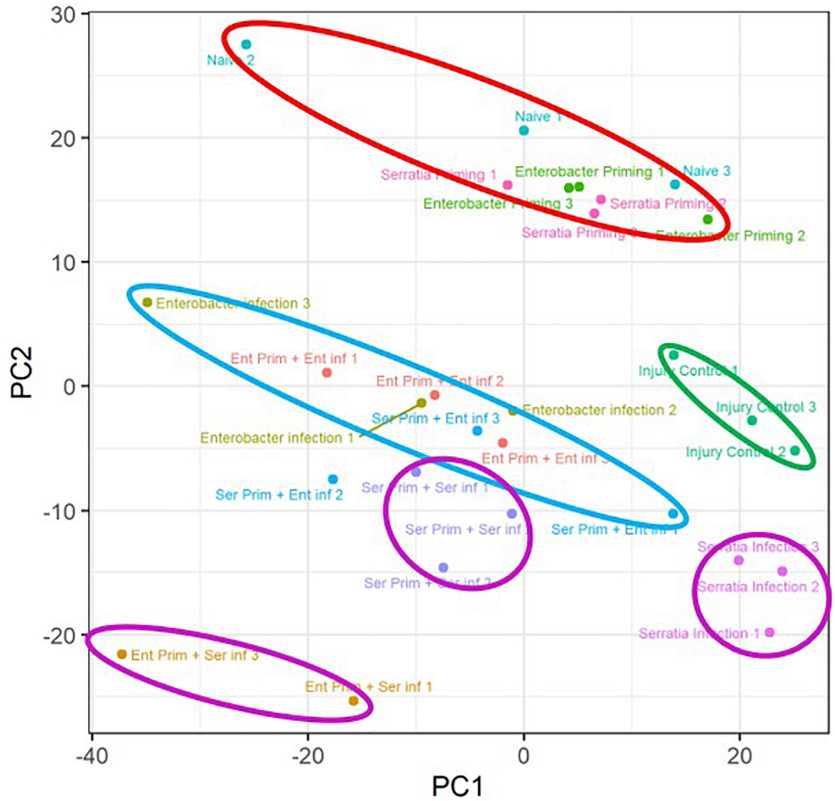

FIGURE 4 | Overview of the priming effects on the transcriptomic response to respective bacterial challenges. Transcriptome replicates from primed cohorts without challenge were marked with a circle in red. Replicates of injury controls were marked with a circle in green. Replicates of *Enterobacter* challenged cohorts with respective priming were marked with a circle in blue. Replicates of *Serratia* challenged cohorts with respective priming were marked with a circle in purple. Naive, cohorts with native microbiota; Enterobacter infection, *Enterobacter* challenged cohort with native microbiota; Serratia infection, *Serratia* challenged with native microbiota.

The mosquitoes that were fed with sugar meals supplemented with *Enterobacter* or *Serratia* were defined as *Enterobacter* or *Serratia* primed cohort. Compared to the native priming, the *Enterobacter* and *Serratia* priming altered expression of 1094 and 1112 genes, respectively, totaling 1562 genes, among them, 644 genes were affected by both priming regimes (**Figures 5A,B**). There were 175 immune genes that were affected by either or both priming regimes, accounting for 11.2% of the affected genes. **Figure 5C** presents a heatmap illustrating 12 upregulated and 14 downregulated immune genes, which were affected by both *Enterobacter* and *Serratia* priming in the same direction. The upregulated genes include the ones that encode three inhibitor of apoptosis proteins (IAPs), two leucine-rich immune proteins, and TEP2, and the downregulated genes include the ones that encode PGRPLD, PPO4, three CLIP serine proteases, two Niemann-Pick proteins, and six FREPs. In the non-immune categories, the upregulated group includes genes encoding seven ATP-binding cassette transporters, three cuticular proteins, 10 cytochrome P450, and 98 unspecified genes, while the downregulated group includes the genes encoding four cuticular proteins, 10 cytochrome P450 proteins, and 135 unspecified genes. The detailed comparison of gene expression in different conditions was provided in **Supplementary Table 3** with gene ID and available gene annotation. Overall, the individual priming affected a set of genes that were uniquely responsive to the priming bacteria as well as a set of genes that were responsive to all priming regimes. The affected genes were dispersed in broad categories with diverse functions, many genes were unspecified, no function information was available yet.

Infection Responsive Genes in the Cohorts With Native Microbiota

To identify infection responsive genes, we compared transcriptomes between the infected mosquitoes and injury control. The mosquitoes with their native microbiota were used for this purpose. The mosquitoes were challenged with *Enterobacter* or *Serratia*, or sterile water. The *Enterobacter* infection altered the expression of 3303 genes while the *Serratia* infection altered the expression of 960 genes. A set of 320 genes were affected by the two infections commonly, 226 were upregulated and 54 were downregulated by both infections (**Figure 6** and **Supplementary Table 3**). In the immune category, 55 genes were induced by both infections, including the antimicrobial genes, such as *Def1, CecA, GNBPs,* and *lysozyme*, and only two immune genes, *PPO9* and *CLIPB12*, were downregulated, indicating that the infections trigger a typical

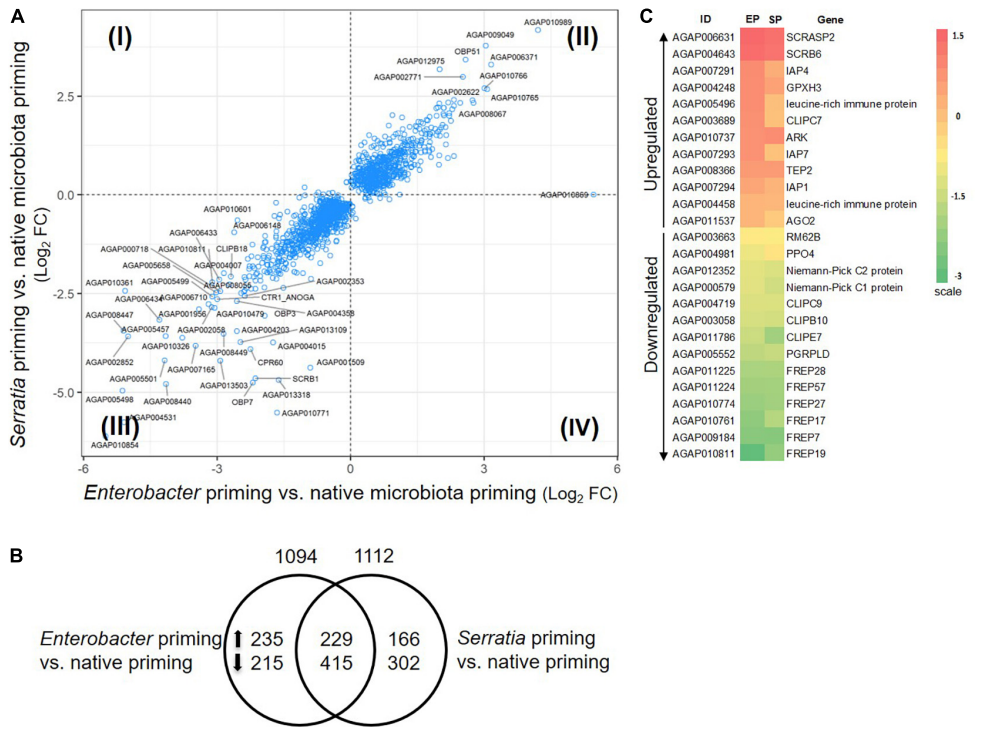

FIGURE 5 | Priming effect of single bacterial diet on the transcriptome without bacterial challenge. **(A)** Transcriptomes in the cohort with *Enterobacter* or *Serratia* priming showed a similar response pattern, majority of genes were regulated in the same direction, plotted in quadrant II and III. Plotted are genes with *q*-value less than 0.05 in either comparison to the cohort with native microbiota. Labeled genes have $\log_2$ fold change (FC) in either comparison greater than 2.5 or less than –2.5. **(B)** Distribution of the genes that were altered by individual priming. Total number of altered genes is placed on top of the circle, the number of up- or down-regulated genes were specified inside the circle. **(C)** The heatmap illustrates the immune genes that were affected by the *Enterobacter* (EP) and *Serratia* (SP) priming compared to the native priming. The color scale represents the $\log_2$ fold change between the EP or SP and native community.

FIGURE 6 | Infection responsive genes. Infection responsive genes were identified by comparison between respective infection vs. injury control with *q*-value < 0.05 as cutoff. Total number of transcriptionally altered genes was specified on top of the circle, number of up- or down-regulated genes were specified inside the circle.

immune response to the bacteria. The presence of different sets of responsive genes between the two infections suggests that different bacteria can induce different responses, these genes are involved in various processes, which may affect infection outcomes in different ways.

Transcriptomic Responses to the Priming Plus Challenge

To identify the effects of prior priming on a particular infection, we compared transcriptomes between the cohort with native microbiota and the cohorts with *Enterobacter* or *Serratia* priming followed by the respective bacterial challenge, as depicted in **Figure 1**. In the case of *Enterobacter* challenges, the cohort with native microbiota and the cohort with *Enterobacter* priming had similar transcriptomic responses, only 133 genes were expressed differentially between, 67 genes were upregulated, and 66 genes were downregulated (**Figures 7A,B**). On the other hand, the *Serratia* primed mosquitoes resulted in a quite different pattern of responses to the *Enterobacter* challenge, 2290 genes were altered, 1152 genes were expressed with a higher level, and 1138 genes were expressed with a lower level (**Figure 7B**). In the case of *Serratia* infections, the

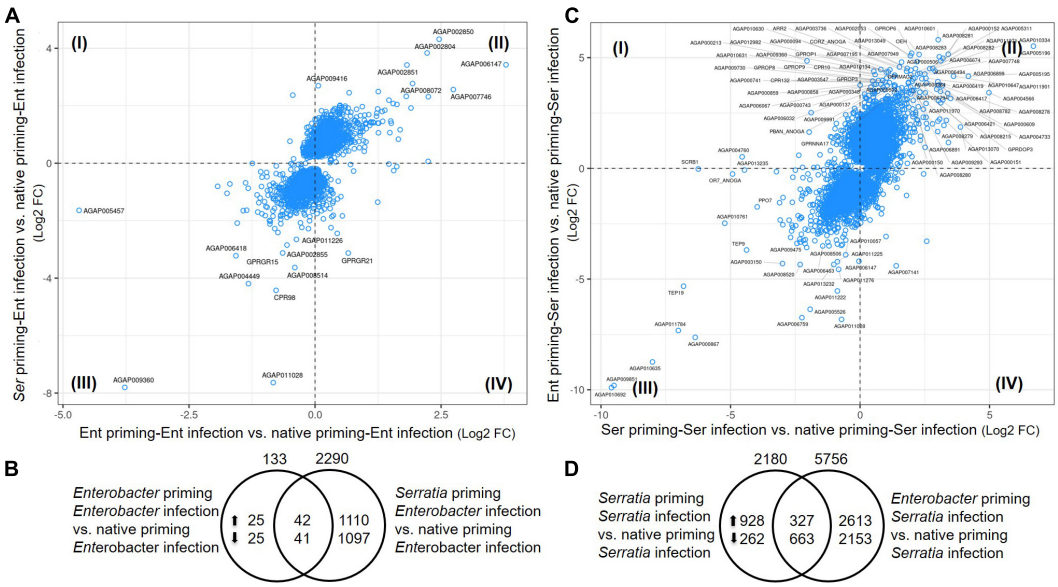

FIGURE 7 | Priming effects on transcriptional response to bacterial challenge. **(A,C)** Comparison between the cohort with *Enterobacter* or *Serratia* priming vs. the cohort with native microbiota. Plotted are genes with *q*-value < 0.05 in either comparison to cohort with native microbiota. Labeled genes have $\log_2$ fold change in either comparison >2.5 or <–2.5 in **(A)** and >3.5 or <–3.5 in **(C)**. The genes in quadrant I and IV were the genes differentially expressed upon different priming regimes. **(B,D)** Distribution of the genes that were altered by priming. Total number of altered genes is placed on top of the circle, number of up- or down-regulated genes were specified inside the circle. The genes in the central area were shared by both cohorts with respective priming.

Serratia primed cohort had 2180 genes altered, 1255 genes were upregulated, and 925 genes were downregulated. The *Enterobacter* primed cohort responded to the *Serratia* challenge with 5756 genes altered, 2940 genes were upregulated, and 2816 genes were downregulated (**Figures 7C,D**). The impact of prior heterogeneous priming on the responsive genes in each challenge was illustrated in **Figure 8**. In the group of infection-upregulated genes with >2-fold change, the prior *Serratia* priming attenuated the induction of four genes upon the *Enterobacter* challenge, and the prior *Enterobacter* priming reduced the induction level of 35 genes upon the *Serratia* challenge. Interestingly, the four genes were affected by the heterogeneous priming in both challenges, including *CLIPA14* and a leucine-rich immune gene (**Figure 8**). In the group of infection-downregulated genes with <2-fold change, the *Enterobacter* priming had mixed effects on 71 genes, either increasing or further decreasing the expression level to the *Serratia* infection. The *Serratia* priming affected 377 genes, the expression level of 373 of these genes was increased. Interestingly, only two genes were affected in both scenarios, the expression level of CLIPB12 was further downregulated in both heterogeneously primed cohorts, and the downregulation of AGAP010340, which encodes a Zink finger protein C2H2 type transcription factor, was reversed in both heterogeneously primed cohorts. In the *Enterobacter* repressed gene group, the genes involved in chromosome structure, chromosome transmission, DNA repair, DNA replication, and ubiquitin-proteasome systems were enriched. In the *Serratia* repressed gene group, the genes encoding seven salivary gland proteins were present.

Responsive Immune Genes

To determine the priming effects on immune genes in response to the bacterial challenges, we compared the transcriptional patterns of the immunoDB gene set (Waterhouse et al., 2007). The transcripts of 342 immune genes were detected in our dataset, 279 of them were responsive to the bacterial challenges in at least one of the six cohorts (**Figure 9**). The gene ID and available annotation were provided in **Supplementary Table 4**. Compared to the native priming, both *Enterobacter* and *Serratia* priming altered a few dozen more genes upon the challenge with a homogeneous bacterium, however, the challenge with a heterogeneous bacterium resulted in three times more downregulated genes (**Figure 9A**). Upon the *Enterobacter* challenge, 101 genes were upregulated in the cohorts with native and *Enterobacter* priming. Among these genes, there were 23 CLIP serine protease genes, (seven CLIPA, 10 CLIPB, four CLIPC, and two CLIPE genes), 11 leucine rich immune genes, nine SRPN genes, 13 FREP genes, five GNBP genes (**Supplementary Table 4**). The *Serratia* priming had a different impact, among the above 101 genes, 36 genes were downregulated upon the *Enterobacter* challenge (**Supplementary Table 4**). Upon the *Serratia* challenge, in the cohorts with native and *Serratia* priming, 72 genes were upregulated by the *Serratia* challenge, including 21 CLIP genes (17 were shared with *Enterobacter* infection), nine FREP genes, six leucine rich immune genes, eight SRPN genes, and nine TEP genes, and the immune signaling pathway genes *TOLL5A*, *Pelle*, *Cactus*, *IKK2* were upregulated as well. The *Enterobacter* priming resulted in 96 downregulated genes, including 11 genes that were upregulated in the other two primed cohorts (**Supplementary Table 4**). In all the cohorts

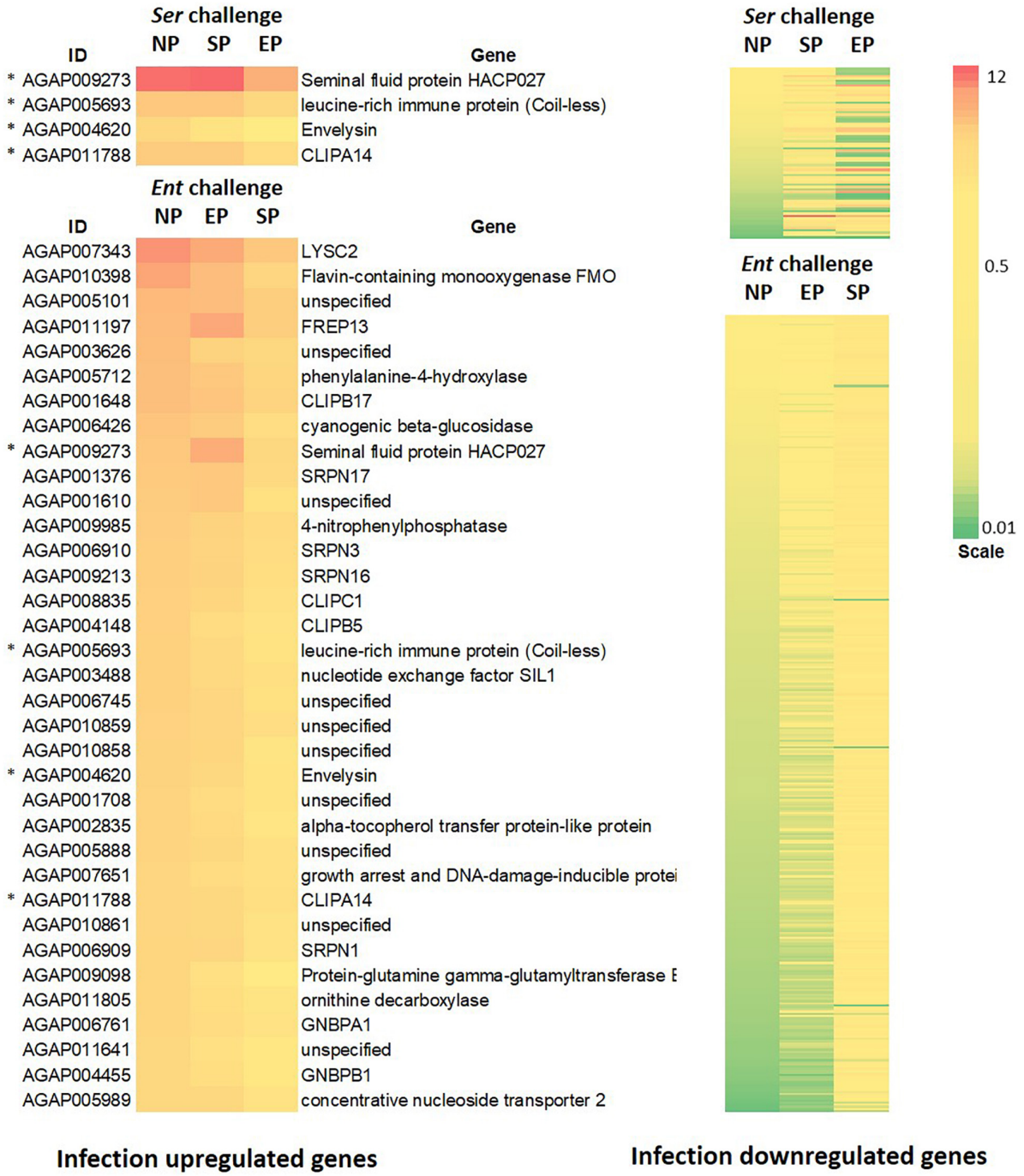

FIGURE 8 | Impact of prior homogeneous and heterogeneous priming on the infection responsive genes. In the *Serratia* challenged cohorts, the infection induced genes (>2-fold) were downregulated by the *Enterobacter* priming, while the infection repressed genes (<2-fold) were either increased or further decreased. In the *Enterobacter* challenged cohorts, the *Enterobacter* infection induced genes were downregulated by the *Serratia* priming, however, most of infection repressed genes were upregulated by the *Serratia* priming. * marks genes that were affected by both challenges. The gene ID and annotation in the downregulated panel were provided in **Supplementary Table 5**. The heatmaps represent genes with significant difference between the NP and homogeneous and heterogeneous priming (q-value < 0.05).

34 genes were upregulated by both *Enterobacter* and *Serratia* challenge, including 13 CLIP genes, five FREP genes, four SRPN genes, and four leucine rich immune genes. These genes were infection inducible, and the priming regimes had little effect on these genes. Interestingly, no downregulated genes were shared by all six cohorts.

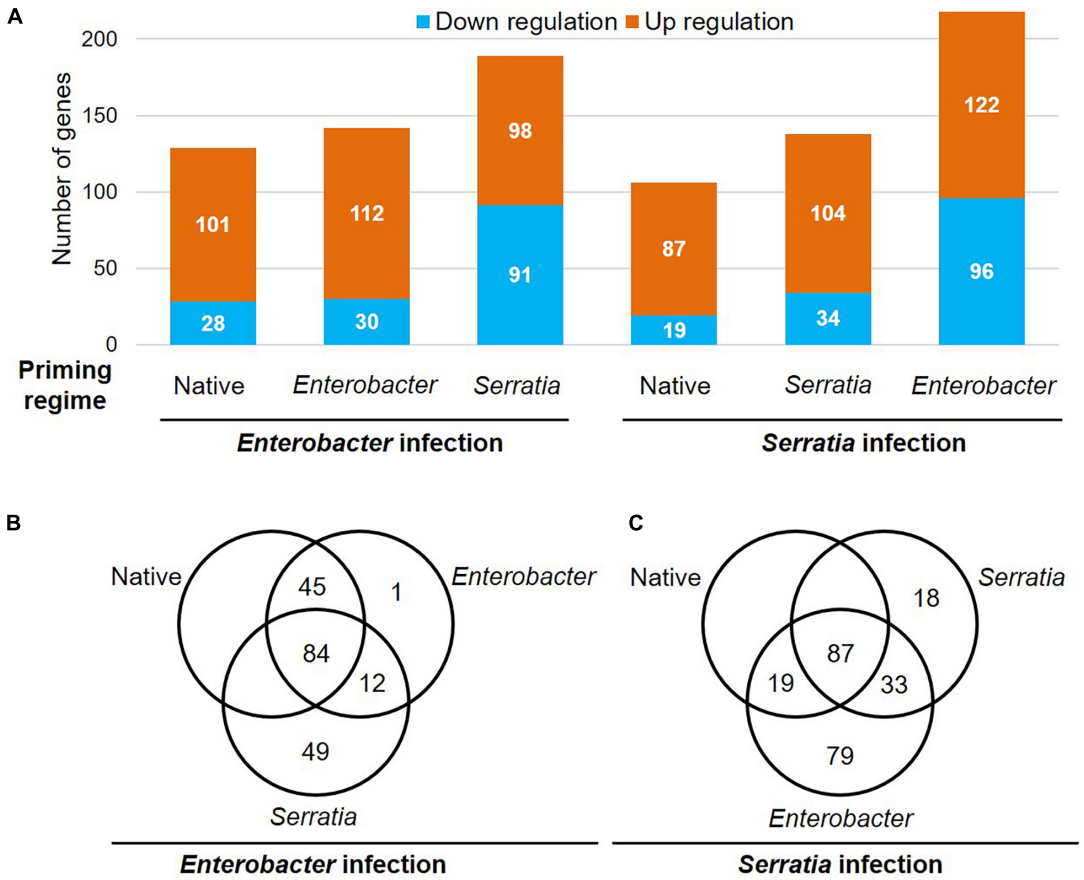

FIGURE 9 | Priming effects on the transcription of the immune genes upon *Enterobacter* and *Serratia* challenge. **(A)** The number of upregulated or downregulated genes is presented in each priming regime upon respective bacterial challenge. **(B,C)** The Venn diagram exhibits genes that were responsive to the respective infection in the cohort with respective priming regime.

In the category of non-immune genes, it worth noting that the genes encoding vitellogenin A1 precursor, vitellogenin and cathepsin B precursor were downregulated by the priming before the challenge, and these genes remained downregulated upon bacterial challenges no matter the priming background (**Supplementary Table 4**).

DISCUSSION

Innate immunity in invertebrates can be trained by priming to execute immune defense in an enhanced mode (Milutinovic et al., 2014; Brown and Rodriguez-Lanetty, 2015; Dhinaut et al., 2018; Gourbal et al., 2018; Ferro et al., 2019), which exhibits the high flexibility and plasticity of innate immunity. The innate immune system in invertebrates has demonstrated a widening spectrum of immune memory and specificity, which have led to the reassessment of the definition of immune memory and specificity (Milutinovic and Kurtz, 2016; Gourbal et al., 2018; Melillo et al., 2018; Sharrock and Sun, 2020). *Anopheles* mosquitoes transmit malaria, and antimalarial immunity has been studied extensively. Bacteria-primed immune enhancement against malaria has been well documented (Dong et al., 2009; Rodrigues et al., 2010; Gendrin et al., 2015; Bai et al., 2019; Cappelli et al., 2019). Antibacterial immunity has become a focus in understanding immune priming and underlying mechanisms (Hillyer and Estevez-Lao, 2010; Moreno-Garcia et al., 2015; Barletta et al., 2019; Brown et al., 2019; Powers et al., 2020). Bacterial symbionts are associated with mosquitoes throughout the evolution, the interactions between bacteria and host mosquitoes have been shaping the mosquito immune system. In this study, we explored the priming effect of gut bacteria on the systemic immune response to the hemocoelic infection caused by these bacteria.

The strains of *Enterobacter* and *Serratia* used in this study are gut commensals, which are naturally associated with mosquitoes. However, once being introduced into hemocoel by injection with approximately 100 CFU per mosquito, both strains cause acute virulent infections (**Figure 2**), and none of the challenged mosquitoes survived beyond 3 days. *Escherichia coli* has been widely used as a representative of Gram-negative bacteria for studying antibacterial immunity in mosquitoes (Moita et al., 2006; Nakhleh et al., 2017; Das De et al., 2018; Brown et al., 2019; Reyes Ruiz et al., 2019; Estevez-Lao et al., 2020; Powers et al., 2020). According to the literature, *E. coli* causes a chronic

infection in the hemocoel. In a recent study, *E. coli* infected *An. gambiae* mosquitoes could have a bacterial load up to >300,000 CFUs per mosquito on day 3 and day 7 post bacterial injection (Powers et al., 2020). It appears that the mosquitoes were tolerant to *E. coli,* but not able to clear the infection, the bacteria were persistent in the hemocoel until all mosquitoes died, though about 5% of infected mosquitoes survived exceeding 31 days post infection (Gorman and Paskewitz, 2000; Powers et al., 2020). We observed a similar infection outcome, in which the mosquitoes were injected with 100 nl of *E. coli* in an amount of OD_{600} = 1, and approximately 20% infected mosquitoes survived through 10 days post injection (data not shown). Therefore, the acute infection caused by the two gut commensal bacteria represents an infection model distinct from the one caused by *E. coli*. Regarding taxonomy, *Escherichia* and *Enterobacter* both belong to family Enterobacteriaceae, and *Serratia* belongs to family Yersiniaceae, the two families are in order Enterobacterales. It would be interesting to elucidate what is behind the difference between acute and chronic infections in terms of bacterial virulence factors and mosquito factors in future studies.

Then we used the infection model to examine the priming effect on infection outcomes from the following hemocoelic challenge. The mosquitoes were primed with a sugar meal supplemented with *Enterobacter* or *Serratia* and later challenged with homogeneous or heterogeneous bacteria. Compared to the infection in the cohorts with native gut community, the single bacterium-primed cohorts exhibit increased survival at 24 h post challenge with homogeneous but not heterogeneous bacterial strain used in priming (**Figure 3**). The data suggest that gut symbionts, when being dominant in the gut community, can train the mosquitoes to enhance immune responses to systemic infection with specificity to a certain degree.

To characterize the transcriptional impact posed by priming, we interrogated the transcriptomes in 10 different conditions, i.e., three priming regimes, six conditions of priming plus challenge with the homogeneous and heterogeneous bacterium, and injury control, as shown in **Figure 1**. In comparison to native priming, each bacterial priming significantly altered the expression of approximately 1100 genes, and 644 genes were affected by both (**Figure 5**). These transcriptional changes demonstrate a measurable priming-mediated systemic impact, suggesting that gut microbial shifts via diet manipulation can be sensed and transduced into a systemic response. This finding is corroborated by a recent study, which showed that two different strains of *Serratia,* once orally introduced into *An. stephensi,* could induce different transcriptomic responses to blood meal (Bai et al., 2019). The microbial structure of gut microbiota is diverse and dynamic during the mosquito life cycle (Wang et al., 2011). These dynamic interactions may have connections with various physiological traits. In the genes that are affected by the priming regimes, besides 40 or 50 immune genes, there are >1000 non-immune genes. Cytochrome P450 proteins are largely involved in xenobiotic defense (Feyereisen, 1999). The P450 system operates xenobiotic sensing and defense in the gut, which plays a critical role in maintaining gut homeostasis (Collins and Patterson, 2020). A recent study demonstrates that gut microbes regulate P450 gene expression and affect host pesticide metabolism in the honey bee (*Apis mellifera*) (Wu et al., 2020). In the primed mosquitoes, some P450 genes were upregulated, and some were downregulated, suggesting that the shift of gut microbial composition can be sensed and the P450 machinery is adjusted accordingly. It is worth noting 15 genes encoding solute carrier transporters (**Supplementary Table 3**). Increasing evidence has emerged that solute carrier transporters play critical roles in nutrient uptake, ion influx/efflux, and waste disposal, which mediates energy and metabolic support for immune activities (Song et al., 2020). Further study is needed to explore the priming effect on immunometabolism, which has become a hot research area recently (Penkov et al., 2019; Samaddar et al., 2020).

To identify the priming effect on transcriptomic responses to the bacterial challenge, we compared the transcriptomes of cohorts with three different priming regimes: native microbiota, *Enterobacter*, and *Serratia*. In the mosquitoes with native microbiota, the *Enterobacter* challenge altered more genes than *Serratia* did (**Figure 6**). In the priming contexts, the *Enterobacter* primed cohorts had a similar response as the native primed cohort did, only 133 more genes were differentially affected. However, the *Serratia* priming affected much more genes than the native priming did (**Figures 7A,B**). In the case of *Serratia* challenge, the *Serratia* priming altered more genes than the native priming, and the *Enterobacter* priming had a much broader influence than the other two priming regimes did (**Figures 7C,D**). It appears that the primed mosquitoes responded more drastically to a heterogeneous bacterial challenge than to a homogeneous challenge. A transcriptomic response involving more genes may reflect chaotic dynamics, which may not necessarily result in a beneficial outcome. Indeed, phenotypically, the primed immune protection is associated only with homogeneous challenges (**Figures 2**, **3**). In the immune gene category, many immune genes are responsive to the challenges (**Figure 9** and **Supplementary Table 4**), including genes encoding microbial pattern recognition, immune signaling, antimicrobial peptides, FREPs, CTLs, PPOs, CLIP serine proteases, and Serpins. Many of these genes play different roles in modulating melanization, one of the defense mechanisms (Christensen et al., 2005; Dong and Dimopoulos, 2009; An et al., 2011; Cao et al., 2017; Gendrin et al., 2017; Meekins et al., 2017; Kumar et al., 2018). The PPOs are activated by proteolytic cleavage via an enzymatic cascade of serine proteases. This process requires complex interactions of different members in the CLIPs B, C, and A as well as serpins (An et al., 2011; Gulley et al., 2013; Povelones et al., 2013; Zhang et al., 2015; Cao et al., 2017; He et al., 2017; Meekins et al., 2017; Nakhleh et al., 2017; El Moussawi et al., 2019; Sousa et al., 2020). It has been shown recently that microbial melanization can be triggered by *E. coli* infection (Sousa et al., 2020). The genes that participate in modulating melanization were enriched in the transcriptomes responsive to the priming regimes. It would be interesting to further investigate priming effects on the modulation of melanization in response to bacterial infections.

Besides typical immune genes, we noticed a set of genes with annotated functions related to lysosomes. In addition to six genes encoding lysozyme C, two genes encoding Niemann Pick type C1 (NPC1) and nine genes encoding Niemann Pick type C2 (NPC2) were responsive to the bacterial challenges

in different priming regimes (**Supplementary Table 4**), and genes encoding cystinosin and mosGILT were responsive to the challenges as well. Cystinosin is a cystine/H(+) symporter that exports cystine out of the lysosomes and is involved in melanin synthesis (Kalatzis et al., 2001; Chiaverini et al., 2012). The mosGILT, INF-γ inducible lysosomal thiol reductase, has been shown to play a critical role in ovarian development. The mosaic mosGILT-mutant mosquitoes exhibit an impaired 20E secretion in the ovaries and downstream vitellogenin synthesis in the fat body (Yang et al., 2020). The reduction of Vg protein, in turn, favors TEP1 mediated *Plasmodium* killing since the Vg interferes with TEP1 binding to ookinetes (Rono et al., 2010). Interestingly, the *Vg* expression is repressed by Rel1 and Rel2 (Rono et al., 2010). In the current study, the *Vg* expression was downregulated by both *Enterobacter* and *Serratia* priming as well as bacterial challenges (**Supplementary Table 4**). In honeybee *Apis mellifera*, Vg plays a dual role in reproduction and immunity. The Vg has immunological binding properties, it can bind to both Gram-positive bacterium *Paenibacillus larvae* and Gram-negative bacterium *E. coli,* and microbial pattern molecules lipopolysaccharide and peptidoglycan as well. More interestingly, pieces of *E. coli* cell wall can be carried into developing eggs by the Vg, which demonstrated the participation of Vg in the *trans*-generational immune priming in the honeybee (Salmela et al., 2015). It would be interesting to investigate the roles of Vg mediated immunity in mosquitoes in different conditions, for example, before, during, and after blood feeding. The Niemann Pick type C1 (NPC1) is an integral transmembrane protein of the limiting membrane of the lysosome. The Niemann Pick type C2 (NPC2) is a soluble cholesterol binding protein. In humans, the mutation of NPC genes can cause lysosomal storage diseases, which result in inflammation and altered innate immune response (Platt et al., 2016; Rigante et al., 2017). Lysosomes process various substrates from phagocytosis, endocytosis, and autophagy. Mosquito Vg is processed by vitellogenic cathepsin B, a lysosomal thiol (cysteine) protease (Cho et al., 1999; Moura et al., 2015). In short, our transcriptome data imply the connections of multiple lysosomal genes to the immunity in mosquitoes.

In this study, we show that the mosquito antibacterial immunity can be enhanced by priming using gut bacterial symbionts via sugar meals. The priming-trained immunity demonstrates certain specificity. The priming effects systemic transcriptomic responses to the following challenges. When primed mosquitoes were challenged by a heterogeneous bacterium, more complex transcriptomic responses occurred, but no phenotypic protection was observed. In addition to typical immune genes, many non-immune genes are affected as well, suggesting that the priming effects are diverse and systemic. Hemocytes are key immune players. In this study, the whole mosquito transcriptomes were profiled, which largely access the transcriptomes in fat body cells and other cells that were sufficiently represented in the samples. Unfortunately, such RNA-seq data do not have the resolution to tease out responses of hemocytes in the context. There are cross-talks between midgut, fat body, and hemocytes during an immune response (Das De et al., 2018). The genes identified in the current study would be the targets of future studies to elucidate the mechanisms behind the priming effects. The infection outcome of the acute hemocoelic infection caused by the two gut commensal bacteria is different from the outcome of the chronic infection caused by *E. coli*. This warrants further studies to elucidate what is behind the differences. Lastly, we would like to emphasize that the *Enterobacter* strain is associated with G3 strain in our insectary, while the *Serratia* strain was derived from wild *Aedes* mosquitoes. And the *Serratia* strain was not detected in the G3 colony (**Supplementary Figure 1**), suggesting that the strain is not associated with the G3 mosquitoes in our insectary. Therefore, the differences in the infection pattern and transcriptomic response between the two bacterial strains may also attributed to the fact that the *Serratia* strain is not a regular gut resident in the G3 mosquitoes. In summary, this study presented novel data that furthered the understanding of mosquito immunity.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI SRA (accession: PRJNA691571).

ETHICS STATEMENT

The animal protocol was reviewed and approved by the NMSU IACUC.

AUTHOR CONTRIBUTIONS

JX conceived the study and wrote the manuscript. JX and AK designed the experiments. AK, AP, WY, JC, AM, and JX conducted the experiments and data analysis. PT, SC, and JX analyzed the transcriptome data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.649213/full#supplementary-material

Supplementary Figure 1 | Bacterial detection in the gut. **(A)** The gut metagenomic DNA was extracted from 3rd to 4th instar larvae, pupae, 5-day old female adults, and 4 days post blood meal. The presence of *Serratia* and *Enterobacter* was examined by PCR targeting two *Serratia* genes (DNA gyrase and glucose phosphatase) and one *Enterobacter* gene (DNA gyrase). Mosquito gene rpS5 PCR was positive for all stages. *Enterobacter* was detected in adult gut only, *Serratia* was not detected in any stages. **(B)** The specificity of bacterial amplicons was determined by respective bacterial genomic DNA. The PCR was positive with respective bacterial DNA. No non-specific amplifications occur.

Supplementary Figure 2 | The expression patterns of five genes were compared between RNA-seq and qPCR. The data were presented as fold change. In the *Enterobacter* challenged mosquitoes, fold changes of (EP-EI)/(NP-EI) and (SP-EI)/(NP-EI) were presented. In the *Serratia* challenged mosquitoes, fold changes of (SP-SI)/(NP-SI) and (EP-SI)/(NP-SI) were presented. In most of the six conditions, the gene expression patterns were comparable between RNA-seq and qPCR. The *PGRPLB* was not consistent in two conditions, and *CLIPB12* was not consistent in two conditions, these inconsistent conditions were marked by arrows. Error bar represents standard deviation.

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Comprehensive Ecological and Geographic Characterization of Eukaryotic and Prokaryotic Microbiomes in African *Anopheles*

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Exposure of mosquitoes to numerous eukaryotic and prokaryotic microbes in their associated microbiomes has probably helped drive the evolution of the innate immune system. To our knowledge, a metagenomic catalog of the eukaryotic microbiome has not been reported from any insect. Here we employ a novel approach to preferentially deplete host 18S ribosomal RNA gene amplicons to reveal the composition of the eukaryotic microbial communities of *Anopheles* larvae sampled in Kenya, Burkina Faso and Republic of Guinea (Conakry). We identified 453 eukaryotic operational taxonomic units (OTUs) associated with *Anopheles* larvae in nature, but an average of 45% of the 18S rRNA sequences clustered into OTUs that lacked a taxonomic assignment in the Silva database. Thus, the *Anopheles* microbiome contains a striking proportion of novel eukaryotic taxa. Using sequence similarity matching and *de novo* phylogenetic placement, the fraction of unassigned sequences was reduced to an average of 4%, and many unclassified OTUs were assigned as relatives of known taxa. A novel taxon of the genus *Ophryocystis* in the phylum Apicomplexa (which also includes *Plasmodium*) is widespread in *Anopheles* larvae from East and West Africa. Notably, *Ophryocystis* is present at fluctuating abundance among larval breeding sites, consistent with the expected pattern of an epidemic pathogen. Species richness of the eukaryotic microbiome was not significantly different across sites from East to West Africa, while species richness of the prokaryotic microbiome was significantly lower in West Africa. Laboratory colonies of *Anopheles coluzzii* harbor 26 eukaryotic OTUs, of which 38% (n = 10) are shared with wild populations, while 16 OTUs are unique to the laboratory

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colonies. Genetically distinct *An. coluzzii* colonies co-housed in the same facility maintain different prokaryotic microbiome profiles, suggesting a persistent host genetic influence on microbiome composition. These results provide a foundation to understand the role of the *Anopheles* eukaryotic microbiome in vector immunity and pathogen transmission. We hypothesize that prevalent apicomplexans such as *Ophryocystis* associated with *Anopheles* could induce interference or competition against *Plasmodium* within the vector. This and other members of the eukaryotic microbiome may offer candidates for new vector control tools.

Keywords: mosquito, *Anopheles*, insect microbiome, eukaryotic microbiology, commensalism, insect immunity

HIGHLIGHTS

- Microbes inhabit the animal digestive tract and body and are generally required for the health of the organism.
- *Anopheles* mosquitoes are responsible for significant human and animal mortality due to the pathogens they transmit.
- New vector control tools are needed because historically effective control methods are declining in effectiveness due to insecticide resistance and other factors.
- Characterization of the eukaryotic and prokaryotic microbes that inhabit mosquitoes could help identify new vector control tools, either as biological control agents or to interfere with pathogen infection and transmission by mosquitoes.

INTRODUCTION

Mosquitoes carry a microbiome of associated eukaryotic and prokaryotic microbes, as well as the viruses that comprise the virome. This assemblage is thought to influence mosquito immunity and the transmission of mosquito borne pathogens, and some taxa could decrease mosquito longevity or pathogen transmission (Pumpuni et al., 1996; Ryu et al., 2008; Dong et al., 2009; Rodrigues et al., 2010; Cirimotich et al., 2011; Boissiere et al., 2012; Broderick et al., 2014; Carissimo et al., 2015; Nanfack-Minkeu et al., 2019; Mitri et al., 2020; Sharma et al., 2020). However, most characterization of the *Anopheles* microbiome to date has focused on the prokaryotic fraction, and the composition and biology of the natural eukaryotic microbiome remains essentially unknown.

Mosquitoes have a deep evolutionary history with the human and animal pathogens they transmit. Insects diverged from other arthropods more than a half billion years ago (Giribet and Edgecombe, 2012). At that time, the Apicomplexa, the phylum including the malaria parasite *Plasmodium*, were already old (Kopecna et al., 2006; Morrison, 2009). Apicomplexans were likely waterborne pathogens of arthropods, and are probably still found in mosquito larval breeding sites today. Thus, the foundational mechanisms of mosquito innate immunity, including the mechanisms addressed against *Plasmodium* today, probably evolved in mosquito common ancestors for protection from ancient arthropod pathogens (Medzhitov and Janeway, 1997; Hoffmann et al., 1999; Mitri et al., 2015; Nanfack Minkeu and Vernick, 2018). However, the candidate natural pathogens, particularly eukaryotic microbes similar to *Plasmodium* and other mosquito-transmitted pathogens, have not been systematically identified.

Profiling the prokaryotic microbiome is simple using amplicon sequencing of the 16S ribosomal RNA (16S rRNA) gene hypervariable regions. In contrast, profiling of the eukaryotic fraction of the microbiome is challenging because both the eukaryotic host as well as the eukaryotic microbiome carry highly related 18S ribosomal RNA (18S rRNA) genes, and the host contribution of 18S rRNA gene sequences in a DNA sample of the organism is in massive excess to that of the microbes. Here, we selectively enrich for amplification of 18S rRNA gene sequences originating in the eukaryotic microbiome. We used derivatized peptide-nucleic acid (PNA) oligonucleotides (called PNA blockers) that bind within the host 18S rRNA gene target and biochemically inhibit amplicon extension, thereby suppressing the generation of host-derived 18s rRNA gene sequences and enriching for the eukaryotic microbes (Belda et al., 2017).

Current vector control tools are being challenged by insecticide resistance and vector behavioral shifts. In the past decade, studies of the mosquito prokaryotic microbiome have led to potential new vector control approaches, including bacteria exhibiting *Plasmodium*-blocking phenotypes (Wang et al., 2017; Shane et al., 2018), the development of biopesticides from mosquito associated bacteria (Lacey, 2007; Caragata et al., 2020), or population replacement using *Wolbachia* (Ryan et al., 2019; Zheng et al., 2019). The eukaryotic members of the microbiome could also be useful to design similar or new approaches, such as interference or competition with *Plasmodium* superinfection, or as biological control agents. In order to explore these and other potential applications, a comprehensive assessment of the eukaryotic composition of the mosquito microbiome is first needed.

Here we sample wild mosquito larvae in West and East Africa, and also from laboratory colonies, in order to comprehensively characterize their eukaryotic and prokaryotic microbiomes using deep sequencing of 18S and 16S rRNA gene hypervariable region amplicons. We analyze association of microbiome parameters with mosquito species, geography, and larval breeding site ecology. Given the paucity of taxonomic database resources for eukaryotic microbes, we also implement an Evolutionary

Placement Algorithm (EPA) for identification of many novel eukaryotic taxa.

MATERIALS AND METHODS

Field Mosquito Samples

Larval samples were collected from the following sites. Samples were stored in 80% ethanol upon collection in the field and prior to DNA isolation.

Burkina Faso

Third and fourth instar mosquito larvae were collected near the village of Goundry in Burkina Faso during the rainy season in 2013. Different larval ecologies were sampled including mud brick pits, puddles, and ponds. Larval samples were stored in 80% ethanol prior to DNA isolation.

Republic of Guinea (Conakry)

Third and fourth instar mosquito larvae were collected across an ecological transect spanning dry savannah to deep forest ecologies in The Republic of Guinea and Mali during the rainy season in 2012 as previously described (Coulibaly et al., 2016). Larval samples were stored in 80% ethanol prior to DNA isolation.

Kenya

Third and fourth instar mosquito larvae were collected at three sites in the Luanda region of Kenya. Emutete village (34°64 E, 00°22 N) is in Emuhaya district in Western Kenya. It is a valley with slow running streams and considered lowland. Itumbu (34°57 E, 00°40 N) and Ebusilaro (34°60 E, 00°02 N) are also villages. However, they are closer to the town Luanda. In all three places, the households have farms that are cultivated almost year-round. The collections were done from a variety of breeding sites e.g., rain puddles, potholes on the roads, fish ponds, irrigation canals that were in the farms and dams for collecting rain water. Larval samples were stored in 80% ethanol prior to DNA isolation.

A summary table of all larval pools, their geographic locations and ecological attributes can be found in **Supplementary Table 1**.

Laboratory Mosquito Samples

All laboratory samples were raised in the same insectary facility, exposed to the same water, food and other environmental variables, at the Institut Pasteur, Paris, France. The colonies M'bita, SDA500 and Ngousso are *An. gambiae, An. stephensi* and *An. coluzzii*, respectively, with origins in Kenya, Pakistan and Cameroon, respectively. The founder (Fd) and isofemale colonies (IML) including Fd03, Fd05, Fd09, Fd33 and IML26, IML29, IML30, IML30-2, and IML69 were previously described (Redmond et al., 2015) and originate from Burkina Faso and Mali. Briefly, Fd colonies were each initiated from the eggs of 6–11 wild-captured female mosquitoes that mated in nature. After oviposition, mothers were genotyped to determine species, and eggs of the same species were combined. IML colonies were initiated from a single mated female originating in an Fd colony.

DNA Isolation

In addition to storage in 80% ethanol, all mosquito larvae were individually rinsed with 80% ethanol to remove surface microbes prior to DNA isolation. Genomic DNA was isolated from individual mosquitoes using DNAzol (Invitrogen, CA, United States). DNAs were resuspended in distilled water and stored at -20°C. All samples were typed by a molecular diagnostic assay to determine species status within the *An. gambiae* species complex (Fanello et al., 2002). In the event this assay failed to yield a diagnostic band, the ribosomal gene ITS2 region was PCR amplified, Sanger-sequenced, and the resulting sequence was used to search the NCBI nr database using blast. Mosquito species calls based on ITS2 sequence used a threshold of >98% nucleotide identity.

DNA pools comprised of 2–8 field-collected mosquito larvae each were constructed by pooling DNA from individual samples at equal volume. DNA pools were assembled after DNA isolation from individuals, because the species of each individual was first determined by molecular diagnostic assays prior to assembling DNA pools of the same species. From Burkina Faso, 17 DNA pools were each comprised of DNA from 2–7 larvae; from Republic of Guinea, 8 DNA pools were each comprised of DNA from 5–8 larvae; and from Kenya, 12 DNA pools were each comprised of DNA from 2–7 larvae. DNA pools were comprised of larval samples of the same mosquito species collected from the same geographic location and the same type of larval site. Water blank controls were co-processed, sequenced and analyzed with experimental samples. Resulting DNA pools were subjected to 18S and 16S rRNA gene amplicon sequencing as described below.

Amplification of Hypervariable Regions of the 18S and 16S rRNA Genes

The prokaryotic and eukaryotic microbiomes of 39 DNA pools comprised of field collected samples and 24 DNA pools of laboratory colony mosquitoes (2 replicates for each of 12 colonies) were characterized by barcoding of the V4 hypervariable region of 16S and the V9 hypervariable region of 18S rRNA genes, respectively. Water blanks were also sequenced to detect any contamination. Samples were amplified using the following PCR recipe: 3 μl template DNA, 1.2 μl 5× KAPA HiFi buffer, 0.18 μl dNTP mix (10 mM), 0.3 μl DMSO, 0.003 μl 1,000× SYBR Green, 0.12 μl ROX (25 μM), 0.06 μl KAPA HiFi HotStart Polymerase (Kapa Biosystems), 0.3 μl V9 forward primer (10 μM), 0.3 μl V9 reverse primer (10 μM), 7.5 mM PNA blocker, nuclease-free water up to a reaction volume of 6 μl. The appropriate PNA blocker concentration was empirically determined previously (Belda et al., 2017). PNA blockers were incubated at 55°C for 5 min and vortexed to fully resuspend prior to adding to the reactions. Reactions were transferred into a 384-well plate and amplified with an ABI7900 thermocycler with the following amplification conditions: 95°C, 5 min and 25 cycles of: 98°C, 20 s, 78°C, 5 s, 55°C, 15 s, 72°C, 1 min. PCR products were diluted 1:100 in nuclease free water, and indexed using the procedure below.

Library Construction From Amplified Products

Indexing PCR reactions were done using the following recipe: 5 μl template DNA, 1 μl nuclease-free water, 2 μl 5× KAPA HiFi buffer, 0.3 μl 10 mM dNTPs, 0.5 μl DMSO, 0.2 μl KAPA HiFi Polymerase, 0.5 μl forward primer (10 μM), and 0.5 μl reverse primer (10 μM). Indexing PCR reactions were carried out in 96-well plates on a Bio-Rad Tetrad two thermocycler, using the following cycling conditions: 95°C, 5 min and 10 cycles of: 98°C, 20 s, 55°C, 15 s, 72°C, 1 min, 72°C, 10 min. The following indexing primers were used (X indicates the positions of eight nucleotide unique indices for demultiplexing): Forward indexing primer: AATGATACGGCGACCACCGAGATCTACACXXXXXXXXTCGTCGGCAGCGTC. Reverse indexing primer: CAAGCAGAAGACGGCATACGAGATXXXXXXXXGTCTCGTGGGCTCGG.

Library Normalization, Pooling, and Quantification

For the PNA blocker experiments, indexing PCR reactions were purified and normalized using a SequalPrep Normalization Plate Kit (Thermo Fisher Scientific). 10 μl of each sample was pooled (V4 and V9 were pooled separately, due to the different sizes of these amplicons) and the amplicon pools were purified and concentrated with a 1× AmPureXP (Beckman Coulter) clean up, followed by elution in 25 μl of Qiagen buffer EB.

The concentrations of the amplicon pools were determined using a Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific) and amplicon sizes were verified on an Agilent Bioanalyzer High Sensitivity Chip. The V9 amplicon pools were independently diluted down to a 2 nM concentration in Qiagen EB buffer, and mixed at a 1:1 ratio.

Library Denaturation, Dilution and Sequencing

10 μl of the 2 nM sequencing library was denatured by adding 10 μl of 0.2 N NaOH and incubating at room temperature for 5 min, then the library was diluted to 8 pM in Illumina HT1 buffer, spiked with 15% PhiX, and sequenced on a portion of a MiSeq 2 × 300 (600 cycle v3) lane. Library construction and sequencing was performed by the University of Minnesota Genomics Center, St. Paul, MN.

Sequence Analyses

Raw paired-end reads were quality trimmed and assembled with Pandaseq (Masella et al., 2012). Primer regions were trimmed using the primer sequences rather than using a fixed value of Q. Following this, low quality amplicons were filtered out using quality scores defined as the geometric mean of their base qualities. Quality profile plots are shown in **Supplementary Figure 1**. Operational taxonomic unit (OTU) clustering and chimera filtering were carried out with QIIME version 1.9.1 (Caporaso et al., 2010b). Only 16S and 18S rRNA gene OTUs that contained more than 10 sequences were retained for subsequent analysis in order to avoid the inclusion of OTUs that were a product of sequencing error. Taxonomic assignment of 16S and 18S rRNA gene OTUs was carried out with QIIME version 1.9.1 using UCLUST (Edgar, 2010) against the 16S and 18S rRNA gene subdivision of the 119 release of the Silva database (Quast et al., 2013). The 16S rRNA gene OTU table was rarified to 10,000 reads per sample to correct for differences in sequencing depth with the *rarefy_even_depth* function of *phyloseq* R package, which was enough to observe saturation in rarefaction analyses (**Supplementary Figure 2A**). Nine field larval breeding sites were excluded after this step, which yielded DNA pools with very small fraction of amplicons joined after the Pandeseq step (mean 7.22% read pairs per DNA pool joined in full-length V4 16S rRNA amplicons in these 9 samples vs 88.99% in retained DNA pools). For 18S rRNA gene data, despite the use of PNA blockers, we observed a large number of sequences coming from the mosquito host, particularly in laboratory colony DNA pools (**Supplementary Figure 3**). After excluding OTUs coming from the mosquito host, the eukaryotic OTU table was rarified to 900 reads per sample with *rarefy_even_depth* function of *phyloseq* R package (McMurdie and Holmes, 2013), which was fixed based on rarefaction analyses (**Supplementary Figure 2B**). 7/24 laboratory colony DNA pools and 32/39 field DNA pools were retained for subsequent analyses (**Supplementary Table 1**). Diversity indexes (Observed species, Shannon, ACE, Chao1) were estimated from rarified OTU tables with the *estimate_richness* function of phyloseq R package (McMurdie and Holmes, 2013). The R package *vegan* (Oksanen et al., 2019) was used to compute Beta-diversity matrix from rarified OTU tables collapsed at genus level (*vegdist* function) and to visualize microbiome similarities using principle coordinate analysis (PCoA) (*cmdscale* function).

To identify covariates with the highest non-redundant explanatory power on 16S and 18S rRNA gene microbiota variation in mosquito DNA pools, first distance-based redundancy analyses was carried out on genus-level community ordination (PCoA based on Bray-Curtis beta-diversity matrix) with six pool covariates (country, mosquito species, 2La inversion, larval site description, larval breeding site status, larval site ecology) with *capscale* function of the *vegan* R package. Individual covariates significantly associated to variations in microbiome composition (*capscale* p-value < 0.05) were subsequently filtered to identify the ones with non-redundant explanatory power with the *env2fit* function of the vegan R package. Differential abundance analysis of prokaryotic and eukaryotic OTUs between conditions was carried out using the *phyloseq* implementation of DESeq2 method (Love et al., 2014). Water blank samples for 18S rRNA gene amplicon sequences included six OTUs, none of which were present among the OTUs from the experimental samples analyzed in the manuscript, and the 16S rRNA gene amplicon sequences included ten OTUs (**Supplementary Figure 4**).

Phylogenetic Analysis

In order to improve the taxonomic annotation of eukaryotic OTUs, 38 unclassified OTUs with at least 100 sequence reads each were placed in a reference phylogeny of 18S rRNA sequences using the EPA of RAxML, which sequentially places each short query sequence (read) at each edge of a reference tree previously

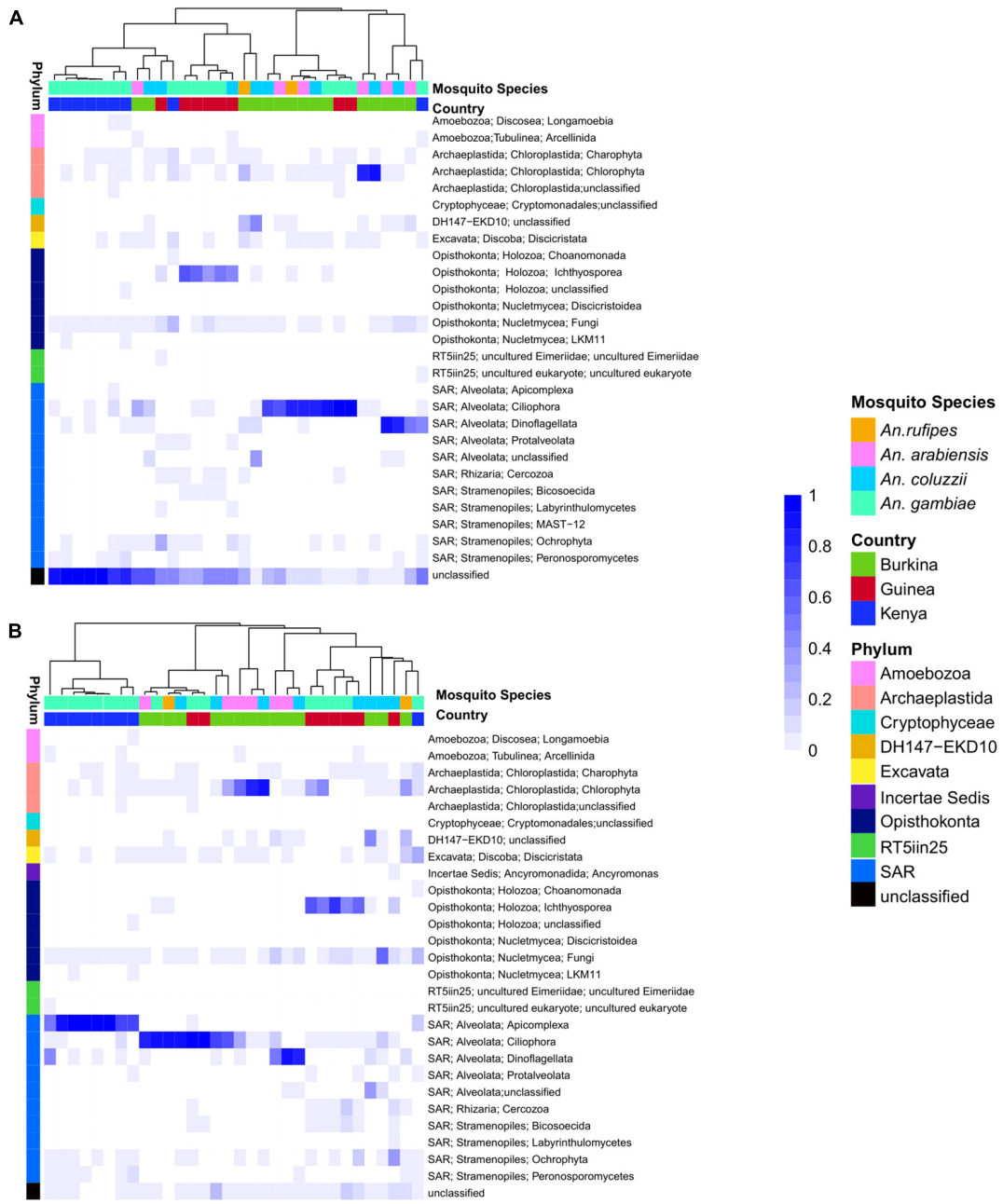

FIGURE 1 | (A) Heatmap depicting the similarity in composition of micro eukaryotic members of the microbiome from field collected larvae displays clustering due to geography. The heatmap is clustered by microbiome similarity and both mosquito species and country of origin are shown above the heatmap for each sample with keys to the right of the heatmap. The dendrogram above the heatmap depicts overall micro eukaryotic microbiome similarity. The colored vertical bar on the left of the heatmap depicts the OTU Phylum membership with a key to the right of the heatmap. Each column represents a single pool of mosquito larvae subjected to 18S rRNA gene V9 amplicon sequencing and each row of the heat map show the relative proportion of one eukaryotic Order. The shade of blue indicates the proportion of the microbiome occupied by a particular Order; key to right of heatmap. **(B)** As in A except after application of the Evolutionary Placement Algorithm (EPA) which was used to assign many of the previously unassigned sequence reads to known phyla based on evolutionary relatedness as described in greater details in the methods. This phylogenetically informed process assigned on average 34.8% of previously unclassified sequences per field sample to known Orders.

constructed with longer sequences and calculates the likelihood of the resulting tree (Berger et al., 2011). For this purpose, the OTUs were aligned with Pynast (Caporaso et al., 2010a) against a curated 18S rRNA sequence alignment template from release 119 of the Silva database (Quast et al., 2013), and the resulting alignment was filtered with the *filter_alignment.py* script of QIIME version 1.9.1 (Caporaso et al., 2010b). This filtered alignment was used to place unclassified eukaryotic OTUs in the

FIGURE 2 | Heatmap depicting the similarity in prokaryotic microbiome composition of field collected larvae shows Betaproteobacteria and Gammaproteobacteria are common members of the prokaryotic microbiome across sampling countries and mosquito species. The heat map is clustered by microbiome similarity and both mosquito species and country of origin are shown above the heatmap for each sample. The dendrogram above the heatmap depicts overall prokaryotic microbiome similarity. The colored vertical bar to the left of the heat map highlight the Phylum membership for identified OTUs with a key to the right of the heatmap. Each column represents a single pool of mosquito larvae subjected to 16S rRNA gene V4 hypervariable region amplicon sequencing. Each row of the heat map indicates the relative proportion of one prokaryotic Class. The shade of blue indicates the proportion of the microbiome occupied by that particular Class level with a key to the right of the heatmap.

reference 18S rRNA tree from release 119 of the Silva database (Quast et al., 2013), using the EPA of RAxML (Berger et al., 2011).

RESULTS

Here we characterize the eukaryotic and prokaryotic microbiomes of *Anopheles* larvae sampled in three countries of East and West Africa: Kenya, Burkina Faso and the Republic of Guinea. For detection of eukaryotic taxa, we implemented a PNA blocking strategy combined with deep sequencing of 18S rRNA gene amplicons to suppress mosquito sequence reads, a technique we have optimized previously (Belda et al., 2017). We also profile the prokaryotic microbiome in the same samples because of the technical simplicity of 16S rRNA gene amplicon sequencing, but most analysis herein is focused on the eukaryotic microbiome, which is novel.

Composition of Eukaryotic and Prokaryotic Microbiomes

The eukaryotic microbiome displays clear sample clustering by country. Specifically, many samples from Burkina Faso cluster together, and all but one Kenyan sample cluster with other samples from Kenya (**Figure 1A**). The most striking observation regarding the eukaryotic fraction of the microbiome is the large number of unclassified operational taxonomic units (OTUs) that do not match any entry in the Silva taxonomic database (the row marked "unclassified" at the bottom of **Figure 1A**). The deepest split in the sample dendrogram separates mosquito larval samples with a large fraction of unclassified OTUs from those that harbor a large fraction of Alveolata. The Alveolata are a major clade of protists that include the phylum Apicomplexa, to which *Plasmodium* belongs.

Among the classified taxa associated with mosquito larvae, some are notable for their abundance in particular samples. Larvae from Burkina Faso harbored high numbers of members of Ciliophora, a phylum of ciliated protozoans within the Alveolata that includes commensal as well as parasitic species, and Chlorophyta, a phylum of green algae that might be present as a larval food source, although there are also commensal and pathogenic species. Larvae from Guinea also displayed high abundance of Ciliophora, as well as Ichthyosporea, a group of Opisthokonta that are mostly parasites, discussed further below. In contrast, the larval samples from Kenya harbored a large proportion of OTUs lacking taxonomic assignment in the Silva database (**Supplementary Table 2**).

In order to extract additional information from the unclassified eukaryotic OTUs, we applied an EPA to classify unidentified OTU sequences based on evolutionary similarity to known OTUs. Prior to analysis using the EPA, an average of 39% (range: 1–98%) of eukaryotic sequence reads from a given sample lacked taxonomic assignment. In contrast,

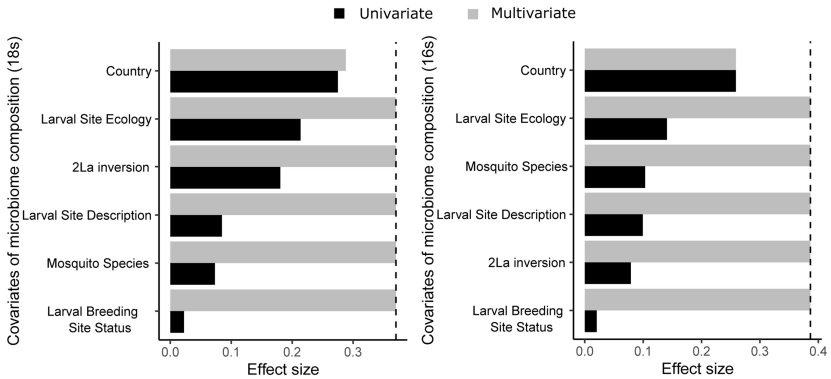

FIGURE 3 | Country of origin is the sample variable showing the greatest correlation with microbiome composition (dbRDA, genus-level Bray-Curtis dissimilarity) for both 18S rRNA gene data (left) and 16S rRNA gene data (right) from field collected samples. Data is shown for both an independent model (black bars; univariate effect sizes, CAP_r2ad) and a multivariate model (gray bars; cumulative effect sizes, RDAcumul_R2.ad). The cut-off for significant non-redundant contribution to the multivariate model is shown by the vertical dashed line. Larval site ecology is the ecology of the geographic region (i.e., deep forest, dry savannah etc.), larval site types are puddle, pond, mud brick pit etc. and larval pool status is whether the pool is temporary, semi-permanent or permanent.

following analysis using the EPA the percent of unassigned reads dropped to 4% (range: 0–25%). The EPA analysis identified an average of 23% of previously unclassified eukaryotic OTUs as related to known OTUs across both field and laboratory samples (**Figure 1B**). Of the 27 field samples harboring unclassified eukaryotic OTUs at >5% abundance, analysis using the EPA improved taxonomic assignments in 19 samples, leaving only eight field samples with >5% unclassified reads at the OTU level.

Among the previously unclassified eukaryotic OTUs placed using EPA analysis, many are novel members of the order Alveolata, followed by a large proportion of novel members of the Chloroplastida (**Figure 1B**). In particular, almost all of the unclassified eukaryotic OTUs in the Kenyan field samples were taxonomically assigned by EPA analysis to the phylum Apicomplexa. The apicomplexan sequences are clustered as OTU38 and placed by EPA analysis as a novel taxon in the genus *Ophryocystis*, hereafter referred to as OTU38_Ophryocystis (**Supplementary Table 3**). Species of *Ophryocystis* have been described as insect pathogens in at least butterflies and beetles (Yaman and Radek, 2017; Gao K. et al., 2020). Application of the EPA also assigned additional OTUs in the Guinea samples to *Ochrophyta*, a group of photosynthetic heterokonts, and also assigned additional OTUs in the Burkina Faso and Guinea samples to the Peronosporomycetes clade in the phylum Oomycota, a group of fungus-like parasites and saprophytes known as water molds.

For the prokaryotic fraction of the microbiome, two families of gram-negative *Proteobacteria*, the *Betaproteobacteria* and *Gammaproteobacteria*, are prevalent across all field captured samples. The deepest root in the dendrogram depicting sample similarity is explained by the distribution of these two families. Many other bacterial phyla are present in all microbiome samples including gram-positive *Firmicutes* and *Actinobacteria*, while individual bacterial families within the gram-negative Chloroflexi phylum tend to be present in just a few samples (**Figure 2**). There is little apparent clustering of the prokaryotic microbiome by either country of origin or mosquito species (**Supplementary Table 4**).

Structuring Influences on Wild *Anopheles* Microbiome Composition

The major correlates of *Anopheles* microbiome compositional differences were determined by testing six attributes of the collected mosquito samples (**Figure 3**). Comparison of genus-level beta-diversity of microbial taxa using the Bray-Curtis dissimilarity statistic indicates that the country of sample collection, a proxy for the most coarse-grained geographic definition of the samples, displays the greatest correlation with the composition of both the eukaryotic and prokaryotic microbiomes. After the variable, country, the second most correlated attribute is larval site ecology (deep forest, dry savannah, etc.), a definition that is shared among and is independent of country of origin. These two attributes, country and larval site ecology, make non-redundant contributions to the multivariate model for either eukaryotic or prokaryotic microbiome. Interestingly, mosquito species displayed little correlation with microbiome composition. Thus, relatively little influence on the microbiome is seen from genetic differences between *Anopheles* species, or 2La chromosome inversion genotypes for *An. gambiae* and *An. coluzzii*, nor from larval breeding site substrate type or permanence. These results indicate that the most important structuring influences on the taxonomic composition of both eukaryotic and prokaryotic microbiomes harbored by *Anopheles* larvae are high-order ecological factors, defined by country of origin and, independently of country, the ecological characteristics of larval sites.

We next compared on a finer scale the eukaryotic and prokaryotic microbiomes across the three sampled countries. Composition of the eukaryotic microbiome displays little similarity across the countries sampled, with only 4% of OTUs present in larval samples from all three countries (**Figure 4A**). An additional ∼12% of OTUs were present in *Anopheles* larval

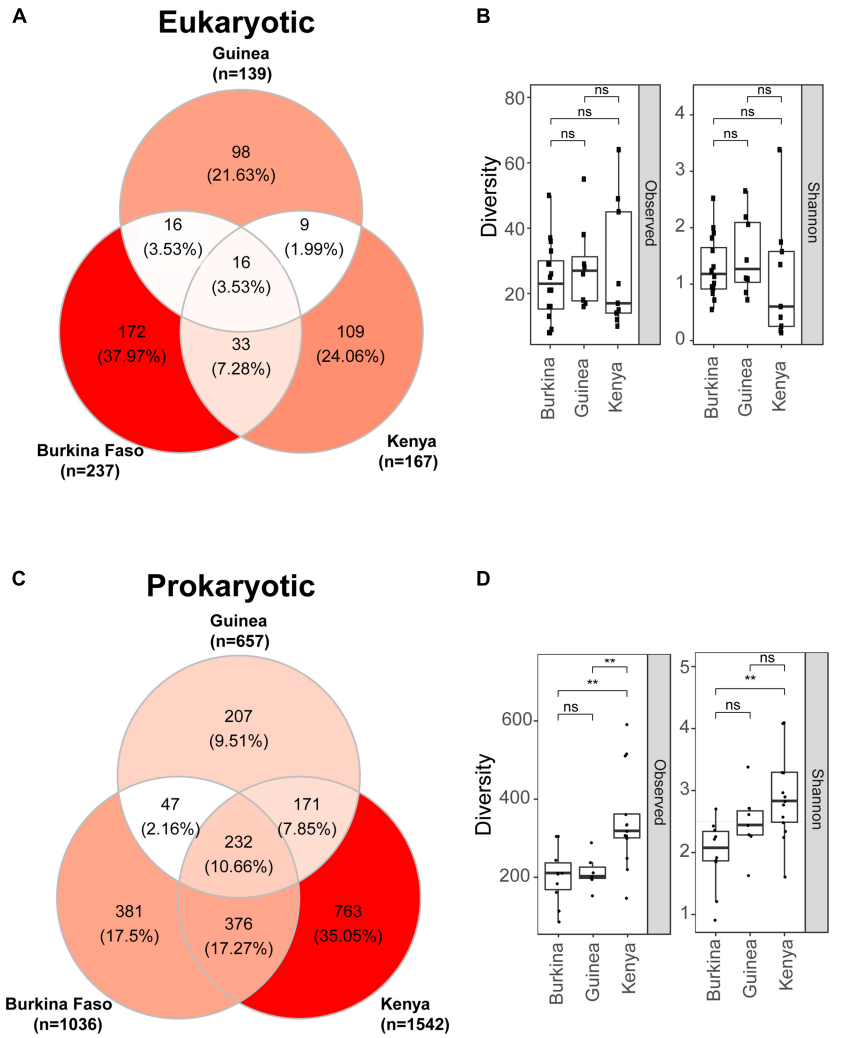

FIGURE 4 | Eukaryotic microbes detected in mosquito larvae are more unique to the geographic location of collection than prokaryotic microbiomes where individuals OTUs are more often detected in mosquito larvae from at least two locations. **(A)** Venn diagram depicting the number of eukaryotic OTUs detected in DNA pools of mosquito larvae across Burkina Faso, Guinea and Kenya. **(B)** Eukaryotic microbiome diversity as a function of geographic origin shown as both observed OTUs (left) and Shannon diversity (right). There is no significant difference in eukaryotic diversity as a function of country of origin; ns = non-significant, $p > 0.05$. For these box plots the upper and lower bounds of the box indicate the third and first quartiles, respectively, and the line within the box indicates the median. Whiskers extends to the largest values no further than 1.5 time the inter-quartile range from the corresponding upper and lower bounds of the box. All individual data points are shown as individual dots. **(C)** Venn diagram depicting the number of prokaryotic OTUs detected in DNA pools of mosquito larvae and shared across Burkina Faso, Guinea and Kenya. **(D)** Prokaryotic microbiome diversity as a function of geographic origin shown both as observed OTUs (left) and Shannon diversity (right). Shannon diversity takes into account both richness and evenness while Observed diversity considered only richness. Mosquito larvae sampled in Kenya have significantly greater prokaryotic diversity measured by either metric, **, $p \leq 0.01$. Box plots as in B.

samples sampled from any two of the three countries. Overall, most of the eukaryotic OTUs (84%) were detected in only one country. *Anopheles* larvae from Burkina Faso display the greatest number of unique eukaryotic OTUs. Eukaryotic microbiome diversity does not significantly differ across countries, but samples from Kenya show a greater range in Observed and Shannon diversity values (**Figure 4B**) (**Supplementary Table 5**).

For the prokaryotic microbiome, *Anopheles* samples from Kenya displayed the greatest taxonomic diversity as well as number of unique OTUs (**Figure 4C**). Approximately 10% of prokaryotic OTUs were shared across all three countries, and an additional 27% of OTUs were shared across any two countries. Thus, the majority of OTUs identified (63%) were sampled from only one country (**Figure 4D**), a finding similar to the eukaryotic microbiome. Diversity of the prokaryotic microbiome measured by either observed or Shannon diversity indicate that Kenyan *Anopheles* larvae display significantly greater prokaryotic microbiome diversity than those from Burkina Faso, while prokaryotic diversity between *Anopheles* from the two West African sites, Burkina Faso and Guinea, is not different. This result for the prokaryotic microbiome is in contrast to the eukaryotic microbiome, where significant richness differences

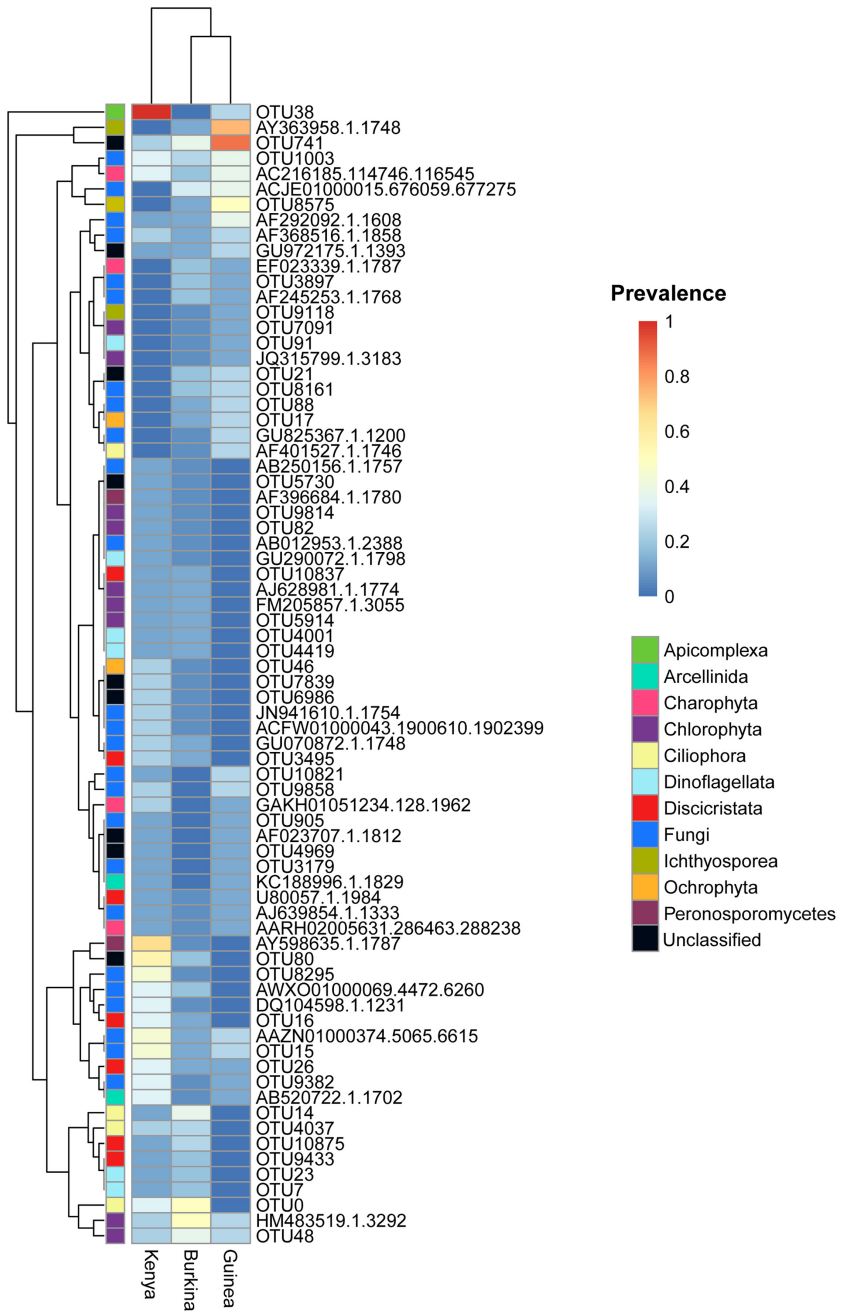

FIGURE 5 | Eukaryotic OTUs detected in mosquito larvae display significant heterogeneity in abundance within and across countries. The heatmap includes the 74 eukaryotic OTUs detected in mosquito larvae collected in at least 2 countries. OTU prevalence is computed as the number of DNA pools with non-zero OTU abundance divided by the number of DNA pools from each country [n = 15 (Burkina), 8 (Guinea), and 9 (Kenya)]. The left most column is colored to indicate taxonomic ranks in the SILVA119 reference taxonomy. Black phylogenetic ranks correspond to unclassified OTUs by both sequence similarity and phylogenetic placement.

were not observed among countries sampled. Interestingly, despite geographic proximity between Burkina Faso and Guinea pools, both share more prokaryotic OTUs with larvae sampled from Kenyan pools than they do with one another which could be explained by the high species richness profiles in Kenyan sample (see **Figure 4D**).

Despite the fact that country of origin was the most significant covariate explaining microbiome composition for both the prokaryotic and eukaryotic microbiome, we also examined the influence of mosquito species after blocking for country. Sample sizes allowed for a comparison of diversity across *A. arabiensis, A. coluzzii*, and *A. gambiae* and showed that there was a significant

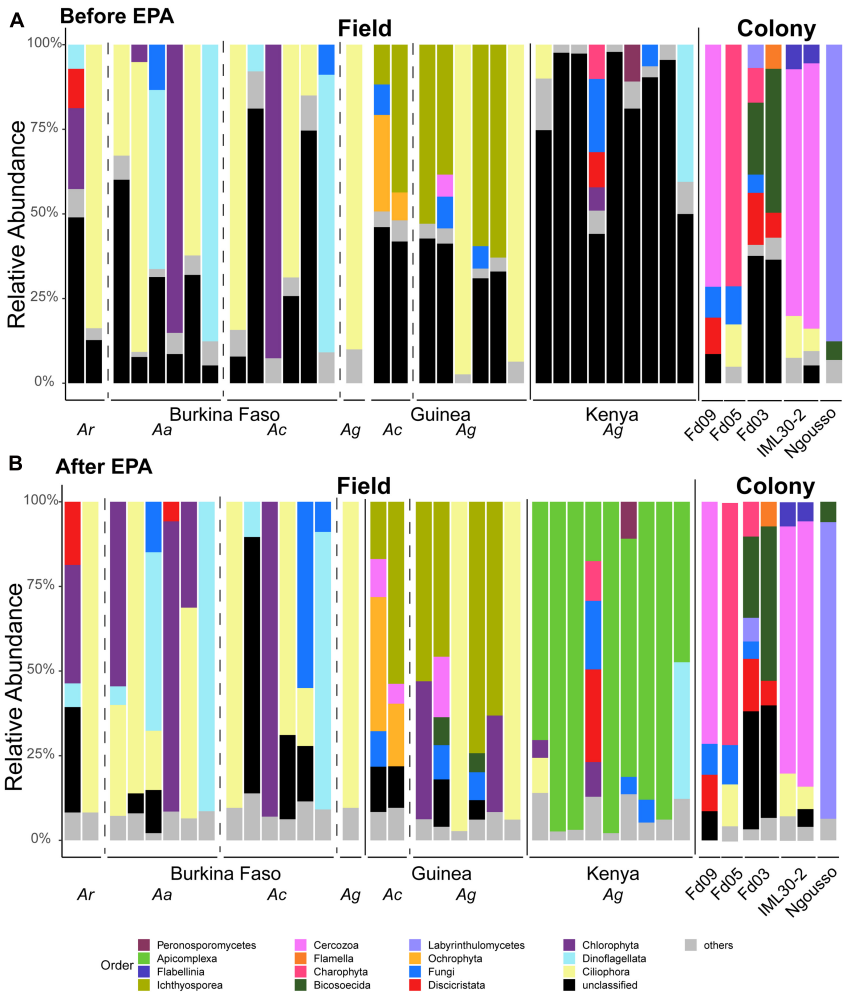

FIGURE 6 | Taxonomic assignments of eukaryotic microbes are improved by employing sequence similarity and phylogenetic placement. Taxonomic Profiles of the micro eukaryotic members of the microbiome before **(A)** and after **(B)** application of the Evolutionary Placement Algorithm (EPA). OTUs are called at the Order level. All OTUs present at less than 5% frequency are grouped together and classified as others. Sequences for which there was not a Silva database match are termed unclassified. Each bar represents the micro eukaryotic taxonomic composition of one pool of larval samples from a given country or laboratory colony. Dashed vertical lines separate samples of different mosquito species (*Ar*, *Anopheles rufipes*; *Ac*, *Anopheles coluzzii*; *Ag*, *Anopheles gambiae*) and solid lines separate samples from different countries and = samples from the field from those from laboratory colonies. All colonies yielding eukaryotic microbes after rarefaction are *A. coluzzii*.

difference in observed diversity for the eukaryotic microbiome, but no significant differences due to mosquito species for Shannon diversity of the eukaryotic microbiome nor for either observed or Shannon diversity of the prokaryotic microbiome (**Supplementary Figure 5**). Mosquito species is confounded with larval site ecology and dissection of their independent effects will require further work.

Ecological Fluctuation of Eukaryotic OTU Abundance

We analyzed the patterns of eukaryotic OTU prevalence among sample DNA pools grouped by country of origin (**Figure 5**). In particular, we wished to identify eukaryotic microbial taxa that could be consistent with an epidemic mode of spread and therefore suggestive of a potentially pathogenic microbe for mosquitoes. We filtered for taxa that fulfill two main criteria: (i) ecologically widespread, indicating taxa that may have had a large generalized impact upon mosquitoes and their ancestors, and (ii) fluctuating prevalence across individuals and geographic sites. The current data cannot exclude other possible explanations for this pattern, for example stochasticity or environmental heterogeneity, but it serves to prioritize candidates for follow-up studies. We also reasoned that ecologically widespread presence of an OTU could be a marker of an efficient colonizer, which could be easier to adapt to culture in the laboratory for biological studies of mosquito immunity and potential development as a biological control agent.

The eukaryotic OTUs display multiple patterns of ecological prevalence. Interestingly, a group of eukaryotic OTUs (**Figure 5**, displayed at the top of the prevalence heatmap) are highly prevalent in *Anopheles* larvae from Kenya and Guinea, and

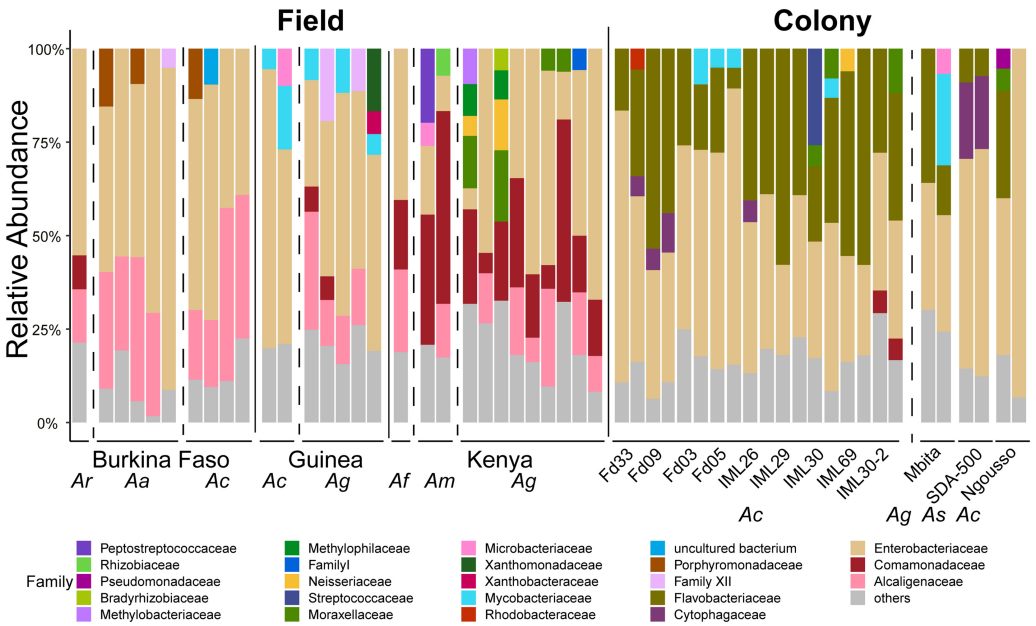

FIGURE 7 | Taxonomic profiles of the prokaryotic microbiome in larvae sampled from the field and from laboratory colonies. OTUs are shown at the Family level. All OTUs present at less than 5% frequency are grouped together and classified as others. Each bar represents the prokaryotic taxonomic composition of one pool of larval samples from a given country or laboratory colony. All larval sample DNA pools contain only samples from a single mosquito species. Dashed vertical lines separate samples of different mosquito species (*Ar*, *Anopheles rufipes*; *Ac*, *Anopheles coluzzii*; *Ag*, *Anopheles gambiae; Af, Anopheles funestus; Am, Anopheles maculatus; As, Anopheles stephensi*) and solid lines separate samples from different countries and samples from the field from those from laboratory colonies.

display fluctuating patterns of ecological prevalence. This group includes in the phylum Apicomplexa, OTU38_Ophryocystis; in the class Ichthyosporea, OTU_AY363958.1.1748; and the unclassified eukaryotic taxon, OTU741.

Each of these three fluctuating OTUs display prevalence at or near 1.0 in collections from at least one country, while at the same time being present across at least two of the three countries (OTU_AY363958.1.1748, DNA pools positive Burkina Faso 2/15, Guinea 6/8, Kenya 0/9; OTU741, DNA pools positive Burkina Faso 6/15, Guinea 7/8, Kenya 2/9; OTU38_Ophryocystis, DNA pools positive Burkina Faso 0/15, Guinea 2/8, Kenya 9/9). The OTU38_Ophryocystis is present in both East and West Africa, suggesting that its absence in the Burkina Faso sequences could be due to undersampling, while OTU_AY363958.1.1748 was only seen in the West African samples, which could be due to undersampling, or could suggest that its geographic range may not include East Africa.

The apicomplexan OTU38_Ophryocystis was described above. The OTU_AY363958.1.1748 belongs to the little-known opisthokont protist clade of Ichthyosporea, which is considered to be near the animal-fungal divergence, and which have been observed as parasites of fish and amphibians but also have relatives that are obligate arthropod gut endosymbionts (Marshall and Berbee, 2010; Reynolds et al., 2017; Borteiro et al., 2018). Finally, the unclassified OTU741 displays an almost complete match to the 18S rRNA gene amplicon sequence of an unidentified eukaryote generated from a soil environmental metagenomic survey [Blast score 193, percent nucleotide identity 99.07%, *e*-value 2e-45, 1701 bp, NCBI nucleotide accession number GenBank: MK945962.1 (Starr et al., 2019)]. Taxonomic placement and identification of OTU741 will require further analysis, as taxonomic databases for analysis of eukaryotic microbes are far less mature than those for prokaryotes.

Effects of Laboratory Colonization and Adaptation on *Anopheles* Microbiomes

We analyzed eukaryotic and prokaryotic microbial composition from larvae of insectary-maintained laboratory colonies and compared these to the composition of the field-caught larval samples (**Figures 6**, **7**, respectively, and **Supplementary Table 1**). We first compare eukaryotic microbial profiles harbored by laboratory colonies to those of the field samples, and next compare microbial overlap between colonies and field samples, and finally compare microbial profiles among the laboratory colonies. Subsequently, the prokaryotic profiles are similarly analyzed.

First, the composition of the eukaryotic microbes found in laboratory colonies is nested within the overall distribution of the field samples and do not form a distinct cluster, when analyzed qualitatively by Bray Curtis Principal Coordinate Analysis (PCoA, **Figure 8A**). Examination of the PCoA indicates that the field samples display greater variation across samples than their laboratory colony counterparts, which suggests less overlapping OTUS in field samples (**Figure 8B**, left panel). Interestingly, the Shannon diversity measure was not significantly different between field and colony samples (**Figure 8B**), indicating that a similarly low number of eukaryotic taxa predominate per sample

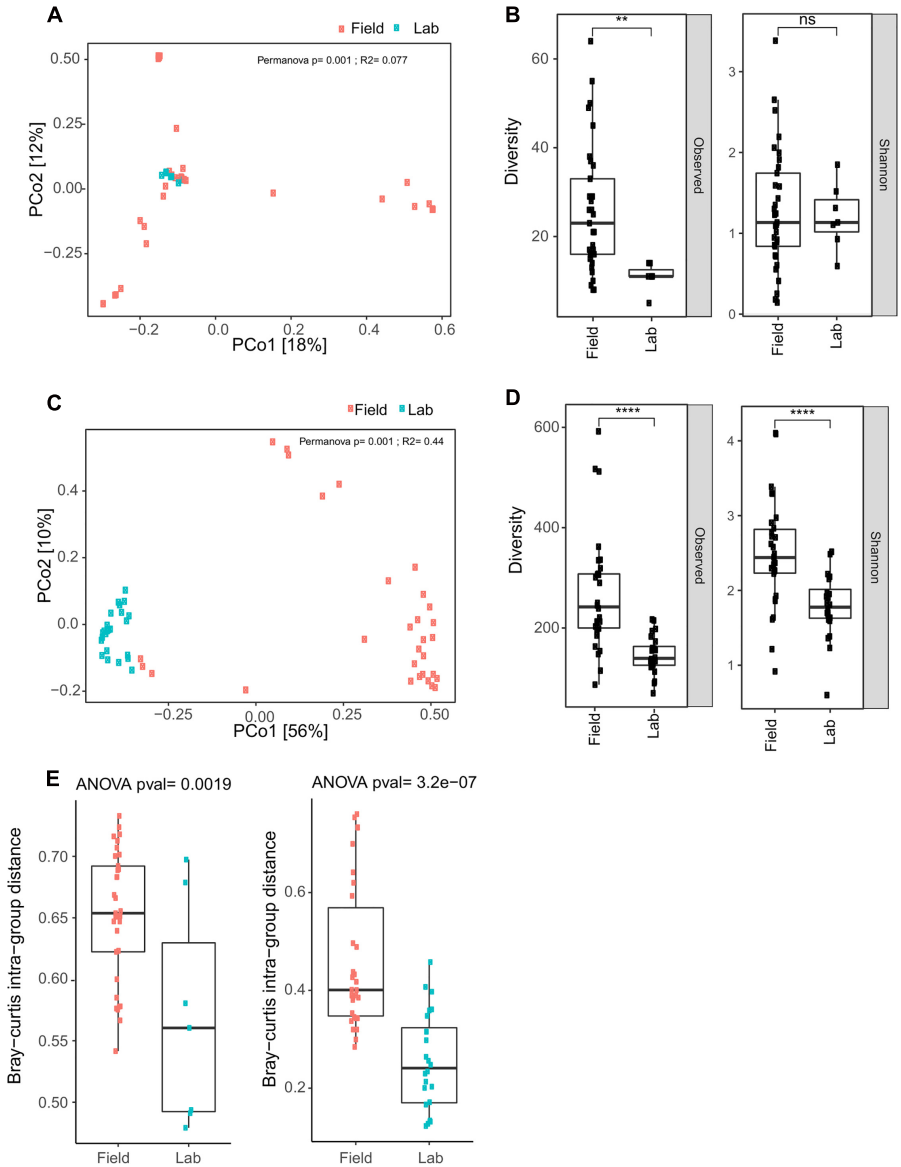

FIGURE 8 | Field collected samples display greater eukaryotic and prokaryotic microbial diversity than laboratory colony samples. **(A)** Bray Curtis Principal Coordinate Analysis (PCoA) for eukaryotic OTU data reveal clustering of field and laboratory colony samples. The cluster of eukaryotic microbes in the colony samples is nested within the distribution of data points for the field samples. **(B)** Observed diversity of eukaryotic taxa is significantly greater in field collected samples as compared to laboratory colony samples, but there is no significant difference in Shannon diversity (ns, non-significant; $p > 0.05$; **, $p \leq 0.01$). Upper and lower bounds of the box indicate the third and first quartiles, respectively, and the line within the box indicates the median. Whiskers extends to the largest values no further than 1.5 time the inter-quartile range from the corresponding upper and lower bounds of the box. All individual data points are shown as individual dots. **(C)** Bray Curtis PCoA for prokaryotic microbiome data displays distinct clustering of field and laboratory samples. Inter-sample diversity is less among colony samples as compared to field collected samples, as indicated by the tighter clustering of individual data points for the colony samples. **(D)** Both observed and Shannon diversity measures are significantly greater in field collected samples as compared to laboratory colony samples (****, $p \leq 0.0001$). **(E)** Bray-Curtis intra-group distance, a measure of within group diversity is significantly greater for field samples as compared to laboratory colony samples for the eukaryotic microbial diversity (left panel) and for the prokaryotic microbial diversity (right panel), indicating greater difference among field samples than larval samples collected from laboratory colonies.

in both field and colony contexts, regardless that the predominant taxa themselves are not necessarily the same between samples. This is also consistent with the impression taken from the taxonomic histograms (**Figures 6A,B**). The Shannon diversity result may suggest an inherent biological property of the eukaryotic microbiome, if these observations mean that only a limited number of eukaryotic microbial taxa can coexist within the ecological niche of a given *Anopheles* larvae. Further studies will be required to elaborate on these ecological and demographic properties of the eukaryotic microbiome.

Second, the overlap of eukaryotic microbial taxa is comprised of ten OTUs present in both field and laboratory colony samples

at an abundance of ≥5 following rarefaction (**Table 1**). Four of the OTUs shared among field and colony samples are fungi. Of these, at least *Aspergillus* is probably present because the spores are ubiquitous aerosol environmental microbes. The other fungi present in both laboratory colonies and field samples are *Pleosporales*, *Wallemia*, and *Malassezia*. The *Pleospora* are a genus of ascomycete fungi. Both *Malassezia* and *Aspergillus* have been previously reported as members of the eukaryotic microbiome in *Aedes* larvae (Shelomi, 2019). The *Anopheles* laboratory colonies harbor an additional 16 OTUs present at an abundance ≥5 following rarefaction that were not detected in field samples in the current study (**Table 1**). These include two OTUs of the genus *Vannella*, which is an ameba found in soil and freshwater environmental samples (Smirnov et al., 2007). Five other taxa unique to the laboratory colonies belong to the clade termed "Stramenophiles, Alveolates and Rhizaria" (SAR), and include two members of the *Ciliophora* and two *Cercozoa*. These 16 colony-specific OTUs were detected in at least one laboratory colony and were not detected in any field samples, thus representing either rare natural taxa that expanded during colonization, or else taxa acquired during colonization.

Finally, comparing eukaryotic microbial profiles among laboratory colonies interestingly reveals that the eukaryotic microbiomes of these mosquito colonies remain distinct (**Figure 9** and **Table 1**), despite the fact that the colonies are co-housed in the same insectary facility, and are exposed to the same water, food and other environmental variables. For example, colonies Fd09 and IML30-2 both harbored members of Cercozoa, a diverse group of heterotrophic protozoa that live in soil and freshwater, include pathogens of agricultural plants and aqua-cultured mollusks, and are also a component of the *Arabidopsis thaliana* eukaryotic microbiome (Braithwaite et al., 2018; Sapp et al., 2018; Irwin et al., 2019; Kang et al., 2019). The two replicate samples from colony Fd03 harbored high proportions of Bicosoecida, an order of unicellular flagellates including notable extremophile members adapted to low oxygen or high salt conditions (Yubuki et al., 2010; Harding and Simpson, 2018). The colony Fd05 predominantly harbored members of *Charophyta*, a group of freshwater green algae, including members with antioxidant activities (Kumar et al., 2015). The widely used Ngousso colony predominately harbored members of Labyrinthulomycetes, a group of protists that acquire resources by means of ectoplasmic slime nets, and include important pathogens of at least aqua-cultured mollusks (Rubin et al., 2014; Iwata and Honda, 2018).

Turning to the *Anopheles* prokaryotic microbial profiles, PCoA reveals that, differently from the eukaryotic microbiome, the prokaryotic microbial composition of the colony samples forms a largely distinct cluster from the field samples (**Figure 8C**). Observed OTU diversity is significantly higher in field as compared to colony samples, similar to the eukaryotic microbes. The observed number of prokaryotic OTUs for field samples averaged 264.31 across 29 samples (range: 81–601). For the laboratory colonies, observed prokaryotic OTUs average 148 across 24 samples (range: 69–219). Prokaryotic Shannon diversity is significantly higher in field samples as compared to colony samples (**Figure 8D**). The greater observed prokaryotic diversity in *Anopheles* field samples is also consistently detected by other indices of alpha diversity, including Chao1 (355 field to 227 laboratory colony), and Ace (367 field to 232 laboratory). These different alpha diversity metrics analyze evenness (Shannon) as well as species richness (Chao1, Ace). Notably, however, the laboratory colonies harbor similar proportions of rare prokaryotic OTUs (grouped together as "others," gray bar in **Figure 7**) as the wild-caught field sample (**Supplementary Table 6**). These diversity results suggest that the prokaryotic microbiome may not display the same biological limit in the number of coexisting taxa per sample, as suggested above by the similarity of eukaryotic Shannon diversity between field and colony samples, and could indicate important differences in the biology of the eukaryotic and prokaryotic fractions of the *Anopheles* microbiome. Further work should focus on elucidating the meaning of these differences.

In comparing prokaryotic profiles between field and colony samples, members of the *Enterobacteriaceae* are predominant at the family level in both sample types (**Figure 7**). The *Enterobacteriaceae* are gram-negative bacteria that include *Salmonella*, *Escherichia*, *Klebsiella* and *Shigella*. The betaproteobacteria families of *Comamonadaceae* and *Alcaligenaceae* are present at >5% in many field samples but are largely absent from laboratory samples. *Flavobacteriaceae*, a family of Bacteroidetes are present above 5% in only laboratory colony samples.

To quantify differences in microbial composition among field and laboratory colony samples, we measured the Bray-Curtis intra-group distance. For both the eukaryotic and prokaryotic microbes, this measure is significantly higher in field samples as compared to laboratory colony samples (**Figure 8E**). Thus, the compositional profiles of both eukaryotic and prokaryotic microbiomes are significantly different between field and laboratory colony samples, with field samples exhibiting greater variability. These results indicate that *Anopheles* larvae sampled in the field display greater inter-sample difference than do larval samples collected from laboratory colonies.

DISCUSSION

Comprehensive mosquito sampling and characterization of the eukaryotic and prokaryotic microbiomes generated a number of new findings. First, geography is the strongest correlate of microbiome composition at both the eukaryotic and prokaryotic levels. Country of sample collection has greater explanatory power for microbial diversity than mosquito species, larval site type or larval site ecology. Second, there are significant differences in both eukaryotic and prokaryotic microbiome diversity both among and between field and laboratory colony samples with field samples showing both greater overall diversity and greater across sample variability. Despite these field and laboratory colony differences, there are shared OTUs present in both field and laboratory colony samples. Wild OTUs that are found in laboratory colonies may provide a convenient opportunity for mechanistic studies of microbiome interactions with the host. The comprehensive description of microbiome

TABLE 1 | Eukaryotic OTUs in *Anopheles* laboratory colonies.

OTU name	Taxonomic assignment							#col	Field samples
AY183888.1.1959	Eukaryota	Amoebozoa	Discosea	Flabellinia	Vannellida	Vannella	NA	1	No
OTU9921	Eukaryota	Amoebozoa	Discosea	Flabellinia	Vannellida	Vannella	NA	1	No
EU186022.1.1834	Eukaryota	Amoebozoa	Gracilipodida	Flamella	Flamella arnhemensis	Flamella arnhemensis	Flamella arnhemensis	1	No
ACUP0100749 8.9215.11012	Eukaryota	Archaeplastida	Chloroplastida	Charophyta	NA	NA	NA	1	No
AGNK010040 45.34055.35850	Eukaryota	Archaeplastida	Chloroplastida	Charophyta	Magnoliophyta	Liliopsida	NA	1	No
U80057.1.1984	Eukaryota	Excavata	Discoba	Discicristata	Tetramitia	Naegleria	NA	1	Yes; BF
AY753597.1.20 86	Eukaryota	Excavata	Discoba	Discicristata	Neobodonida	Rhynchomonas	Rhynchomonas nasuta	2	No
OTU189	Eukaryota	Excavata	Discoba	Discicristata	Tetramitia	NA	NA	1	No
DQ104591.1.1252	Eukaryota	Opisthokonta	Holozoa	NA	NA	NA	NA	1	Yes; K
ACJE01000015. 676059.677275	Eukaryota	Opisthokonta	Nucletmycea	Fungi	Trichocomaceae	Aspergillus	NA	1	Yes; BF, G
OTU1003	Eukaryota	Opisthokonta	Nucletmycea	Fungi	Dothideomycetes	Pleosporales	NA	1	Yes; BF, G, K
OTU3897	Eukaryota	Opisthokonta	Nucletmycea	Fungi	Incertae Sedis	Wallemia	NA	1	Yes; BF
OTU8161	Eukaryota	Opisthokonta	Nucletmycea	Fungi	Incertae Sedis	Malassezia	Uncultured fungus	2	Yes; G
OTU9	Eukaryota	Opisthokonta	Nucletmycea	Fungi	Eurotiomycetes	NA	NA	2	No
OTU2074	Eukaryota	SAR	Alveolata	Ciliophora	Cyrtophoria	NA	NA	1	No
OTU3066	Eukaryota	SAR	Alveolata	Ciliophora	Colpodida	NA	NA	1	No
AJ514867.1.1767	Eukaryota	SAR	Rhizaria	Cercozoa	Rhizaspididae	Rhogostoma	NA	2	Yes; G
OTU1001	Eukaryota	SAR	Rhizaria	Cercozoa	Thecofilosea	uncultured	NA	1	No
OTU8372	Eukaryota	SAR	Rhizaria	Cercozoa	Rhizaspididae	Rhogostoma	NA	1	No
OTU2896	Eukaryota	SAR	Stramenopiles	Bicosoecida	NA	NA	NA	1	Yes; G
OTU69	Eukaryota	SAR	Stramenopiles	Bicosoecida	CH1-2B-3	Uncultured stramenopile	Uncultured stramenopile	1	Yes; G
OTU94	Eukaryota	SAR	Stramenopiles	Bicosoecida	Siluaniidae	Paramonas globosa	Paramonas globosa	1	No
OTU741	Unassigned	NA	NA	NA	NA	NA	NA	1	Yes; G
OTU18	Unassigned	NA	NA	NA	NA	NA	NA	3	No
OTU2085	Unassigned	NA	NA	NA	NA	NA	NA	1	No
OTU5832	Unassigned	NA	NA	NA	NA	NA	NA	1	No

OTUs with abundance ≥5 following rarefaction.

#col, number of laboratory colonies with the OTU; field samples, present in samples from BF, Burkina Faso, K, Kenya, G, Guinea.

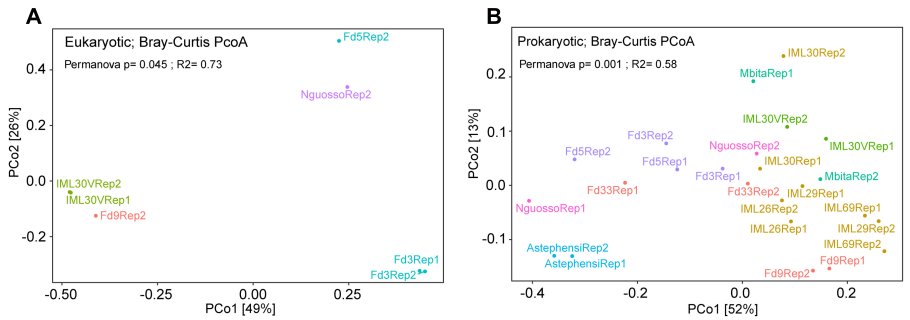

FIGURE 9 | Principal Coordinate Analysis (PCoA) for microbial diversity in laboratory colonies of *Anopheles*. Co-housed *Anopheles* colonies maintain diversity for their **(A)** eukaryotic and **(B)** prokaryotic microbiomes. PCoA for the eukaryotic microbiome of seven colony samples with greater than 900 sequence reads following rarefaction. **(B)** PCoA for the prokaryotic microbiome of laboratory colonies. All colonies were sampled for both eukaryotic and prokaryotic microbiomes, but only those samples yielding adequate sequence information were analyzed (10,000 sequences per sample for 16S rRNA gene data and 900 sequences per sample for 18S rRNA gene data). Both plots highlight the similarity eukaryotic and prokaryotic microbial profiles across experimental replicates (all data points are labeled either Rep1 or Rep2). The variable tested in the Permanova was the colony of origin of lab pools.

composition presented may offer candidates for new malaria control tools, including classic biological control agents.

The findings of both the eukaryotic and prokaryotic microbiomes confirm trends that have been reported previously only for the mosquito prokaryotic microbiome. Environment appears to exert a larger influence on shaping the mosquito microbiome than does the genetics of species differences (Yun et al., 2014; Rothschild et al., 2018). Nevertheless, genetically distinct laboratory colonies maintained in a shared controlled environment may display stable microbiome differences based on genetics or on vertical transmission via the larval rearing environment. Multiple pieces of evidence support the finding that mosquitoes acquire their microbiome each generation from their environment. This evidence includes extensive overlap of microbial composition between mosquito larval and their aquatic habitat (Boissiere et al., 2012; Coon et al., 2014; Gimonneau et al., 2014; Buck et al., 2016), a lack of microbes in the gut of newly hatched larvae (Coon et al., 2014) and extensive variability in mosquito midgut communities which would be unlikely if microbiomes were acquired from parents (Boissiere et al., 2012; Gimonneau et al., 2014; Buck et al., 2016). Thus, although environment may play a predominant role in determining the array of ambient taxa that a newly hatched larva will encounter, our current findings also suggest that host genetics may play an important part in shaping the precise community of taxa that persist within the host. The possibility and basis of stable differences for microbial profiles among co-housed mosquito colonies deserves attention in further work.

Deriving Value From the Eukaryotic Microbiome

With comprehensive characterization of eukaryotic microbiome members in both field samples and laboratory colony samples, efforts can shift to more mechanistic understandings of the role of these microbiome members and how they are balanced with prokaryotic and viral members of the microbiome. Future vector control efforts could potentially use apicomplexan members of the mosquito eukaryotic microbiome that may compete with *Plasmodium* superinfection, or as biological control agents for population control. The apicomplexan OTU38_Ophryocystis is widespread in Kenyan larval samples, but was not detected in laboratory colony samples. There are other eukaryotic taxa associated with laboratory colonies that could be exploited in mechanistic studies, including ten eukaryotic OTUs that are also present in field samples.

Little work has been published on the eukaryotic microbiome of mosquitoes. This is largely due to the greater technical challenge in separating eukaryotic microbial 18S rRNA sequences from the mosquito host or vertebrate bloodmeal (Belda et al., 2017). There are reports of fungal isolation from *Anopheles* larvae and adults using standard microbiological techniques (Ricci et al., 2011a,b; Bozic et al., 2017) as well as published results suggesting fungus can have negative (Bargielowski and Koella, 2009) and positive effect on *Plasmodium* infectivity (Anglero-Rodriguez et al., 2016). The current work examines the composition of eukaryotic microbiome taxa through similarity with available 18S rRNA sequence databases, which are less mature as compared to those for prokaryotes. To circumvent this limitation, we generated taxonomic assignments based on sequence similarity and phylogenetic placement. Application of this analytical approach allowed identification of a large fraction of novel eukaryotic microbiome OTUs found in *Anopheles*. Moreover, these results highlight that almost all of the unassigned OTUs detected in field samples from Kenya belong to the phylum Apicomplexa as OTU38_Ophryocystis.

New vector-based tools are needed to bolster efforts toward malaria elimination and eradication. The *Anopheles* prokaryotic microbiome has been reasonably well characterized, and has yielded candidates for malaria vector or transmission control. Both natural and genetically modified microbes as well as Wolbachia have been shown to block parasite transmission (Gao H. et al., 2020). In comparison, studies of the eukaryotic microbiome are in their infancy and more studies are necessary to understand the biology of the eukaryotic microbiome. More data on the eukaryotic microbiome as well as the mosquito virome will inform the tripartite interactions between prokaryotes, eukaryotes and viruses that together

shape the ecological community of commensal, symbiotic and pathogenic microorganisms affecting mosquito physiology and vectorial capacity.

DATA AVAILABILITY STATEMENT

All sequence files are available from the EBI European Nucleotide Archive database (http://www.ebi.ac.uk/ena/) under ENA study accession number PRJEB40885. Assembled eukaryotic OTU 18S rRNA amplicon sequences are available in this article, **Supplementary File 1**, and assembled prokaryotic OTU 16S rRNA OTU sequences, **Supplementary File 2**.

ETHICS STATEMENT

The animal study was reviewed and approved by the research animal ethics committee of the Institut Pasteur, 'C2EA-89 CETEA Institut Pasteur' as protocol number B75-15-31. The Institut Pasteur ethics committee is authorized by the French law N° 2001-486, which is aligned with Directive 2010/63/EU of the European Commission on the protection of animals used for scientific purposes.

AUTHOR CONTRIBUTIONS

EC, KV, and MR: designed the research and wrote the manuscript. EC, BC, TB, KE, MC, SZ, MB, AG, WG, AB, ST, N'FS, and MR: performed the research. EC and MR: analyzed the data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.635772/full#supplementary-material

Supplementary Figure 1 | Quality profile plots of raw sequence files and assembled amplicons of pools included in the study from FASTQC quality checking. **(A)** Quality profile of forward (R1) and reverse (R2) Illumina 16s MiSeq reads before the Pandaseq assembly step. Each line depicts the mean quality score of read positions with 10 and 90% quantiles depicted as whiskers. Background color signifies FASQC standards for good (green), reasonable (yellow) and poor (red/pink) quality. **(B)** Quality profile of the 16S amplicons assembled by Pandaseq. **(C)** Quality profile of forward (R1) and reverse (R2) Illumina 18s MiSeq reads before the Pandaseq assembly step. **(D)** Quality profile of the 18S amplicons assembled by Pandaseq.

Supplementary Figure 2 | Rarefaction analyses of field and laboratory colony DNA pools. Rarefaction plots of 16S rRNA gene **(A)** and 18S rRNA gene **(B)** datasets based on 4 alpha-diversity indexes (Observed Species, ACE, Chao1, Shannon). Curves are colored by the source of the sample (laboratory colony samples, orange; field samples, blue). Vertical dashed lines correspond to the threshold of rarefaction fixed in the analyses to correct for differences in sequencing depth (10,000 sequences per sample for 16S rRNA gene data and 900 sequences per sample for 18S rRNA gene data).

Supplementary Figure 3 | Evaluation of PNA blocking efficiency on 18S rRNA gene data: Bar plots representing the relative abundances of Insecta (orange) and non-Insecta (blue) sequences in 18S datasets of mosquito DNA pools stratified by source (laboratory colony, field). Those samples with Xs above the bars had inadequate numbers of 18S reads for analysis and were not analyzed further. The failure of laboratory colony samples to provide ample non-mosquito 18S sequence reads was not due to low sequence depth as colony samples were sequenced to equal or greater depths than field samples.

Supplementary Figure 4 | Water blanks show no significant contamination affecting either eukaryotic or prokaryotic microbiome results. **(A)** The 18S read abundance of non-insecta reads from all samples for all taxa detected in water blank samples. **(B)** The 18S read abundance for only those samples that passed rarefraction and were analyzed in the paper. In neither **A** nor **B** are there any 18S eukaryotic microbial reads in experimental samples that were derived from water controls. **(C)** 16S read abundance for all reads with reads separate by those also detected in water control samples and those absent from water control samples. **(D)** As in C, but only for those samples that passed rarefraction and were analyzed in the paper.

Supplementary Figure 5 | Diversity profiles of field pools across different mosquito species. **(A)** 18s OTU richness distributions across mosquito species. **(B)** 18s Shannon diversity distributions across mosquito species. **(C)** 16s OTU richness distributions across mosquito species. **(D)** 16s Shannon diversity distributions across mosquito species. Statistical comparisons were carried out between A. coluzzii, A. arabiensis and A. gambiae (those species with sample sizes greater than three per group) by means of a Kruskal–Wallis non-parametric test blocked for country of origin using the independence_test function of the coin R package.

Supplementary Table 1 | Description of pooled larval samples used for 16S and 18S amplicon sequencing.

Supplementary Table 2 | Eukaryotic OTUs before application of the EPA.

Supplementary Table 3 | Eukaryotic OTUs after application of the EPA.

Supplementary Table 4 | Prokaryotic OTUs.

Supplementary Table 5 | Diversity measures of eukaryotic OTUs.

Supplementary Table 6 | Diversity measures of prokaryotic OTUs.

Supplementary File 1 | Fasta file of eukaryotic OTU sequences.

Supplementary File 2 | Fasta file of prokaryotic OTU sequences.

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Diet–Microbiota Interactions Alter Mosquito Development

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Gut microbes and diet can both strongly affect the biology of multicellular animals, but it is often difficult to disentangle microbiota–diet interactions due to the complex microbial communities many animals harbor and the nutritionally variable diets they consume. While theoretical and empirical studies indicate that greater microbiota diversity is beneficial for many animal hosts, there have been few tests performed in aquatic invertebrates. Most mosquito species are aquatic detritivores during their juvenile stages that harbor variable microbiotas and consume diets that range from nutrient rich to nutrient poor. In this study, we produced a gnotobiotic model that allowed us to examine how interactions between specific gut microbes and diets affect the fitness of *Aedes aegypti*, the yellow fever mosquito. Using a simplified seven-member community of bacteria (ALL7) and various laboratory and natural mosquito diets, we allowed larval mosquitoes to develop under different microbial and dietary conditions and measured the resulting time to adulthood and adult size. Larvae inoculated with the ALL7 or a more complex community developed similarly when fed nutrient-rich rat chow or fish food laboratory diets, whereas larvae inoculated with individual bacterial members of the ALL7 community exhibited few differences in development when fed a rat chow diet but exhibited large differences in performance when fed a fish food diet. In contrast, the ALL7 community largely failed to support the growth of larvae fed field-collected detritus diets unless supplemented with additional protein or yeast. Collectively, our results indicate that mosquito development and fitness are strongly contingent on both diet and microbial community composition.

Keywords: ***Aedes aegypti*, microbiota diversity, host–microbiota, microbe–microbe, diet–microbe**

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INTRODUCTION

The digestive tract of multicellular animals is the location of nutrient acquisition and absorption, while it is also an ecosystem that hosts communities of microorganisms that are capable of altering animal metabolism, physiology, and development (Sommer and Bäckhed, 2013; Moran et al., 2019). These gut-associated microbial communities can modify animal nutrition by directly serving as a food source or producing factors that have nutritive, digestive, or signaling functions

Abbreviations: ALL7, a simplified 7-member community of bacteria including *Acinetobacter* sp., *Sphingobacterium* sp., *Flectobacillus* sp., *Serratia* sp., *Rahnella* sp., *Microbacterium* sp., and *Escherichia coli*; **RCM**, rat chow mix (laboratory mosquito diet consisting of equal parts (w/w) rat chow, heat-killed torula yeast (*Cyberlindnera jadinii*), and lactalbumin); FF, fish food (TetraColor Tropical Granules).

(e.g., vitamins not found in the diet, digestion of inaccessible compounds in the diet) (Dadd, 1973; Claesson et al., 2012; Russell et al., 2013; Wong et al., 2014; Yamada et al., 2015; Singh et al., 2017; Bing et al., 2018). Animals often consume diets that vary in macronutrient composition that affects the overall nutritional content and the gut microbiota can vary in species membership, community complexity, and abundance (Moeller and Ochman, 2013; Martinson et al., 2017; Fast et al., 2018; Youngblut et al., 2019). Interactions between gut microbes and diet can affect a range of physiological processes in both vertebrates and invertebrates; however, it is often difficult to discern how hosts are affected by microbe–microbe vs. microbe–diet interactions because of the complex microbial communities that many animals harbor and variable diets they consume (Ezenwa et al., 2012; Engel and Moran, 2013; McFall-Ngai et al., 2013; Kohl et al., 2014; Wong et al., 2014; Martino et al., 2018; Guilhot et al., 2019; Kovatcheva-Datchary et al., 2019; Zimmermann et al., 2019). Thus, species that are amenable to simplifying and manipulating microbiota composition while controlling diet can help advance understanding of how microbes and diet interact to affect host fitness (Virk et al., 2016; Keebaugh et al., 2018).

In the case of insects, some species feed on highly specialized diets while others including many detritivores consume variable diets that range from nutrient rich to nutrient poor (Douglas, 1998). Most insect detritivores also host communities of microbes in their gut that are acquired from the environment by feeding (Engel and Moran, 2013). Mosquitoes (family Culicidae) are a diverse group (∼3,500 species) of insects that are primarily aquatic detritivores during their juvenile stages while feeding on vertebrate blood (females) and/or carbohydrates (males and females) as adults (Clements, 1992). Blood feeding by adult females can also result in the transmission of pathogens. Mosquitoes host environmentally acquired gut microbiotas that consist primarily of bacteria, but can also include fungi, algae, protozoa, and viruses (Strand, 2018). Microbiota community composition varies greatly within and between mosquito species as a function of collection site and date, life stage, and sex; all factors that alter the microbes that are encountered by individuals (e.g., females encounter blood, while males do not) (Boissiere et al., 2012; Osei-Poku et al., 2012; Yee et al., 2012; Duguma et al., 2013; Minard et al., 2013; Gimonneau et al., 2014; Kim et al., 2015; Yadav et al., 2015; Buck et al., 2016; Coon et al., 2016b; Muturi et al., 2016a,b; Dickson et al., 2017; Thongsripong et al., 2018; Villegas et al., 2018; Malassigné et al., 2020). Regardless of the highly variable microbial communities observed across mosquitoes and similar to many other insects, the species richness of an individual mosquito's microbiota is much lower than in mammals, which makes them tractable organisms to study how differences in microbial diversity can affect development and nutrition (Yee et al., 2012; Strand, 2018).

Aedes aegypti is a mosquito that prefers subtropical–tropical, urban habitats where it blood feeds on humans and can vector the viruses that cause yellow fever, Dengue fever, and Zika syndrome (Kyle and Harris, 2008; Enserink, 2015). Adult females preferentially lay eggs in small, water-holding containers where larvae develop under physical conditions that include non-freezing temperatures and seasonal photoperiods (Service, 1995; Washburn, 1995; Vezzani, 2007). In the laboratory, *A. aegypti* and other mosquitoes are reared by feeding larvae nutrient-rich diets that also support the growth of microbial communities in the aquatic environment (Bond et al., 2017). While laboratory diets differ widely among research groups, a diet consisting of equal parts (w/w) rat chow, heat-killed torula yeast (*Cyberlindnera jadinii*), and lactalbumin (hereafter named RCM diet) has been used to rear *A. aegypti* and several other mosquito species (and aquatic microorganisms) at the University of Georgia Entomology Department since the 1970s (Foster and Lea, 1975). In contrast, the plant-based detritus diets larvae consume in the field contain much lower amounts of protein and other macronutrients such as fats, but also support microbial communities (Merritt et al., 1992; Kaufman et al., 2002; Xu et al., 2008; Wang et al., 2018).

Studies of detritivorous *Drosophila* spp. indicate that the gut microbiota is either non-essential or only minimally affects the growth of larvae fed protein-rich laboratory diets, whereas consumed microbes benefit larvae fed protein-poor diets by serving concurrently as a protein source and promoting signaling activities that regulate growth functions (Shin et al., 2011; Storelli et al., 2011; Yamada et al., 2015; Bing et al., 2018; Keebaugh et al., 2018). Experimental manipulation of the microbiota indicates that *A. aegypti* larvae do not develop beyond the first instar when reared axenically (germ-free), even when fed the nutrient-rich RCM diet (Coon et al., 2014). However, the addition of an individual bacterial species to generate monoxenic, gnotobiotic larvae generally leads to the rapid develop into adults (Coon et al., 2014). Restoration of development is not restricted to a particular species or community of bacteria, but bacteria must be viable (Coon et al., 2014). Addition of living bacteria also activates several signaling pathways in larvae with functions in nutrient sensing and development (Coon et al., 2017; Vogel et al., 2017; Valzania et al., 2018a,b). Altogether, these findings suggest *A. aegypti* requires a gut microbiota when fed the RCM diet and the nutrient-rich composition of this diet suggests this benefit is not due to bacteria serving as a source of protein but instead viable microbes produce factors that dead microbes cannot provide. In contrast to these results, it was shown recently that autoclaved *E. coli* can promote larval mosquito growth in axenic conditions when fed at very high concentrations in combination with additional high-nutrient dietary components; however, development is delayed and fecundity is reduced relative to mosquitoes conventionally reared (Correa et al., 2018).

To further define the community of microbes that *A. aegypti* larvae require when fed RCM diet, we developed a simplified bacterial community that we could manipulate. We also assessed whether microbes that promote growth when larvae are fed RCM diet also similarly do so when fed (1) commercially prepared tropical fish food (FF), which can also be used to rear *A. aegypti* and other mosquito species in the laboratory, or (2) detritus diets that mimicked diets encountered in the field. Our results indicated that microbiota composition minimally affected larvae fed RCM diet, strongly affected larvae fed FF,

and largely failed to support development when larvae were fed detritus diets unless supplemented with protein or yeast. Yet strikingly, bacteria grew to comparable abundances in cultures across all diets we tested. Our results overall identify a range of fitness outcomes for *A. aegypti* that depend on both microbiota composition and diet and provide a framework for future studies to identify the microbial-derived components that promote mosquito development.

RESULTS

A Simplified Community of Bacteria Produces Progeny of Comparable or Superior Quality With Conventional Rearing

We assembled a microbiota (ALL7) composed of seven taxonomically diverse species of bacteria (**Supplementary Table 1**) that had previously been identified as gut community members in the field or laboratory populations (Coon et al., 2014, 2016b). Each of these species could also be distinguished from the others by colony morphology, differences in resistance to particular antibiotics, or other visual traits (**Supplementary Table 1**). RCM and FF diets differ in terms of specific ingredients but contained near identical amounts of protein, fat, and fiber (**Supplementary Table 2**). Culture flasks containing sterile water were thus inoculated with axenic first instars, RCM or FF diet that had been sterilized by gamma irradiation, and either no microbiota, an ALL7 microbiota, or a conventional microbiota that was collected in March 2018 from a rearing pan containing fourth instars from our laboratory culture of *A. aegypti*. Previous studies indicated that the conventional microbiota in our laboratory culture contained ∼200 species of bacteria (Coon et al., 2014). Axenic larvae fed FF diet with no microbiota under standard rearing conditions (see "Materials and Methods") remained first instars and died after several days without ever molting, which was identical to what occurs when axenic larvae are fed RCM diet (Coon et al., 2014). Using 1/10 diluted 869 agar (Eevers et al., 2015) to estimate the abundance of bacteria, results indicated that the ALL7 and conventional microbiota grew to comparable densities (10^9 cfu/ml) in cultures containing RCM or FF diet regardless of whether mosquito larvae were present or absent (**Supplementary Figure 1A**). Two measures of mosquito fitness, development time to pupation and adult size as estimated by wing length, were also equivalent or superior for progeny in cultures inoculated with the ALL7 microbiota and either RCM or FF diet when compared with cultures inoculated with a conventional microbiota (**Supplementary Figure 1B**). No significant differences were detected in the adult size of males or females or in the development time to pupa of mosquitoes reared 6 months later using the ALL7 microbiota (**Supplementary Figure 1C**). In contrast, *A. aegypti* reared with a conventional microbiota collected in September 2018 exhibited small but significant differences in development time and size when compared with the conventional microbiota collected in March 2018, which potentially reflected changes in community composition (**Supplementary Figure 1C**). Overall, these results indicated that ALL7 is a simplified microbial community that functionally recapitulates or outperforms the microbiota from our conventionally reared culture on two laboratory diets.

Monoxenic Rearing on FF Diet Adversely Affects Mosquito Fitness

We determined that each bacterium, when cultured individually in flasks with axenic larvae and RCM or FF diet, grew to densities (**Supplementary Figure 2A**) that were comparable with the estimated density of total bacteria present in cultures inoculated with the ALL7 community (**Supplementary Figure 1A**). Although each bacterial species alone generally grew to similar densities in cultures containing RCM and FF diet, *Acinetobacter* grew to about 10-fold lower abundance in RCM (**Supplementary Figure 2A**). Using FF diet further indicated that each species grew to similar densities in both the presence and absence of larvae (**Supplementary Figure 2B**). We therefore asked if larvae inoculated with individual bacterial species could develop into adults when fed RCM or FF diet.

There were developmental changes in larvae grown with different bacterial species or grown on different diets (**Figure 1**). No larvae grew beyond the first instar in cultures inoculated with only *Microbacterium*, which similarly occurred in an earlier study where larvae were fed RCM diet and correlated with *Microbacterium* being unable to persist in the larval gut in the absence of other community members (Coon et al., 2014, 2016a). For the other six species in the ALL7 community, monoxenic rearing showed that each supported larval growth to the adult stage (**Figure 1**). When fed RCM diet, no differences in adult size were detected between these monoxenic treatments and the ALL7 control, but development time to pupation was longer in cultures inoculated with *Serratia*, *Rahnella*, or *Escherichia* (**Figure 1**). When fed FF diet, development time to pupation was longer than the ALL7 control in cultures individually inoculated with each bacterial species (**Figure 1**). Cultures inoculated with *Rahnella* or *Escherichia* exhibited especially long delays ($\bar{x}$ = 17.1 and 12.8 days, respectively). Adult females from all monoxenic cultures fed FF diet were also significantly smaller than females from ALL7 control while males in some monoxenic cultures (*Acinetobacter*, *Sphingobacterium*, *Serratia*) were also significantly smaller (**Figure 1**).

For the preceding assays, the individual mosquitoes within each treatment served as the unit of replication when comparing performance metrics between treatments. However, for a subset of these treatments, we also compared progeny from different culture flasks to assess whether outcomes were consistent with the results presented in **Figure 1**. Larvae inoculated with the ALL7 community, which exhibited rapid development times and large average adult sizes when fed either RCM or FF diet, exhibited similar developmental times and sizes when progeny from different flasks were compared with one another (**Supplementary Figure 3**). Further, larvae inoculated with only *Rahnella* or *Escherichia* and fed FF diet exhibited among the longest development times in **Figure 1**, while comparing progeny from different flasks also showed that

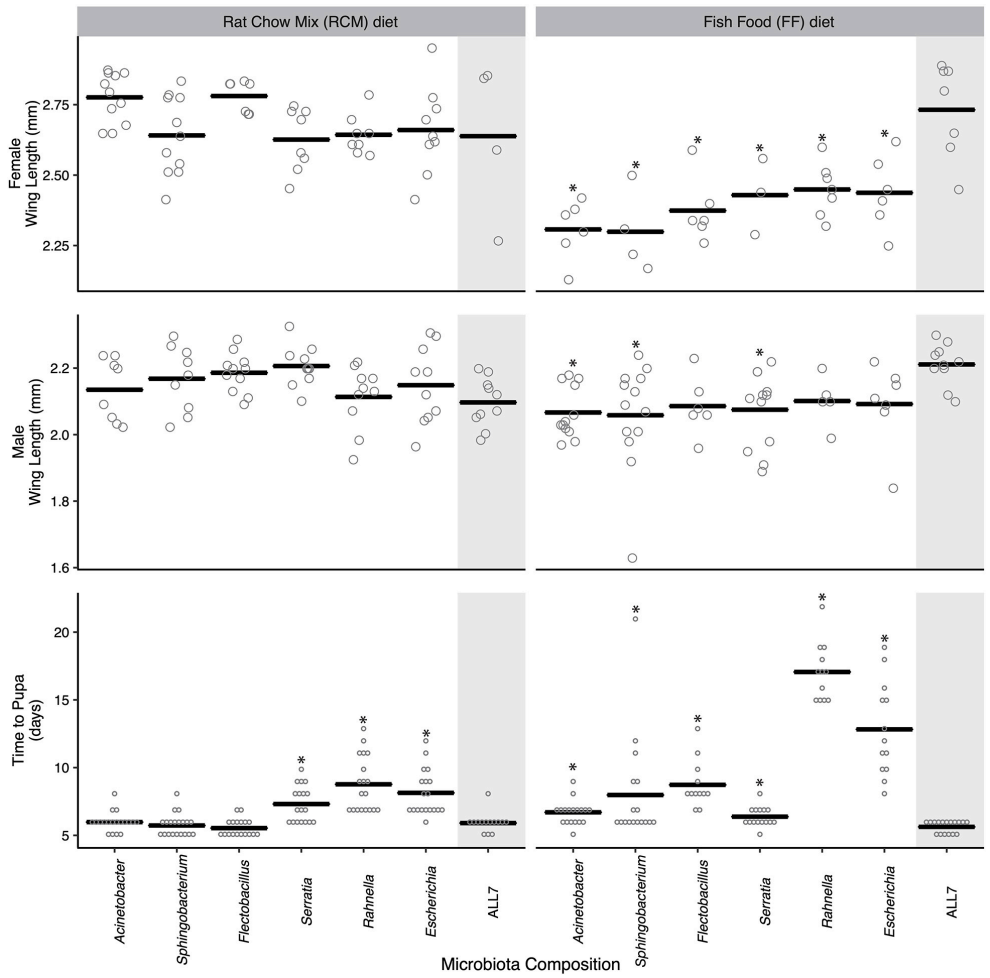

FIGURE 1 | Mosquito development time and adult size when reared monoxenically with different microbiotas on RCM or FF diet. Bars indicate mean abundance for the treatment. Asterisks indicate a significant difference for treatment relative to the community microbiota control = ALL7 [wing length, Dunnett's ($p < 0.05$); pupation time, Steel's ($p < 0.05$)].

developmental rates and adult sizes were similar to one another (**Supplementary Figure 3**). Thus, development times and adult sizes were generally consistent within each of the aforementioned treatments, while our between-treatment comparisons overall suggested that species composition of the microbial community affected *A. aegypti* development more when larvae were fed FF diet than when fed RCM diet.

Certain Two-Member Microbiotas Produce Mosquitoes of Similar Quality to the ALL7 Community

To further study the outcome of microbial community on *A. aegypti* development, we fed axenic first instars the FF diet and inoculated cultures with the 21 possible pairwise combinations of bacteria from the ALL7 microbiota. Pairwise cultures grew to comparable densities as the ALL7 or monoxenic cultures (**Supplementary Figure 4**). However, unlike cultures inoculated with only one bacterial species, certain pairwise combinations resulted in development times and adult sizes that did not differ from cultures inoculated with the ALL7 community (**Figure 2**). Many pairs that included *Microbacterium* had densities at or above that found in the ALL7, yet these microbial communities often resulted in smaller adults and delayed development (**Figure 2**), indicating that microbial density alone does not explain differences in mosquito development. Pairs that included *Acinetobacter* exhibited development times and adult sizes that were most similar to the ALL7 treatment, while pairs that included *Rahnella* or *Sphingobacterium* exhibited delayed pupation times although delays were shorter than those observed in monoxenic rearing with *Rahnella* or *Escherichia* (**Figure 2**).

For within treatment comparisons, we selected *Acinetobacter–Escherichia* and *Acinetobacter–Flectobacillus* as examples of two-member communities that performed similarly to the ALL7 community and *Acinetobacter–Rahnella* as an example of a two-member community that exhibited longer development times than the ALL7 community. For each of these two-member communities, development times, and adult sizes were similar

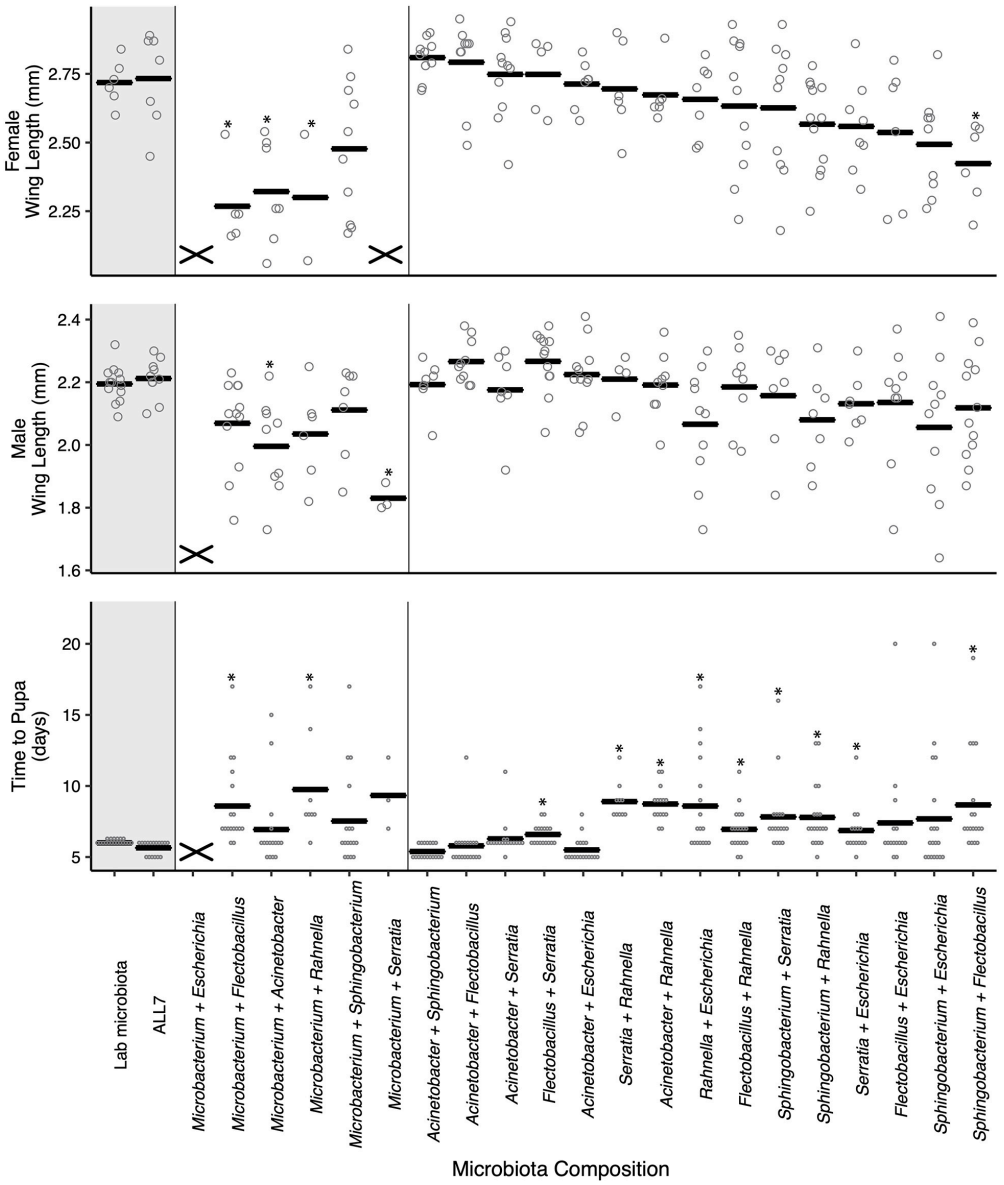

FIGURE 2 | Paired microbial communities largely restore mosquito performance to that of individuals reared with the ALL7 or conventional microbiotas on FF diet. Two-member communities containing *Microbacterium* produced mosquito phenotypes more similar to those monoxenically reared. The "X" marks at the bottom of data columns for "Time to Pupa" and "Wing Length" indicate that larvae did not develop into pupae or adults, respectively. Bars indicate mean abundance for the treatment and asterisks indicate a significant difference for treatment relative to the ALL7 control as in **Figure 1**.

when progeny from different flasks were compared with one another (**Supplementary Figure 3**), which overall provided further support that certain two-member microbial communities promoted development of larvae better than others.

Manipulating Microbial Community Composition Over Time Affects Mosquito Fitness

Since *Acinetobacter* showed evidence of promoting larval growth in two-member communities while *Rahnella*, *Escherichia*, and *Sphingobacterium* showed evidence of slowing larval growth, we assessed whether manipulating the abundance of these bacteria at different times during development affected *A. aegypti*. This was approached in one set of experiments by inoculating cultures with axenic first instars, FF diet, and either *Escherichia* or *Rahnella* and then adding *Acinetobacter* at the same time (time 0), day 2 post-inoculation, or day 4 post-inoculation (**Supplementary Figure 5A**). In a second set of experiments, cultures containing axenic larvae and FF diet were inoculated with *Acinetobacter* and either *Sphingobacterium* or *Flectobacillus* at time 0 followed by addition of kanamycin at time 0, day 2, or day 4, which selectively affected *Acinetobacter* (**Supplementary Figure 5A**).

Together, these approaches allowed us to either increase or decrease community diversity in cultures at particular times during larval growth.

Density estimates showed that adding or removing a second bacterial species resulted in similar colony-forming unit abundances (**Supplementary Figure 5B**) as found for monoxenic and paired cultures (see **Supplementary Figures 2A, 4**). Introducing *Acinetobacter* into a monoxenic culture also resulted in it reaching a stable titer within 1 day post-inoculation (**Supplementary Figure 5B**). The earlier *Acinetobacter* was added to cultures containing only *Escherichia* or *Rahnella*, the greater its effect on reducing larval development times and increasing adult female size (**Figures 3A,B**). For example, adding *Acinetobacter* to a culture containing *Rahnella* at time 0 decreased mean development time by 44% (16.8 ± 1.01–7.4 ± 0.36 days), while adding at day 2 or 4 reduced development time by 35 and 27% (10.89 ± 0.3 days, 12.33 ± 0.46 days).

Kanamycin treatment of two-member communities resulted in complete elimination of *Acinetobacter* when paired with *Sphingobacterium*, but only partially reduced *Acinetobacter* from a density of $\sim 10^8$ to 10^5 cfu/ml when paired with *Flectobacillus* (**Supplementary Figure 5B**). Despite the loss or reduction of *Acinetobacter* after kanamycin addition, overall colony-forming units in both treatments changed little because of the high abundance of *Sphingobacterium* or *Flectobacillus* which remained at densities of 10^8–10^9 cfu/ml (**Supplementary Figure 5B**). However, the loss or reduction of *Acinetobacter* significantly affected *A. aegypti* larvae, which exhibited longer development times and smaller adult sizes (**Figures 3C,D**). For example, even partially reducing the *Acinetobacter* titer at time 0, day 2, or day 4 when paired with *Flectobacillus* still increased development time by 26, 15, and 5%, respectively, relative to untreated *Acinetobacter*–*Flectobacillus* cultures (5.22 ± 0.1 days). The use of the antibiotic kanamycin was the only method available to selectively kill *Acinetobacter*; however, we cannot fully eliminate the possibility that kanamycin directly inhibits mosquito development rather than the elimination of *Acinetobacter*. That said, larvae treated with kanamycin still developed into adults. Further, *Escherichia* and *Rahnella* may act to slow larval development; however, this experiment did not directly test this hypothesis and future studies should be performed to identify if certain microbes have growth-inhibiting effects on *A. aegypti*.

Dead Bacterial Amendments Also Affect Mosquito Fitness

Previously, axenic larvae were shown to not grow beyond the first instar when dead microbes were added to RCM and other nutrient-rich laboratory diets under standard rearing conditions (27°C and photoperiod (16 h light:8 h dark) (Valzania et al., 2018a). However, adding dead microbes to cultures did cause first instars to live longer than unfed larvae or larvae fed RCM diet alone, which suggested dead microbes provide nutrients that extend the longevity of larvae (Valzania et al., 2018b). We thus revisited our previous two-member community experiments where larvae were fed FF diet, and asked if adding one species that was dead enhanced development of larvae into adults if the second species is viable. Adding living *Acinetobacter* to *Rahnella* or *Sphingobacterium* that were killed by autoclaving or sonication resulted in larval development times that did not differ from cultures inoculated with living *Acinetobacter* and *Rahnella* or *Sphingobacterium* (**Figure 4**). However, adult sizes trended smaller in cultures containing one living and one dead bacterium (e.g., female A[L]-Sp[s] 2.73 ± 0.02 mm, A[L]-Sp[a] 2.66 ± 0.02 mm, A[L]-R[a] 2.72 ± 0.03 mm, A[L]-R[s] 2.64 ± 0.03 mm) vs. cultures where both bacteria were living (A[L]-Sp[L] 2.8 ± 0.02 mm; A[L]-R[L] 2.73 ± 0.03 mm) (**Figure 4**). In reciprocal experiments, time to pupation was significantly longer when larvae were inoculated with living *Rahnella* or *Sphingobacterium* regardless of whether dead *Acinetobacter* was heat-inactivated or sonicated (**Figure 4**).

Addition of autoclaved *Acinetobacter* to living *Sphingobacterium* resulted in longer development times, significantly smaller females, and smaller males, whereas sonicated *Acinetobacter* restored development times and adult sizes to levels that were similar to larvae inoculated with living *Sphingobacterium* and *Acinetobacter* (**Figure 4**). Adding living *Rahnella* plus autoclaved *Acinetobacter* produced larger females, whereas adding sonicated *Acinetobacter* produced smaller females than cultures inoculated with living *Rahnella* and *Acinetobacter* (**Figure 4**). We also assessed whether increased resources associated with adding a dead bacterium affected the population of the second living bacterium. Results showed that *Acinetobacter* populations increased less when dead *Rahnella* or *Sphingobacterium* were added than when these species were added as living bacteria (**Supplementary Figure 6**). There was also little change in *Rahnella* and *Sphingobacterium* abundances between living–living or living–dead combinations (**Supplementary Figure 6**). We thus concluded that some combinations of living and dead bacteria mimicked outcomes when both community members were viable, but most combinations did not.

ALL7 and Endemic Microbiotas Generally Failed to Support *A. aegypti* Development When Larvae Were Fed Plant-Based Natural Diets

As previously noted, while RCM, FF, and other diets used to rear mosquitoes in the laboratory are nutrient rich, larvae feed upon detritus in the field, which primarily consists of plant debris that is comparatively nutrient poor (Anderson et al., 2016). However, many bacteria grow in environments that have nutrients that are inaccessible to animals because of their diverse catabolic metabolisms and biosynthetic abilities to produce essential amino acids, vitamins, and other factors required for growth. We thus assessed how six gamma-irradiated plant-based diets (**Supplementary Table 3**) affected microbiota growth and the development of *A. aegypti* larvae into adults. Results showed that the ALL7 microbiota grew to a similar density ($\sim 10^8$ cfu/ml) in the six plant-based diets (with or without mosquito larvae) as previously observed for the RCM or FF diets (compare **Supplementary Figure 7** with **Figure 4**).

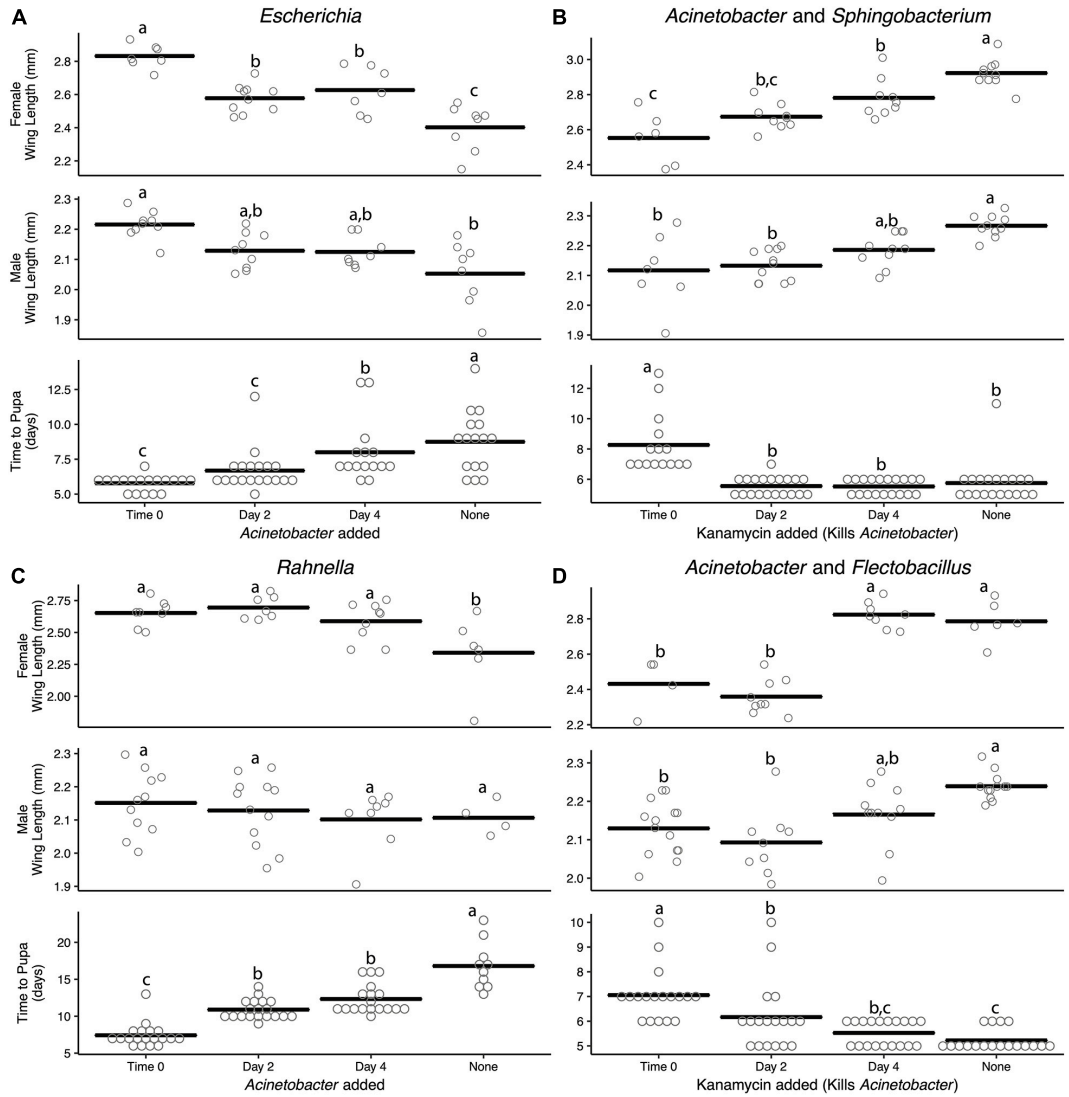

FIGURE 3 | Addition or removal of a second bacterial species during development alters adult size and time to pupa. *Acinetobacter* was added to **(A)** *Escherichia* or **(C)** *Rahnella* or removed with kanamycin from **(B)** *Sphingobacterium* or **(D)** *Flectobacillus*. All experiments were conducted using FF diet. Bars indicate mean abundance for the treatment and significance level assigned by ANOVA with Tukey's HSD for wing length and Steel–Dwass test for pupation time.

Regardless of the plant-based diet provided, most *A. aegypti* larvae did not develop into adults when either the ALL7 microbiota was added or when the endemic microbiota was added to the Tree Hole or Discarded Tire diets (**Supplementary Table 3**). Tulip tree (*Liriodendron tulipifera*) leaves with the ALL7 microbiota supported development of 4/20 larvae into adults, but development times were much longer (>18 days to pupa, $\bar{x}$ = 21.5 days) relative to larvae fed RCM or FF diet. The one adult female and three males that eclosed were also much smaller (**Supplementary Table 4**). The endemic microbiotas from the Discarded Tire and Tree Hole resulted in slightly more larvae developing into adults (3/40 larvae) than the ALL7 microbiota (0/40) but also exhibited long development times and small adult sizes (**Supplementary Tables 3, 4**). Thus, the six plant-based diets largely failed to support development of *A. aegypti* larvae despite growth of the ALL7 microbiota to a similar density as observed when fed laboratory diets.

Adding RCM Components to Detritus Promotes *A. aegypti* Development

Several mosquito species have been observed to exhibit higher growth rate, survivorship, and adult size when plant detritus, as commonly fed upon by mosquito larvae in the field, is supplemented with animal tissues that contain higher amounts of protein (Yee and Juliano, 2006; Yee et al., 2007). We therefore added two RCM components: (1) lactalbumin that provides protein or (2) torula yeast that provides protein plus other macro- and micronutrients. These components were provisioned at two concentrations (1×, 2×) to the Discarded Tire detritus. We then assessed effects on microbial and *A. aegypti* growth in cultures

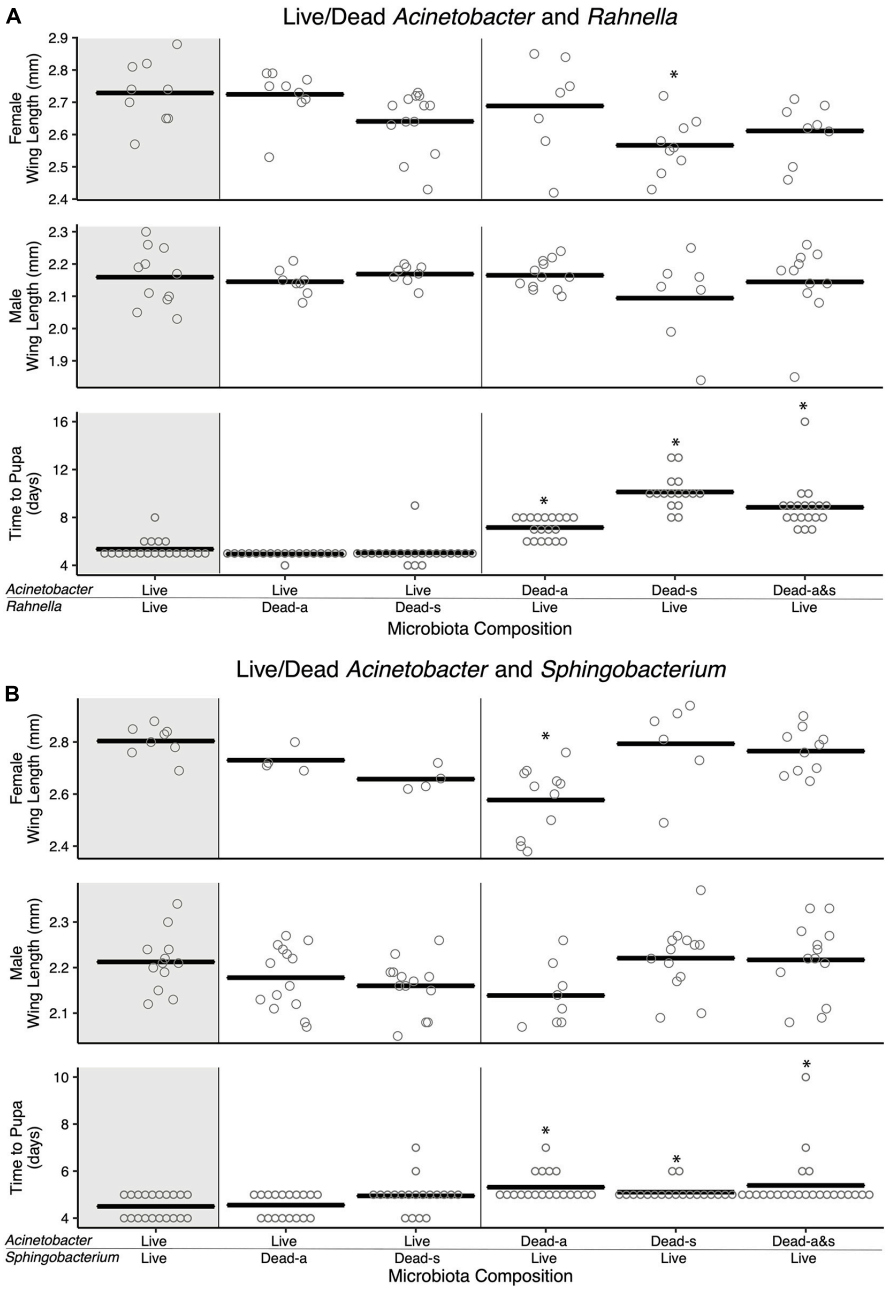

FIGURE 4 | Amendment with dead bacterial cell components shows species-specific and context-dependent ability to restore adult size and development time. Pairwise combinations of living and dead cells were performed for two sets of bacteria: **(A)** *Acinetobacter* and *Rahnella*, and **(B)** *Acinetobacter* and *Sphingobacterium*. Bars indicate mean abundance for the treatment and asterisks indicate a significant difference from control (paired living bacterial microbiota) by Dunnett's test for wing length and Steel's test for pupation time. Dead-a (autoclaved) or Dead-s (sonicated and 0.2 μm filtered) cells were added to living cultures (Live).

containing the ALL7 microbiota, *Acinetobacter* alone, or *Rahnella* alone. Supplemented alone, lactalbumin, torula yeast, and Discarded Tire detritus served as low-nutrient dietary controls, while RCM diet served as a high-nutrient dietary control.

Assessment of bacterial growth indicated that the ALL7 microbiota and *Rahnella* alone grew to $\sim 10^8$ cfu/ml across most treatments with the exception of cultures containing only lactalbumin where densities were $>10^7$ cfu/ml (**Supplementary Figure 8**). Monoxenic *Acinetobacter* treatments in contrast grew to $< 10^7$ cfu/ml in all treatments except with RCM diet (**Supplementary Figures 5, 8**). Assessment of mosquito growth showed that no larvae developed into adults when fed lactalbumin or tire detritus alone, but adding lactalbumin to tire detritus plus the ALL7 microbiota resulted in

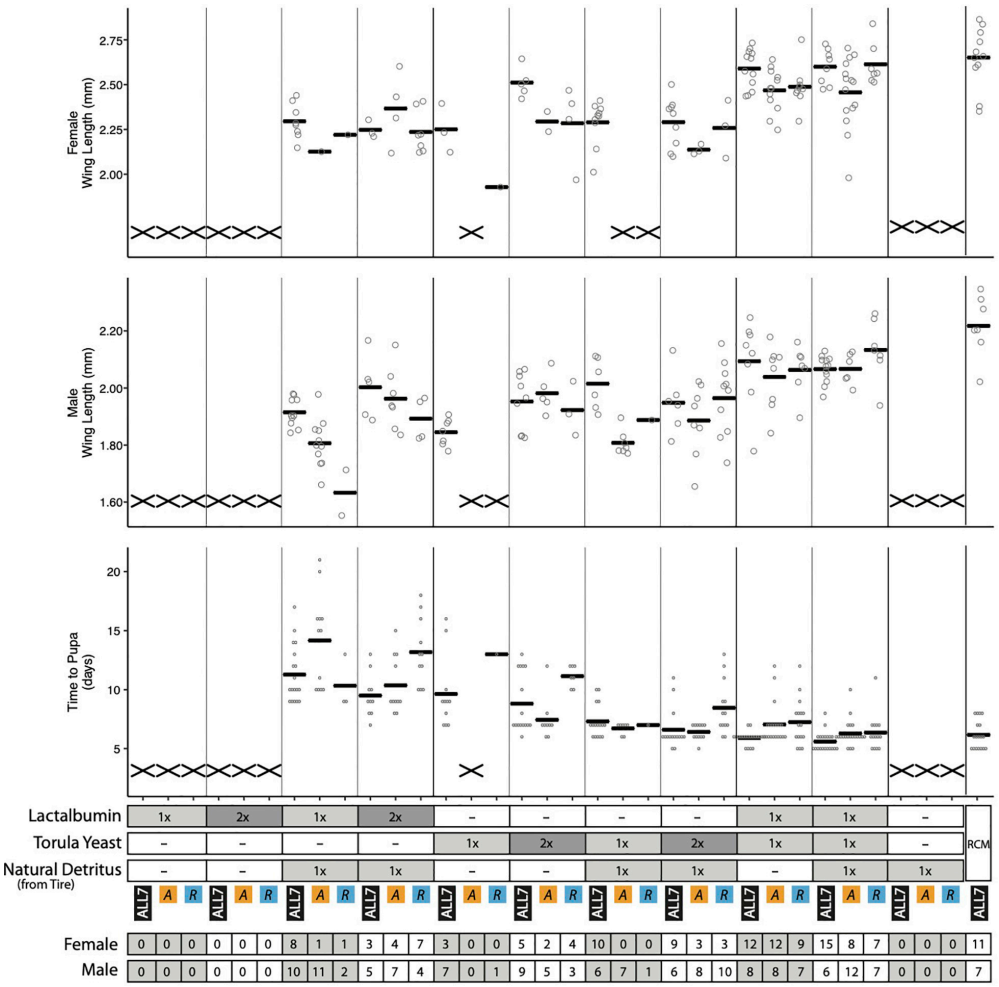

FIGURE 5 | Performance metrics of *A. aegypti* reared on diet combinations of lactalbumin, torula yeast, and natural detritus. For each diet composition, mosquitoes were reared with three microbiotas: ALL7, *Acinetobacter* (A), and *Rahnella* (R). Bars indicate mean abundance for the treatment. The "X" marks at the bottom of data columns for "Time to Pupa" and "Wing Length" indicate that larvae did not develop into pupae or adults, respectively. Positive control mosquitoes were reared on RCM diet with the ALL7 microbiota. 1×, diet provisioned at 1/3 quantity of RCM diet; 2×, diet provisioned at 2/3 quantity of RCM diet; –, omitted from diet. The number of adult male and female mosquitoes is listed at the bottom for each treatment.

most larvae developing into adults (**Figure 5**). Lower proportions of larvae developed into adults when lactalbumin was added to tire detritus in monoxenic *Rahnella* or *Acinetobacter* treatments (**Figure 5**). Surviving progeny across these treatments exhibited longer development times and smaller adult sizes when compared with progeny from the positive control (**Figure 5**).

Half of the progeny fed 1× torula yeast alone developed into adults in the presence of the ALL7 microbiota (10/20), whereas almost no progeny developed into adults when fed 1× torula yeast in cultures containing monoxenic *Rahnella* (1/20) or *Acinetobacter* (0/20) (**Figure 5**). Increasing the amount of torula yeast to 2× resulted in most progeny developing into adults when the ALL7 community was present (14/20), whereas only ∼40% of larvae developed into adults in cultures containing monoxenic *Rahnella* (7/20) or *Acinetobacter* (7/20). Similar to RCM-fed larvae, most progeny developed into adults regardless of the microbiota composition (ALL7, *Rahnella*, *Acinetobacter*) when fed a mix of torula yeast and lactalbumin (with or without the addition of detritus) (**Figures 1**, **5**). Development times and adult sizes for treatments fed a mix of lactalbumin and torula yeast were also similar to progeny from the RCM diet control (**Figures 1**, **5**). Taken together, addition of a single protein (lactalbumin) to plant-based detritus strongly promoted development of *A. aegypti* into adults in the presence of the ALL7 microbiota, whereas addition of mix of proteins and micronutrients (torula yeast) to either detritus or lactalbumin promoted development in the presence of ALL7, *Rahnella*, or *Acinetobacter*.

DISCUSSION

The goal of this study was to characterize general patterns in regard to whether (1) microbes that promote growth of *A. aegypti* when fed nutrient-rich RCM diet similarly promote growth when larvae are fed other diets, and (2) single species of bacteria

similarly or differentially support larval growth when compared with conventional or simplified communities of bacteria. Overall, our results suggest that the growth-promoting effects of microbes vary with nutrient environment. For larvae fed nutrient-rich RCM and FF diets, our results corroborate previous findings showing that axenic larvae require living bacteria for growth, while no differences in development time or adult size were detected between larvae that were reared with an ALL7 vs. a conventional microbiota. However, experiments using individual or paired members of the ALL7 community revealed differences between RCM and FF diet in the growth-promoting potential of particular bacteria (**Figure 1**). For larvae fed plant-based detritus, axenic larvae also do not grow. ALL7 and endemic microbiotas grow to comparable densities in water containing detritus diets as observed with laboratory diets (**Supplementary Figures 1A,2,7,8**), but largely do not support larval growth into adults unless additional protein or yeast is added (**Figure 5**).

Microbiota composition in *A. aegypti*, like other mosquito species, varies greatly between populations (Coon et al., 2016b; Muturi et al., 2016b; Dickson et al., 2017; Thongsripong et al., 2018). In turn, no core microbiota consisting of particular genera or species has been identified in *A. aegypti* or other mosquito species although bacteria in certain higher order taxa such as Actinobacteria, Sphingobacteria, and Gammaproteobacteria are commonly present (Coon et al., 2016b; Dickson et al., 2017; Thongsripong et al., 2018). Variability in microbiota composition further mirrors data showing that the microbial communities in the aquatic habitats where mosquitoes develop are also highly variable in composition, and that most microbes detected in larvae from a particular collection site and date are also present in the aquatic habitat from which they came (Strand, 2018).

The species of bacteria we selected for inclusion in the ALL7 community are known gut community members in *A. aegypti* but could also be distinguished from one another by colony morphology and growth dynamics which made it easier to monitor their abundance in the water where larvae feed. Larvae failed to grow when fed RCM diet axenically but had very similar developmental rates and adult sizes when fed RCM diet plus a conventional community, the ALL7 community, or most individual members of the ALL7 community (**Figure 1**). These results corroborate previous findings that larvae require microbes for development under standard rearing conditions but that this requirement is not species specific (Coon et al., 2014, 2016b). In contrast, while having similar overall abundances in rearing water (**Supplementary Figures 1**, **2**), no single bacterial species supported larval growth rates or adult sizes equal to that of the ALL7 community in mosquitoes fed FF diet (**Figure 1**), suggesting an important role for microbe–microbe interactions in this nutrient environment. These results also suggest nutritional differences exist between RCM and FF diets despite their overlap in macronutrient ingredients (**Supplementary Table 2**). The observation that conventional and ALL7 communities grow to densities between 10^7 and 10^9 cfu/ml in cultures fed plant-based detritus or laboratory diets (i.e., RCM, FF) indicates that nutrients are sufficient to comparably support microbial growth (**Supplementary Figures 1A,7**). On the other hand, the failure of most larvae to develop into adults indicates that plant-based detritus and the bacteria present in these cultures provide inadequate resources for *A. aegypti* (**Figure 5**). One of these inadequacies is insufficient protein since adding lactalbumin to cultures containing tire detritus plus the ALL7 community substantially rescues larval development into adults (**Figure 5**). This outcome also supports the previous suggestion that animal detritus can be an important source of nutrition for mosquito larvae in the field (Yee and Juliano, 2006; Yee et al., 2007). However, it is also possible other species of bacteria or other microorganisms provide essential resources when only plant-based detritus is available. One candidate that could be especially important are yeasts and other fungi, which have been identified in the aquatic habitats and microbiotas of several mosquito species (Chandler et al., 2015; Muturi et al., 2016a; Steyn et al., 2016). Results of this study also support this suggestion since adding heat-killed torula yeast to cultures containing the ALL7 community promotes larval growth into adults.

While a few studies identify negative effects of microbiota diversity on multicellular animals (Krams et al., 2017; Napflin and Schmid-Hempel, 2018), most studies identify benefits with increased diversity for both invertebrates (Newell and Douglas, 2014; Callens et al., 2018; Gould et al., 2018) and vertebrates (Knutie et al., 2017; Ellison et al., 2019). Increased diversity can create novel microbe–microbe interactions that change gene expression in the overall microbial community or in particular community members, which can result in emergent properties that change the overall metabolism or ecological interactions among microbial species (Ibberson et al., 2017; Kešnerová et al., 2017; Gould et al., 2018). Previous studies with mosquitoes identify conditions where more diverse microbial assemblages promote survivorship and development (Díaz-Nieto et al., 2016; Travanty et al., 2019). In contrast, our results suggest the benefits of increasing microbiota diversity for larval growth rates and adult size in *A. aegypti* are contextual, with diet being a key variable as to whether increased diversity promotes development or not. While we observed striking variation in growth-promoting activity of different microbes in the FF diet assays, all species in our ALL7 community grew to similar densities across all diets we tested (**Supplementary Figure 2**) and there was no clear relationship between higher density and increased mosquito development. For example, *Microbacterium* grew to densities near 10^9 cfu/ml but was unable to support mosquito development, whereas *Acinetobacter* grew to a density near 10^7 cfu/ml in RCM diet which enabled full mosquito development (**Supplementary Figure 2**). This further suggests that currently unknown traits in particular species of bacteria (e.g., vitamin production) found in association with *A. aegypti*, rather than differences in abundance, are important for larval growth. Microbial growth rate and turnover or the digestibility of a microbe might be possible features that affect mosquito nutrient acquisition and development. Our finding that certain two-member microbial communities promote growth rates and adult sizes that are very similar to the ALL7 community when larvae are fed FF diet (**Figure 2**) further indicate that even extremely simple communities can generate emergent properties under certain dietary conditions that enhance larval performance.

Developmental differences among insects and other multicellular animals can result from absent or imbalanced nutrients including inorganic micronutrients (e.g., salts, trace metals), amino acids found in protein, and sterols which most bacteria cannot synthesize (Huang and London, 2016; Wei et al., 2016) but may be accessible from plant and animal tissues, or microbial eukaryotes including fungi (Baker, 1992; Gray et al., 2006; Piper et al., 2014; Simpson et al., 2017). Our studies using FF diet indicate that two-member communities containing *Acinetobacter* promote larval growth comparably to the ALL7 community (**Figure 2** and **Supplementary Figure 9**), while providing one heat-killed member in a two-species community also comparably supports larval growth in some cases (**Figure 4**). This result is consistent with the suggestion that microbes, in part, serve as food for *A. aegypti* but the inability of dead bacteria to support development under a typical light:dark photoperiod indicates that larval growth also involves factors that living microbes provide. Similar to the results from Keebaugh et al. (2018), our assays using detritus diets also indicate that adding dead torula yeast to cultures containing living bacteria likely provides macronutrients like protein plus micronutrients such as vitamins, sterols (e.g., ergosterol), and trace metals that enable larvae to develop without any detritus (**Figure 5**). In contrast, lactalbumin and detritus are unable to support larval growth into adults when supplied individually with living bacteria but do support growth to the adult stage when provisioned together. This complementarity potentially stems from the juxtaposition of trace metals, salts, and plant sterols (e.g., sitosterol, stigmasterol), which are common in natural detritus, with added protein from lactalbumin plus other unknown factors provided by viable ALL7 community members. The recent report that *A. aegypti* can be reared axenically only when fed a high-nutrient diet with very high densities of autoclaved bacteria (Correa et al., 2018) further suggests that living microbes may provision factors, in addition to macronutrients, that are at extremely low concentrations in dead bacteria or that rapidly degrade after bacterial death.

In natural mosquito habitats, microbe populations experience events that alter community diversity and abundance (e.g., drought, algal blooms, agricultural runoff). Microbes also rapidly transit the larval gut in association with feeding (Allison and Martiny, 2008; Green et al., 2008; Coon et al., 2017). Thus, mosquito larvae may experience frequent changes in microbiota composition as a function of environmental conditions or foraging activity in the water column that can disrupt development. Alternatively, this rapid turnover of the microbiota may enable larvae to select and consume advantageous microbe communities. Stochastic variation in the microbial community of our insectary's rearing water over 6 months was enough to change host development (**Supplementary Figure 1C**), underscoring the potential for natural microbial variation to affect wild mosquito populations. More generally, changes in the environmental or larval gut microbial communities may have cascade effects on mosquitoes that extend to their adult life phase and have consequences for disease transmission.

Using *Drosophila melanogaster* as a model for host–microbe interactions, researchers have identified numerous growth-promoting factors produced by the microbiota, which include structural components of the cell membrane and catabolic and anabolic metabolites (acetic acid, ribonucleotides, vitamin co-factors) (Blatch et al., 2010; Shin et al., 2011; Storelli et al., 2011; Piper et al., 2014; Matos et al., 2017; Sannino et al., 2018; Consuegra et al., 2020a,b). Among these factors, B vitamins are cofactors critical to the central metabolism of animals (tricarboxylic acid cycle; aerobic respiration; metabolism of amino acids, fatty acids, DNA), but cannot be synthesized by animals and must be obtained from the diet or supplied by microbes inhabiting the animal body (Douglas, 2017). Riboflavin (Wong et al., 2014), thiamine (Sannino et al., 2018), and pantothenate (Consuegra et al., 2020a,b) have been demonstrated to be supplied by the microbiota and directly influence growth in *D. melanogaster* larvae, a terrestrial species. However, microbiota-produced B vitamins may be more important to aquatic animals like mosquito larvae because of their instability in liquid media, where light, oxygen, high temperature, or changes in pH can rapidly degrade them into forms that cannot be used (Sheraz et al., 2014; Schnellbaecher et al., 2019). Further, work using holidic diets that excluded individual micronutrients showed direct evidence that microbes promote the growth of mosquito larvae through the production of various B vitamins (Wang et al., 2021). In addition, this work suggests that completely axenic rearing of mosquito larvae was previously thwarted by the degradation of B vitamins via photodegradation in the aquatic rearing environment (Correa et al., 2018; Wang et al., 2021).

A major pattern observed across the experiments performed in this study was that mosquito larvae reared with individual bacteria grew slower and to smaller adult sizes than those reared with two or more bacterial species, suggesting there is a benefit to harboring a more diverse microbiota. This pattern follows theoretical predictions (Lozupone et al., 2012; Friedman et al., 2017) and has also been observed in other systems (Newell and Douglas, 2014; Knutie et al., 2017; Callens et al., 2018; Gould et al., 2018; Ellison et al., 2019; Consuegra et al., 2020a,b); however, the underlying mechanisms driving this process are less understood. Recently, it was shown that the exchange of metabolites between *Acetobacter pomorum* and *Lactobacillus plantarum*, common members of the *D. melanogaster* microbiota, induced the production of B vitamins and cofactors that is not observed in the monoculture of either species, which resulted in increased larval growth (Consuegra et al., 2020a). This emergent property of co-culturing promoted *D. melanogaster* larval growth even in a low nutrient diet and demonstrates how cross-feeding (syntrophic) interactions, especially involved with vitamin biosynthesis, can result in major changes to host animal growth. Environmental multi-species communities (18 species) of non-host-associated bacteria have also been shown to depend on syntrophic interactions to share B vitamins and their precursor metabolites, with all members relying on a shared pool of micronutrients to survive (Romine et al., 2017). Altogether, there are data coming from both host–microbe interaction studies and microbial community ecology that suggests the biosynthetic repertoires (particularly in B vitamins) of interacting bacteria may dramatically alter the nutritional

composition present in an environment, which in turn affects host nutrition and growth.

CONCLUSION

Our results indicate that growth of *A. aegypti* larvae is strongly affected by both microbes and diet. While our work used only one strain of *A. aegypti* (UGAL), it seems likely that developmental responses to macronutrients may be conserved in mosquitoes and research on different species will be very informative. Moving forward, defined (holidic) diets will be required to determine how individual microbial species alter larval homeostasis and to identify specific nutrients or growth-promoting factors produced by viable microbes. These rearing techniques will further facilitate comparative studies, such as identifying differences in microbiota-based nutrient requirements among mosquito species and how these differences influence mosquito life-history ecology (e.g., container vs. running-water breeders; detritivorous vs. carnivorous larvae) and competition between mosquitoes in natural habitats. Better understanding of host–microbe interactions in mosquitoes is important because of the implications for vectoring human disease; however, mosquitoes also present a powerful system for studying the effects of microbiota alteration on host performance and fitness because microbes and diet can be easily manipulated in their aquatic rearing arrangement. Finally, assessing microbial interactions within the microbiota and subsequent changes in metabolic networks, niche partitioning, and resource allocation will be generally informative to microbial community assembly and stability in host-associated ecosystems.

MATERIALS AND METHODS

Mosquitoes and Diets

UGAL *Aedes aegypti* were originally collected in Athens, GA (Valzania et al., 2018b). Non-sterile (conventional) larvae were reared at 27°C under a 16 h light:8 h dark photoperiod in 2-L pans containing water and fed RCM diet which contained equal parts (w/w) powdered rat chow diet (LabDiet 5012, St. Louis, MO, United States), heat-killed torula yeast (Frontier Scientific Services, Newark, DE, United States), and lactalbumin (Sigma, St. Louis, MO, United States) (Coon et al., 2014; Bond et al., 2017). Larvae can also be reared by feeding them FF diet which consisted of TetraColor Tropical Granules (Tetra, Blacksburg, VA, United States). Adults of both sexes were maintained in Plexiglas cages at 27°C and a 16 h light:8 h dark photoperiod and fed 10% sucrose (w/v) in water. Adult females laid eggs after consuming commercially purchased rabbit blood (Hemastat Laboratories, Dixon, CA, United States) using artificial feeders. For experiments, larvae were maintained under the same physical conditions as our conventional culture and fed RCM diet, FF diet, or plant-based natural diets collected from locations near the University of Georgia that consisted of leaves, leaf litter, or wet detritus from a sweet gum (*Liquidambar styraciflua*) tree hole or a discarded automobile tire (**Supplementary Table 3**). Any invertebrates including mosquito larvae were removed from these materials to minimize the possibility of animal tissue contamination before drying at 60°C for 48–72 h. Each material was then ground into a fine powder with a blade grinder (Hamilton Beach, Glen Allen, VA) followed by sterilization via gamma irradiation at 10 kGy as previously described (Coon et al., 2014). Endospores of *Bacillus* species have been shown to be reduced 10-fold when exposed to ∼2 kGy of gamma radiation (Cote et al., 2018). The 10 kGy used on our diets would reduce the spore population by approximately 10^5. Our main laboratory cultures as well as all experiments were maintained at 27°C under a 16 h light:8 h dark photoperiod.

Bacterial Isolates

Water samples were collected from two rearing pans containing fourth instar UGAL *A. aegypti* and four outdoor containers containing mosquito larvae that were located within 2 km of the laboratory in the fall of 2017 (**Supplementary Table 1**). Larvae and organic debris (e.g., leaf tissue) were first removed from samples to minimize carryover of potential nutrients. The microbial communities in each water sample were next centrifuged at 6,000 rpm (rad) for 15 min. The resulting pellets were then resuspended in a 1:1 mixture of sterile glycerol:1× PBS and cryopreserved at −80°C. Strains of bacteria from the laboratory or field collection sites were isolated on minimal medium of 1/10 diluted 869 agar plates (Eevers et al., 2015). Unique colony morphologies were selected and passaged three times to new agar plates to ensure individual isolates. Isolates were then suspended in a 1:1 mixture of sterile glycerol:1× PBS and cryopreserved at -80°C. Template DNA was extracted from each isolate with the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, United States) and used to amplify a portion of the 16S rRNA gene with the primer set 27fshort-1507r, HotMaster Taq DNA polymerase (Quantabio, Beverly, MA, United States), and previously described PCR conditions (Martinson et al., 2011). Amplicons were visualized on a 1% agarose gel and cleaned with the QIAquick PCR purification kit (Qiagen, Valencia, CA, United States) before submitting for Sanger sequencing at Eurofins Genomics (Louisville, KY, United States). Isolates were identified to genus by blasting the 16S rRNA sequence to the NCBI nr database.

Six isolates were selected as representatives of environmental microbes that were found in water that contained wild or laboratory-reared mosquito larvae (**Supplementary Table 1**). These bacterial species were selected based on (1) the ability to grow relatively quickly on a common medium (1/10 diluted 869 agar), (2) the ability to differentiate species by colony morphology (size, margin shape, color), and (3) that they were a taxonomically diverse set of species. Colony morphology for each bacterium at ∼24, ∼48, and > 48 h growth (on 1/10 diluted 869 agar) was used to differentiate species when they were grown in two-member communities and images of these differences can be found in Presentation S1. Bacterial isolates were assayed for resistance to antibiotics to help design the experiments presented in **Figures 3B,D** using 1/10 diluted 869 agar plates with kanamycin (50 μg/ml), ampicillin (100 μg/ml), spectinomycin (50 μg/ml), chloramphenicol (25 μg/ml), and

tetracycline (10 μg/ml) (**Supplementary Table 1**). We also selected *Escherichia coli* K-12 substr. MG1655 because this species is a known gut community member in *A. aegypti* (Thongsripong et al., 2018) and was also used previously in gnotobiotic rearing assays (Coon et al., 2014, 2017; Valzania et al., 2018a,b). The combination of these seven bacterial species was designated the ALL7 microbiota. To obtain dead cell additives, bacteria were grown in 1/10 diluted 869 liquid media to near stationary phase. A dilution series was performed to determine the colony-forming units per milliliter for subsequent concentration to 10^9 cfu/ml. Bacteria were pelleted at 2,000 × *g* and resuspended in sterile water to a concentration of 10^9 cfu/ml. Dead bacteria were created in two ways: (1) autoclaved, which does not preserve certain heat-instable nutrients, or (2) sonicated and filter sterilized, which is capable of preserving heat-instable nutrients (Valzania et al., 2018b).

Mosquito Rearing Conditions

Axenic first instars were produced by surface sterilizing eggs using previously established methods (Coon et al., 2014). Larvae were reared in two types of containers: 25-cm^2 cell culture flasks (Corning, Corning, NY, United States) or 6-well plates that served as rearing containers (Genesee Scientific, San Diego, CA, United States). Flasks contained 20 ml of sterile water, 60–65 mg of FF diet (3–3.25 mg/larva), 20 axenic larvae, and 10 μl of a given bacterial suspension. Individual wells in culture plates contained 5 ml of water, 10 axenic larvae, 5 μl of bacterial suspension, and RCM diet on a feeding schedule (3.3 mg at hour 0, 24; 8.3 mg at hour 72, 96; total = 2.32 mg/larva) (Valzania et al., 2018b). Rearing with natural diets was also performed in 6-well plates with 5 ml water and 10 axenic larvae, but diet was added *ad libitum* to encourage larval development.

For experimental treatments, bacterial isolates were grown on 1/10 dil. 869 agar plates for 24–48 h at 37°C until individual colonies were visible to ensure single species growth. Colonies were collected off plates with sterile disposable loops (Genesee Scientific) and diluted into 1 ml of sterile 1× PBS. A dilution series was performed for each bacterium using the SP-SDS method to calculate the colony-forming units per milliliter and initial concentration of cells in each experimental treatment (Thomas et al., 2015). This serial dilution method was also used for subsequent counts of bacterial abundance.

Assays where particular bacteria were added or eliminated were conducted in 25-cm^2 flasks using the aforementioned methods and microbiota composition/abundance was monitored by dilution series. *Acinetobacter* was selected as the target bacterium because bacterial pairs including it produced mosquitoes robust in performance metrics and it was sensitive to at least one antibiotic, unlike *Sphingobacterium* and *Flectobacillus*. Addition or removal of the second bacterium (*Acinetobacter*) occurred on days 2 and 4 of larval development. Removal of *Acinetobacter* was performed with the addition of kanamycin at 50 mg/ml, which was able to kill or severely decrease the growth of *Acinetobacter* while not inhibiting the growth of *Sphingobacterium* or *Flectobacillus*. Tests of the effects of dead bacteria on mosquito development used the 25-cm^2 cell culture flasks with 20 axenic larvae and FF diet experimental design described previously. Living cells were inoculated at time 0 at concentrations near 10^6 cfu/ml. Dead cell homogenates or filtrates were provisioned daily at concentrations similar to those found in living cells (following are the final concentrations in the rearing container): time 0—10^6 cfu/ml, day 1—5×10^6 cfu/ml, day 2—10^7 cfu/ml, day 3—10^7 cfu/ml, and day 4—10^7 cfu/ml.

Assays where two components of the RCM diet (i.e., lactalbumin, torula yeast) were added to tire detritus were performed in 6-well plates with 5 ml sterile water, 10 axenic larvae, and 10^6 cfu/ml of the ALL7 microbiota, *Acinetobacter*, or *Rahnella*. Detritus was added to each well on a feeding schedule (3.3 mg at hour 0, 24; 8.3 mg at hour 72, 96; total = 2.32 mg/larva). Lactalbumin and torula yeast were added to DI water at a concentration of 10 mg/ml and autoclaved before use. Each component was added to a well at 1 × (1.1 mg at hour 0, 24; 2.76 mg at hour 72, 96; total = 0.77 mg/larva) or 2 × (2.2 mg at hour 0, 24; 5.52 mg at hour 72, 96; total = 1.54 mg/larva). Provisions of the lactalbumin and torula yeast at 1 × level were equivalent to one-third the total weight of complete RCM diet (see above).

Microbiota Composition

Each of the seven bacteria used in pairwise microbiota treatments could be distinguished from each other by colony morphology, including colony size, margin shape, and color (at 24, 48, > 48 h) (**Supplementary Table 1**). *Acinetobacter*, *Rahnella*, and *Microbacterium* were also readily identifiable in the ALL7 mixture due to their morphology and abundance, but the other species could not be distinguished because they were present at lower abundance. Rearing water was sampled at two timepoints during development of mosquito larvae (3 days post-inoculation; at fourth instar) to determine the microbiota composition and abundance. A dilution series was performed as described previously, and colony morphologies were observed under a dissection microscope at × 1–4 magnification. These assessments further confirmed or refuted contamination and the expected microbiota composition in each treatment. Contaminated samples were removed.

Mosquito Fitness Measures

The time to pupation for conventionally reared *A. aegypti* fed RCM diet is 5 days. Larval development was observed daily, and the number of pupae and adults was recorded. Adults were aseptically collected, sex was determined, and wings were removed and placed onto microscope slides. Photographs of the wings were taken with a dissection microscope and wing length was measured using the LASX software (Leica Microsystems, Wetzlar, Germany). The distance between the alular notch to the apex of the radius vein 3 was measured as a well-established proxy for adult size (Yeap et al., 2013). Individual mosquitoes exposed to a given treatment served as the unit of replication when comparing development times with pupation and adult sizes between treatments. Within select treatments, development times and adult sizes were also compared between larvae that developed in different culture flasks. Statistical tests were performed in JMP pro14

(SAS Institute, Cary, NC, United States). Wing length data were analyzed by *t*-test, or ANOVA followed by Dunnett's or Tukey's HSD *post-hoc* comparison test. Because time to pupation was binned into 1-day intervals (non-parametric distribution), data were evaluated with Wilcoxon, Steel, Kruskal–Wallis, or Steel–Dwass tests.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: Accession numbers for the 16S rRNA gene sequences of the ALL7 microbiota members are deposited in GenBank (MN544614–MN544619) and individually listed in **Supplementary Table 1**. All other data are available upon request to the authors.

AUTHOR CONTRIBUTIONS

VM and MS designed the experiments, collected the data, and contributed to the writing of the manuscript. VM analyzed the data. All authors read and approved the final article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.650743/full#supplementary-material

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Larval Diet Abundance Influences Size and Composition of the Midgut Microbiota of *Aedes aegypti* Mosquitoes

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The midgut microbiota of the yellow fever mosquito *Aedes aegypti* impacts pathogen susceptibility and transmission by this important vector species. However, factors influencing the composition and size of the microbiome in mosquitoes are poorly understood. We investigated the impact of larval diet abundance during development on the composition and size of the larval and adult microbiota by rearing *Aedes aegypti* under four larval food regimens, ranging from nutrient deprivation to nutrient excess. We assessed the persistent impacts of larval diet availability on the microbiota of the larval breeding water, larval mosquitoes, and adult mosquitoes under sugar and blood fed conditions using qPCR and high-throughput 16S amplicon sequencing to determine bacterial load and microbiota composition. Bacterial loads in breeding water increased with increasing larval diet. Larvae reared with the lowest diet abundance had significantly fewer bacteria than larvae from two higher diet treatments, but not from the highest diet abundance. Adults from the lowest diet abundance treatment had significantly fewer bacteria in their midguts compared to all higher diet abundance treatments. Larval diet amount also had a significant impact on microbiota composition, primarily within larval breeding water and larvae. Increasing diet correlated with increased relative levels of *Enterobacteriaceae* and *Flavobacteriaceae* and decreased relative levels of *Sphingomonadaceae*. Multiple individual OTUs were significantly impacted by diet including one mapping to the genus *Cedecea*, which increased with higher diet amounts. This was consistent across all sample types, including sugar fed and blood fed adults. Taken together, these data suggest that availability of diet during development can cause lasting shifts in the size and composition of the microbiota in the disease vector *Aedes aegypti*.

Keywords: ***Aedes aegypti*, microbiota, diet, nutrition, microbe, mosquito**

INTRODUCTION

Mosquito-borne arboviruses like dengue virus, yellow fever virus and Zika virus are an ongoing public health concern, causing hundreds of millions of infections each year and tens of thousands of deaths (WHO, 2020). The vast majority of arboviral diseases have no vaccine and treatment is limited to supportive care. Additionally, novel arboviruses continue to emerge, presenting

a "moving target" for public health intervention. One commonality for all these pathogens is transmission by vector arthropods such as the yellow fever mosquito *Aedes aegypti*, and disease prevention efforts focus on reducing transmission through vector population reduction or replacement strategies (Wilson et al., 2020).

The mosquito midgut microbiota has been shown to influence factors relevant to vector-borne disease transmission (Minard et al., 2013; van Tol and Dimopoulos, 2016; Guégan et al., 2018) including larval development (Coon et al., 2014, 2017; Correa et al., 2018), susceptibility to arboviral infection (Xi et al., 2008; Apte-Deshpande et al., 2012, 2014; Ramirez et al., 2012, 2014; Wu et al., 2019; Möhlmann et al., 2020), blood digestion (Gaio et al., 2011), egg production (Gaio et al., 2011; Gendrin et al., 2015), and longevity (Bahia et al., 2014; Ramirez et al., 2014; Gendrin et al., 2015, 2016). These observations have led to growing interest in manipulation of the midgut microbiota for vector control and/or prevention of disease transmission. Progress in this regard would be greatly facilitated by a more complete understanding of the factors governing microbiota size and composition in the mosquito.

Previous work suggests that the environment is an important determinant of mosquito microbiota formation. For example, bacterial populations in breeding sites have been shown to correlate with midgut microbiota composition in larvae (Coon et al., 2014, 2016; Gimonneau et al., 2014), and larvae have been shown to ingest bacteria regularly during development (Coon et al., 2017). These data support the hypothesis that the larval microbiota is orally acquired and reflective of the bacterial community in the larval breeding water. The adult microbiota is also influenced by breeding site (Gimonneau et al., 2014; Buck et al., 2016), and bacteria found in larvae are commonly also found in adults (Coon et al., 2014; Guégan et al., 2018), suggesting adult microbiota is influenced at least in part by the microbial community in the larval breeding water.

Environmental factors influencing the microbial communities in larval breeding water could, therefore, have a long-lasting impact on the mosquito microbiota. It remains unclear, however, what aspects of the larval breeding habitat influence microbiota formation in larvae or adults. At least two studies have assessed the impact of larval diet on microbiota formation in mosquitoes. One found that the type of food (e.g., fish food flakes vs. fish food pellets) available to larvae had a significant and lasting impact on the amount of *Enterobacteriaceae* and *Flavobacteriaceae* in larval and adult *Anopheles gambiae* and a correlated impact on susceptibility to infection by *Plasmodium* parasites (Linenberg et al., 2016). Another found that varying the amount of diet provided to *Culex nigripalpus* larvae in outdoor mesocosms had no significant effect on microbiota composition in larvae or adults, though they did identify indicator species that corresponded with high and low organic matter treatments (Duguma et al., 2017). To our knowledge, there is no comparable information in *A. aegypti,* a critical vector of human arboviruses.

We assessed how the amount of diet available during development influences both the composition and size of the microbiota during different life history stages for *A. aegypti* mosquitoes. To test this, we reared *A. aegypti* mosquitoes with access to varying amounts of a complex larval diet and assessed the total bacterial load as well as the composition of the microbial community in breeding water, larvae, and the midguts of sugar fed and blood fed adult females. We found that lower diet abundance was generally predictive of a smaller bacterial community in larvae and that this effect persisted through adulthood and even after a blood meal. We also found that microbiota composition was significantly affected by larval diet availability. This was primarily observed in breeding water and larvae, though for some bacterial taxa the effects persisted into adulthood. Among all sample types, estimates of within-sample diversity (alpha diversity) were positively correlated with diet abundance. Additionally, diet was a significant predictor of diversity between samples (beta diversity), i.e., the microbiota composition of samples from the same feeding regimens were more similar to each other than to samples from different feeding regimens. These findings suggest that, for *A. aegypti*, the amount of food in breeding water can significantly influence the microbiota over multiple stages of development and into adulthood, when these mosquitoes are capable of transmitting pathogens to humans.

MATERIALS AND METHODS

Experimental Design, Sample Collection, and Sample Processing

Mosquito Strain Maintenance

Aedes aegypti Singapore (Sing) mosquitoes were established from larvae collected in the field in Singapore in 2010 (Sim et al., 2013). For strain maintenance, we reared Sing strain larvae at 27°C and 80% residual humidity on a 14:10 light:dark photocycle. We reared larvae in reverse osmosis (RO) water with *ad libitum* access to larval food (liver powder, tropical fish flake food, and rabbit food pellets mixed in a 2:1:1 ratio and autoclaved) and provided adults *ad libitum* access to 0.22 μm filter-sterilized 10% sucrose.

Experimental Design and Replication Structure

For each full biological replicate of the experiment, we reared one tray of larvae per diet treatment and transferred pupae from each tray to a single cage (4 total trays/cages). We set up three full biological replicates for a total of 12 trays/cages ($4_{\text{feeding regimens}} \times 3_{\text{biological replicates}} = 12_{\text{trays/cages}}$). To rear mosquitoes for the experiment, we bleached eggs once with 3% bleach then rinsed 2X with RO water, and hatched them in a vacuum. Development of larvae from the lowest R1 feeding regimen is delayed by 1 day, so we hatched eggs for this treatment 1 day earlier than for the other treatments. Eggs for all treatments were laid by the same two generations (F24–F25) of Sing strain adults. For all treatments, we thinned larvae immediately after hatching to 200 larvae per tray and to each tray added 2L RO water. In addition, we added 500 μL of a 25% glycerol stock of breeding water collected from an *Aedes* mosquito breeding site (tire pile) in Baltimore, MD on September 23, 2016, to standardize the starting breeding water microbiota

across diet regimens and make it more reflective of a natural *Aedes* container breeding site. Since collection, the glycerol stock has been continuously stored at −80°C and handled under sterile conditions. Larval food, prepared as described above, was replenished daily under the food regimens shown in **Table 1**, which were based on those described in Yeap et al. (2011) and ranged from nutritional deprivation to excess. When the larvae were 4th instars, we collected $n = 2$ water samples from each tray and $n = 3$ larval samples (5 larvae/sample) from each tray. We then transferred pupae from each tray to a separate cage and allowed adults to eclose. Adults were maintained on 10% sucrose until dissection. From each cage, we collected $n = 3$ pooled midgut samples from sugar fed adult females (8 midguts/sample) and $n = 1$–3 pooled midgut samples from blood fed adult females (average six midguts/sample). Our design therefore included biological replicates (i.e., separate trays/cages) as well as technical replicates (i.e., replicate samples taken from the same tray or cage). A listing of all samples used in the experiment can be found in **Supplementary Table 1**. All samples were used for qPCR analysis. Samples from R1, R2, and R4 were used for 16S amplicon sequencing. All technical replicate pools were sequenced from R1 and R2 to verify that microbiota composition did not vary by technical replicate. Final 16S profiling analysis was performed using the first technical replicate from all treatments to ensure the same number of samples were used from all treatment groups.

Water and Larval Collection and Sample Preparation

When larvae were 4th instars, we collected two samples of 5 mL of larval water from each tray in a conical bottom tube. We then centrifuged all samples at 5,000 × g for 20 min at 4°C, removed the supernatant, and froze the pellet at −20°C for storage. On the same day, we removed 15 4th instar larvae from each tray and transferred them by treatment to separate wells of a 6-well cell culture plate. We then immobilized larvae on a cold block, surface sterilized them with 70% EtOH, and washed them twice with sterile 1X PBS. We transferred three pools of five larvae from each group to 200 μl lysis solution from the Zymobiomics DNA extraction kit (Zymo Research, Irvine, CA, United States), homogenized with sterile pestles, and stored the samples at −80°C. We also collected contamination control buffer blanks (lysis buffer handled identically to an experimental sample but without tissue added) for each biological replicate. We collected water and larval samples before supplementing the larval breeding water with food for the day.

Adult Blood Feeding, Sample Collection, and Preparation

For blood feeding, we starved females overnight and then provided them a blood meal consisting of 45% human red blood cells and 55% heat-inactivated human serum. The blood meal was also supplemented with 1% 100 mM ATP. Sugar fed females were also starved and then provided 10% sucrose meals the following morning. Adult females were dissected at 4–6 days post eclosion and 24 h after blood feeding. Sugar fed females were dissected in parallel with blood fed females. We first removed the right wing from eight sugar fed females from each larval diet/replicate combination for a total of 24 wings per larval diet treatment. We then returned females to their respective groups and externally sterilized all adult females with 70% EtOH for a minimum of 30 s, then washed them twice with filter-sterilized 1X PBS. We dissected midguts from each mosquito on glass slides (sterilized with 70% EtOH) in sterile 1X PBS. We cleaned forceps with 70% EtOH between pools of mosquitoes and used clean 1X PBS for each dissection pool. We transferred dissected midguts to 200 μL lysis solution from the ZymoBIOMICS DNA Miniprep Kit (Zymo Research, Irvine, CA, United States) in microcentrifuge tubes on ice. We also collected contamination control buffer blanks (lysis buffer handled identically to an experimental sample but without tissue added) for each biological replicate. Samples were then frozen at −80°C until DNA extraction. Our goal was to dissect three pools of eight females from each diet/replicate/adult feeding status combination, which we were able to achieve for nearly all samples from the sugar fed treatment. For blood fed females, however, we were unable to obtain three pools of eight in many instances because not enough mosquitoes took a blood meal. Those differences are documented in **Supplementary Table 1**.

DNA Extraction

DNA was extracted using the ZymoBiomics DNA Miniprep Kit according to the manufacturer's instructions, with the following adjustments: all samples were homogenized manually using sterile pestles before the bead beating step. Pestles were treated with DNA erase (Sigma) and rinsed with sterile water prior to use. DNA was eluted in 100 μL filter-sterilized water heated to 60°C.

Wing Measurement

We measured wing length as a proxy for body size to compare size of adults between feeding regimens (Christophers, 1960; Bock and Milby, 1983; Van Handel and Day, 1989). We mounted wings on microscope slides using double sided tape and used ImageJ to measure the distance between the alular notch and the distal end of the right mosquito wing (i.e., the termination of the R3 wing vein), according to Bock and Milby (1983).

qPCR

To quantify the bacterial load in our samples, we performed qPCR targeting the bacterial 16S rRNA gene. For all samples, in each well we combined 7.5 μL SYBR master mix (Applied Biosystems), 0.35 μL of each primer (primer starting concentrations were all 10 μM), 1 or 5 μL template (as described below), and MilliQ water to a final volume of 15 μL. qPCR conditions were as follows: 95°C for 10 min, (95°C for 15 s then 60°C for 1 min) × 40 cycles. A melt curve was performed after all reactions to verify single product amplification. Primers used for qPCR can be found in **Supplementary Table 2**.

For water samples, we diluted gDNA 1:100 and used 1 μL of gDNA as template, and all water samples were run in quadruplicate. This was necessary for water samples because in an initial qPCR run with duplicated wells, multiple samples had more than 1 CT difference between duplicate wells and had to be discarded. We then repeated the qPCR run to obtain either two or four high quality technical replicates for each sample. 16S

copy number per microliter was determined using a standard curve generated from gel-purified PCR product of the *E. coli* 16S gene. We quantified the amount of DNA in ng/μL in our PCR product using a NanoDrop 2000 (Thermo Scientific) then, using the length of the PCR product (466 bp, Nadkarni et al., 2002) and assuming an average weight per base pair of 660 Da, we determined the copies per μL in our undiluted PCR product and subsequent dilutions. We then compared the CT values of our water samples to this standard curve to determine 16S copy number per microliter for each water sample. Technical replicates were averaged for each sample and the average copy number concentrations (copies per μL) were then used for data analysis (see below).

For tissue samples (larvae, adult sugar fed midguts, adult blood fed midguts), we diluted gDNA 1:50 then used 5 μL of gDNA as template. We performed qPCR targeting both the bacterial 16S rRNA gene and the mosquito S7 gene (AAEL009496), and all reactions were run in duplicate. See **Supplementary Table 2** for primer sequences. All technical replicates were averaged, and S7 CT values were subtracted from 16S CT values for each sample to obtain delta CT values. Delta CT values were used for data analysis (see below). Inverse delta CT values were used in plots.

16S Amplicon Sequencing and Data Processing

The concentration of DNA samples was determined using a NanoDrop 2000 (Thermo Fisher Scientific), and used to dilute samples to 1 ng/μl; 5 ng of DNA was used as template in a PCR to amplify the V4 region of the bacterial 16S rRNA gene. Primers were 515F and 806R with Illumina adapters and eight basepair dual indices (Kozich et al., 2013), and all reactions were performed in triplicate using GoTaq (Promega) and including 10 μg BSA (New England Biolabs). We also added 0.1 femtomole 515F and 806R without adapters or barcodes. This was done to overcome initial primer binding inhibition. PCR reaction conditions were as follows: 95°C for 2 min, then 30 cycles of 30 s at 95.0°C, 60 s at 50.0°C, and 60 s at 72.0°C, followed by final extension as 72.0°C for 10 min. Four samples (samples 10, 21, 22, and 23, **Supplementary Table 1**) did not amplify under these conditions, so an additional five cycles were performed for these samples. PCR products were quantified and visualized using the QIAxcel DNA Fast Analysis (Qiagen). Negative buffer controls (generated by performing a DNA extraction on lysis solution handled identically to tissue samples during larval and adult sample collection) failed to amplify but were still included in the sequencing reaction to account for any potential contamination. PCR products were normalized based on the concentration of DNA from 350 to 420 bp then pooled using the QIAgility liquid handling robot. The pooled PCR products were cleaned using the Mag-Bind RxnPure Plus (Omega Bio-tek) according to the manufacturer's protocol. The cleaned pool was sequenced on a MiSeq system using the MiSeq Reagent Kit V2 (Illumina, Inc.).

Sequences were demultiplexed using onboard bcl2fastq. Demultiplexed sequences were processed in Mothur v. 1.39.4 following the MiSeq SOP (Kozich et al., 2013), and exact commands can be found here: https://github.com/krmaas/bioinformatics/blob/master/mothur.batch. Merged sequences that had any ambiguities or did not meet length expectations were removed. Sequences were aligned to the Silva nr_v119 alignment (Quast et al., 2013). Taxonomic identification of OTUs was done using the RDP Bayesian classifier (Wang et al., 2007) against the Silva nr_v119 taxonomy database. OTUs were determined using the opti clustering method with a distance cutoff of 0.03 (97% similarity).

Data Analysis

Wing Length Data Analysis

To assess the effect of diet regimen on wing length, we fit a linear mixed-effect model using lme in the package "nlme" in R (Pinheiro et al., 2019). The response variable was wing length in millimeters and we used diet regimen as a fixed effect and biological replicate as a random effect. We measured wings from eight individuals per diet regimen per biological replicate, for a total of 24 measurements per diet regimen. After fitting the overall model, we performed an ANOVA querying the effect of diet regimen followed by a Tukey's test using the glht function in the package "multcomp" in R (Hothorn et al., 2008) to compare wing lengths between each diet regimen. Models and outputs can be found in **Supplementary File 1**. Raw data can be found in **Supplementary File 2**.

qPCR Data Analysis

To assess the effect of diet regimen on bacterial 16S copy number, we fit linear mixed-effect nested models using lme in the package "nlme" in R (Pinheiro et al., 2019). Separate models were fitted to breeding water data, larval data, and adult data. Both sugar fed and blood fed adults were analyzed together since they were sampled at the same time. The full model included average 16S copy number as the response variable, larval diet as a fixed effect and biological replicate and technical replicate (pool) as random effects. For adult data, we also included adult feeding status as a fixed effect. Biological replicate is defined as larval tray/cage per diet level (3 trays/cages = 3 biological replicates). Technical replicates are the individual pools taken per tray/cage. Technical replicate was nested within biological replicate in all analyses. After fitting the overall model, we performed an ANOVA to determine the overall significance of fixed effects, and then performed a Tukey's test using the glht function in the package "multcomp" in R (Hothorn et al., 2008) to assess pairwise differences between diet regimens. Models and outputs can be found in **Supplementary File 1**. Raw data can be found in **Supplementary File 2**.

Sequencing Data Analysis

Sequencing data analysis was primarily performed using the phyloseq and vegan packages in R (McMurdie and Holmes, 2013; Oksanen et al., 2019). Sequences were filtered to remove any taxa that did not map to bacteria and all taxa that contained fewer than 0.005% of all reads in the dataset (Bokulich et al., 2013). To assess the potential for contamination, Bray Curtis dissimilarity values were calculated and a non-metric multidimensional scaling (NMDS) analysis performed to compare the buffer blanks to all experimental samples. To determine whether sequencing was repeatable across technical

replicates, a PERMANOVA was performed on all samples assessing the effect of technical replicate nested within biological replicate. No significant effect of technical replicate was detected, suggesting microbiota composition is consistent across technical replicates. To standardize sample number across all treatments, for all downstream analyses only the first technical replicate was used. Therefore, the final sequencing dataset included three diets (R1, R2, and R4) and four sample types (breeding water, larvae, adult sugar fed, adult blood fed), and for all combinations, three biological replicates were analyzed. Each biological replicate consisted of a pool of 5–8 individuals; samples used in final analysis and number of individuals per pool can be found in **Supplementary Table 1**. Effect of diet and sample type on relative abundance of individual OTUs was performed using Analysis of Composition of Microbiomes (ANCOM) (Mandal et al., 2015). This analysis method allows for the inclusion of random effects (biological replicate in our case) and corrects for multiple comparisons to control for the fact that we are testing the effect of diet on each OTU in our dataset. This was first performed on all samples combined and then, given the dramatic differences in composition between sample types, on breeding water/larval samples alone. ANCOM would not successfully run on adult samples, likely due to the highly unbalanced nature of the adult sample dataset (most reads fall into very few OTUs). This persisted even after repeated attempts to trim the dataset to reduce low frequency and zero-count OTUs, suggesting it is an inherent problem in the structure of the dataset and not a filtering issue. The dataset was then scaled to standardize the number of reads in each sample using a method developed by Denef et al., 2016. This approach achieves the same result as rarefying the dataset. The scaled dataset was used for alpha and beta diversity analyses. Alpha diversity indices were generated using the estimate_richness command in phyloseq. Main effects of diet and treatment and an interaction between the factors was assessed for each index using an ANOVA. To assess beta diversity, Bray Curtis dissimilarity values were calculated and NMDS analysis performed using the ordinate function in phyloseq. PERMANOVA was performed using adonis in vegan. All R code, notes, and outputs for microbiota composition analysis can be found in **Supplementary File 3**.

RESULTS

Mosquitoes Reared With Lower Abundance of Larval Diet Host a Smaller Microbiota Even After Accounting for Reduced Mosquito Body Size

First, it was important to assess whether larval diet abundance affects body size, because differences in body size among the diet treatments could influence total bacterial load as well. We measured wing length of adults from the alular notch to the distal end to estimate overall body size. We used a linear mixed-effects model to assess the impact of diet on overall wing size and, as expected (Yeap et al., 2011), found that diet significantly predicted wing size ($F = 46.11$, $p < 0.0001$). Pairwise comparisons using a Tukey's test showed that adults reared with the lowest access to food (the "R1" group, **Table 1**) had significantly shorter wings than all other groups (**Figure 1**). Similarly, individuals with the next highest access to food (the "R2" group, **Table 1**) had significantly longer wings than R1 individuals but significantly shorter wings than individuals from R3 and R4 (**Figure 1**). Individuals reared under higher larval food abundance (R3 and R4) were not significantly different from each other. Significant differences in wing size range from a 2.7% increase (R2 vs. R4) to an 8.9% increase (R1 vs. R3) (**Supplementary Table 3**), which does reflect a potential difference in size of the midgut between treatments. To account for this issue, all 16S qPCR values were corrected for expression of S7, a housekeeping gene, which controls for potential differences in tissue amounts.

TABLE 1 | Amount of larval food per larva per day for four feeding regimens.

Feeding regimen	Days 0–1 post hatching Diet conc. (mg/larva/day)	Day 2 post hatching – pupation Diet conc. (mg/larva/day)
R1	0.125	0.25
R2	0.375	0.75
R3	0.5	1
R4	1	2

Next, we quantified total bacterial load in breeding water, whole larvae, and adult mosquito midguts from all larval feeding regimens using qPCR targeting the bacterial 16S rRNA gene. We then used linear mixed effect models to determine the effect of feeding regimen on total bacterial load in each sample type. In larval breeding water, we found that diet was a significant predictor of bacterial 16S rRNA gene copy number (**Figure 2A**;

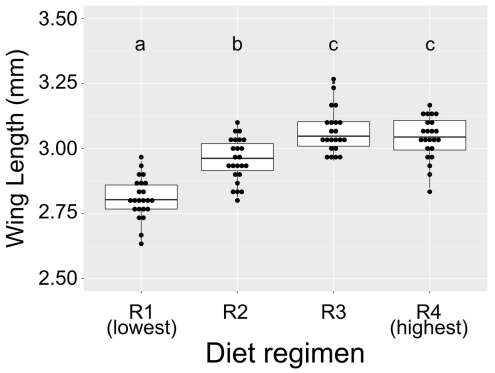

FIGURE 1 | Diet abundance during larval development significantly affects adult wing size. We measured wing length (in mm) of adult female *A. aegypti* reared under different larval diet amounts. Amount of diet was lowest in R1 and increased through R4 (**Table 1**). Results are from eight individuals per biological replicate ($n = 24$ per treatment). Data were analyzed using a linear mixed model with diet regimen as a fixed effect and biological replicate as a random effect. ANOVA indicated a significant effect of diet on wing length ($F = 46.11$, $p < 0.0001$). Significant pairwise differences ($p < 0.01$) between treatments were assessed using Tukey's test and are denoted by letters above each treatment.

F-value = 5.537, p = 0.0092). When comparing all diet regimens pairwise, we found that R4 samples (the highest diet amount) had significantly higher 16S copy number than samples reared in both R1 ($p < 0.001$) and R2 ($p = 0.016$) diet regimens. No other pairwise comparisons were significant. Among larvae, we found that larval diet was a significant predictor of bacterial 16S relative abundance (**Figure 2B**; F-value = 16.734, $p < 0.0001$). In pairwise comparisons, we found that the bacterial load of whole larvae did not differ significantly between individuals from the R1 and R4 treatments ($p = 0.092$), but was significantly higher in individuals from the R2 and R3 treatments compared to those from both the R1 and R4 treatments (**Figure 2B**; R1 vs. R2, $p < 0.0001$; R1 vs. R3, $p < 0.0001$; R4 vs. R2, $p = 0.004$; R4 vs. R3, $p < 0.0001$). Among adults, we found that both larval diet (F-value = 15.934, $p < 0.0001$) and blood feeding status (F-value = 168.036, $p < 0.0001$) significantly predicted relative 16S rRNA levels but that there was no interaction between the two factors, suggesting that the effect of diet is consistent regardless of blood feeding status (**Figure 2C**). We therefore removed the interaction from the model and performed pairwise comparisons between diet levels regardless of blood feeding status. We found that individuals from the R1 treatment had significantly lower midgut bacterial loads than individuals from all other treatments and that the other treatments were not significantly different from one another (**Figure 2C**; R1 vs. R2, $p < 0.0001$; R1 vs. R3, $p < 0.0001$; R1 vs. R4, $p < 0.0001$; R2 vs. R3, $p = 0.770$; R2 vs. R4, $p = 0.995$; R3 vs. R4, $p = 0.589$).

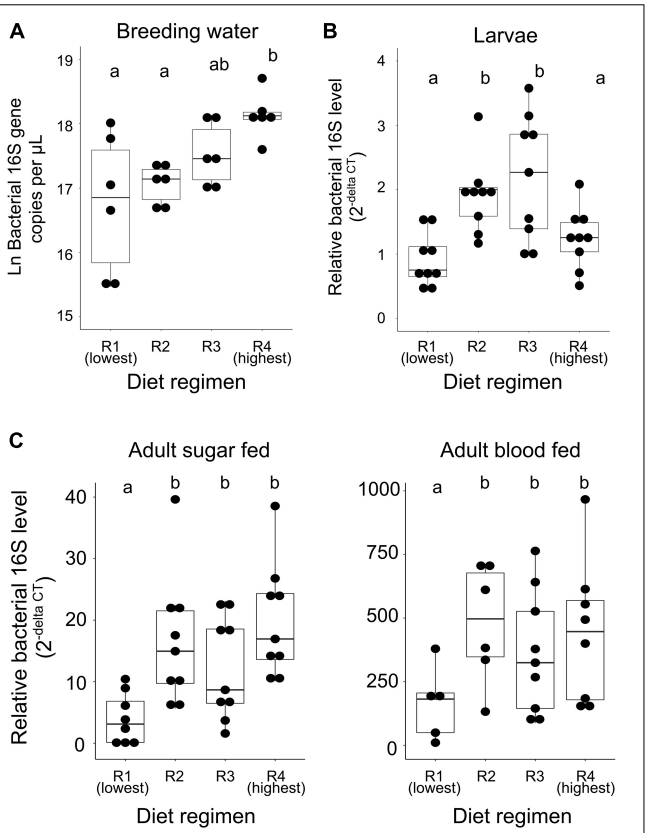

FIGURE 2 | Microbial community size is restricted by low diet availability during larval development. We reared *A. aegypti* larvae with varying access to larval diet (R1 = lowest abundance, R4 = highest, **Table 1**) and used 16S qPCR to quantify bacterial load in breeding water, whole larvae, sugar fed adult midguts, and blood fed adult midguts. Data were analyzed using mixed effect nested linear models to determine the effect of diet regimen on 16S level. Results of pairwise comparisons are denoted by letters above each treatment. **(A)** Higher diet availability results in higher 16S levels in breeding water (F-value = 5.537, p = 0.0092). For each diet, N = 6 (two samples from each of three replicate breeding trays). **(B)** Food availability significantly impacts 16S levels in larvae (F-value = 16.734, $p < 0.0001$). For each diet, N = 9 (three samples from each of three replicate breeding trays). **(C)** Low food availability during larval development results in lower 16S levels in sugar fed and blood fed adult midguts (F-value = 15.934, $p < 0.0001$); 16S levels are also significantly predicted by blood feeding status (F-value = 168.036, $p < 0.0001$). For each diet/feeding status combination, N = 9 (three samples from each of three replicate cages) with the exceptions of R1/sugar fed, N = 8; R1/blood fed, N = 5; R2/blood fed, N = 6; R4/blood fed, N = 8.

Larval Diet Abundance Induces Significant Shifts in Bacterial Community Composition

In order to assess the effects of larval diet abundance on microbial community composition, we performed high throughput 16S amplicon sequencing on breeding water, whole larvae, sugar fed adult midguts, and blood fed adult midguts from feeding regimens R1, R2, and R4. Average number of sequences obtained per experimental sample after all filtering steps was 44,180.2 and samples ranged from 6,647 to 83,125 total reads (**Supplementary File 3**). The blank samples we collected (which consisted of lysis solution handled identically to larval and adult tissue samples during collection) failed to amplify in PCR and yielded very few sequences (mean = 144.5; range: 66–361 total reads) which suggests that contaminants are likely to be, on average, approximately 0.3% of the reads in our experimental samples. We performed a NMDS analysis to evaluate similarity between our experimental samples and blanks, which showed that blanks cluster together and separately from experimental samples (**Supplementary Figure 1**). We therefore proceeded with analysis despite the presence of this minor contamination.

Our initial OTU assignment at 97% similarity resulted in 76 OTUs but 99.9% of the total reads fell into 19 OTUs, indicating simple microbial communities among all experimental samples (**Figure 3**). Communities were composed primarily of the phyla Proteobacteria (44.3% of all reads) and Bacteriodetes (55.5% of all reads). Families most commonly found among our samples were *Flavobacteriaceae* (50.2% of all reads), *Moraxellaceae* (20.8% of all reads), *Enterobacteriaceae* (15.8% of all reads), *Cytophagaceae* (5.0% of all reads), *Oxalobacteraceae* (1.8% of all reads), Neisseriaceae (1.6% of all reads), *Sphingomonadaceae* (1.6% of all reads), and *Acetobacteraceae* (1.1% of all reads) (**Figure 3**). All other families accounted for less than 1% of all reads. Initial examination of relative abundance data at the family level showed that bacterial communities were more diverse in water and larval samples than in adults, which primarily consisted of only two families (*Flavobacteriaceae* and *Enterobacteriaceae*). Taxa from the family *Enterobacteriaceae* increased in relative abundance with increased larval diet

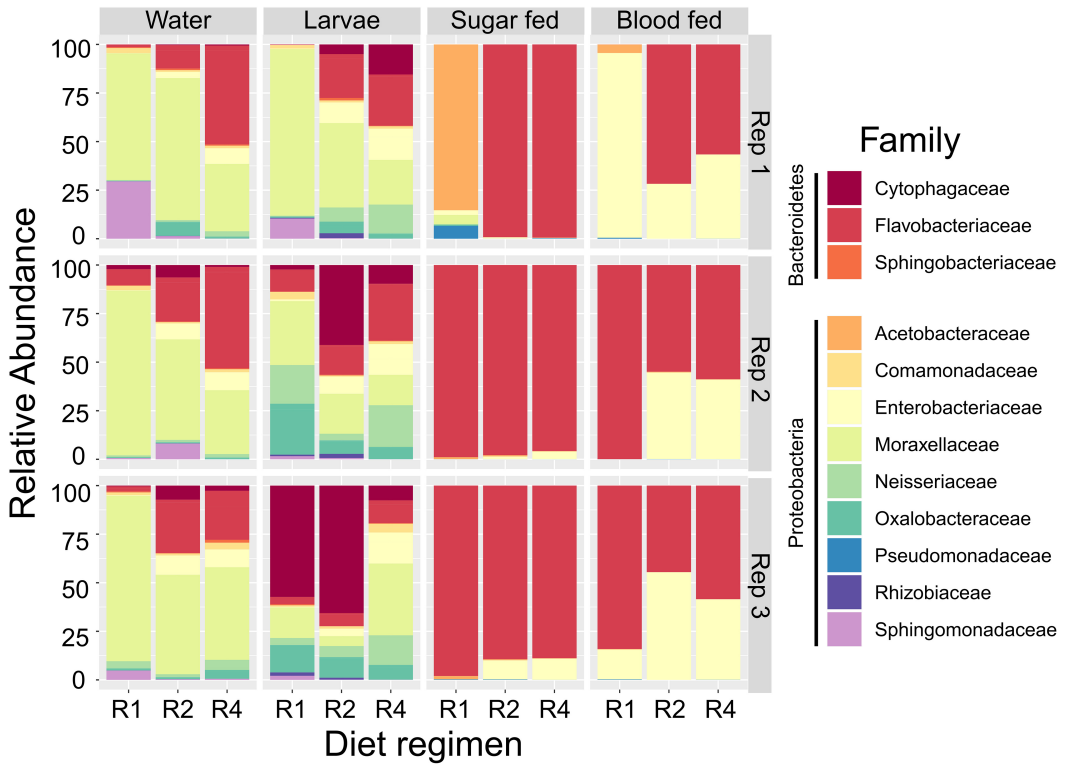

FIGURE 3 | Larval diet abundance impacts microbiota composition. Percent composition of bacterial families in larval breeding water, larvae, adult sugar fed midguts, and adult blood fed midguts from three feeding regimens (R1, R2, and R4, **Table 1**) and three biological replicates. Taxa containing fewer than 0.1% of all reads in the entire dataset were excluded.

amount, as did taxa from *Flavobacteriaceae*, but only in breeding water and larvae (**Figure 3**).

We assessed whether alpha diversity (i.e., microbial community diversity within samples) differed between diet regimens and sample types, using measures of richness (observed OTUs and Chao1 index) and Simpson's index (1-D), which accounts for richness and evenness. Observed richness did not significantly differ by diet regimen (F = 0.296; p = 0.746) but sample type was highly significant (F = 106.45; $p = 4.38 \times 10^{-16}$; **Figure 4**). The observed number of OTUs was highest in larval breeding water and larvae and substantially lower in sugar fed and blood fed adult midguts (**Figure 4**). For Chao1, we observed a significant interaction between diet regimen and sample type (F = 3.847; p = 0.008), indicating that the effect of diet regimen on estimated richness differed by sample type. Upon further inspection of the data, we found that Chao1 index values were higher in treatments with higher larval diet concentrations, but this effect was primarily limited to the breeding water (**Figure 4**). Diet regimen significantly predicted Simpson's Index value (F = 4.74, p = 0.016) and sample type was highly significant (F = 20.19, $p = 2.35 \times 10^{-7}$); there was no significant interaction between diet regimen and sample type, suggesting any effect of diet regimen is consistent across sample types (**Figure 4**). Simpson's index was lowest in samples from the R1 treatment and higher in R4 samples (**Figure 4**).

We also assessed beta diversity (i.e., differences in microbial community between samples) in our samples and the effects of diet and sample type on beta diversity. Using the subsampled dataset, we performed a NMDS analysis on all samples using Bray Curtis dissimilarity as the distance metric. This revealed clustering by sample type, with breeding water and larvae clustering together and sugar fed and blood fed adults clustering together (**Figure 5A**). This analysis also suggested clustering by diet, and this effect appeared to be mostly limited to breeding water and larval samples (**Figure 5B**). To test whether larval diet or sample type significantly predicted differences in microbiota composition between samples, we performed a PERMANOVA on Bray Curtis dissimilarity values to assess the effect of diet (R1, R2, or R4) and sample type (breeding water, larvae, sugar fed adult, blood fed adult). We found a significant overall effect of diet (p = 0.003) and sample type (p = 0.001) and the interaction between these factors was not significant (p = 0.856).

Our beta diversity analysis indicated that overall microbial community composition varied significantly between diets. We therefore determined whether the prevalence of specific OTUs was significantly affected by diet using ANCOM. When we analyzed all sample types together (breeding water, larvae, sugar fed adults, and blood fed adults), we found that diet significantly affected the abundance of only one OTU (OTU0003, W = 58, significant at 0.8 cutoff threshold) which mapped to the family *Enterobacteriaceae* and the genus *Cedcea* (**Figure 6**).

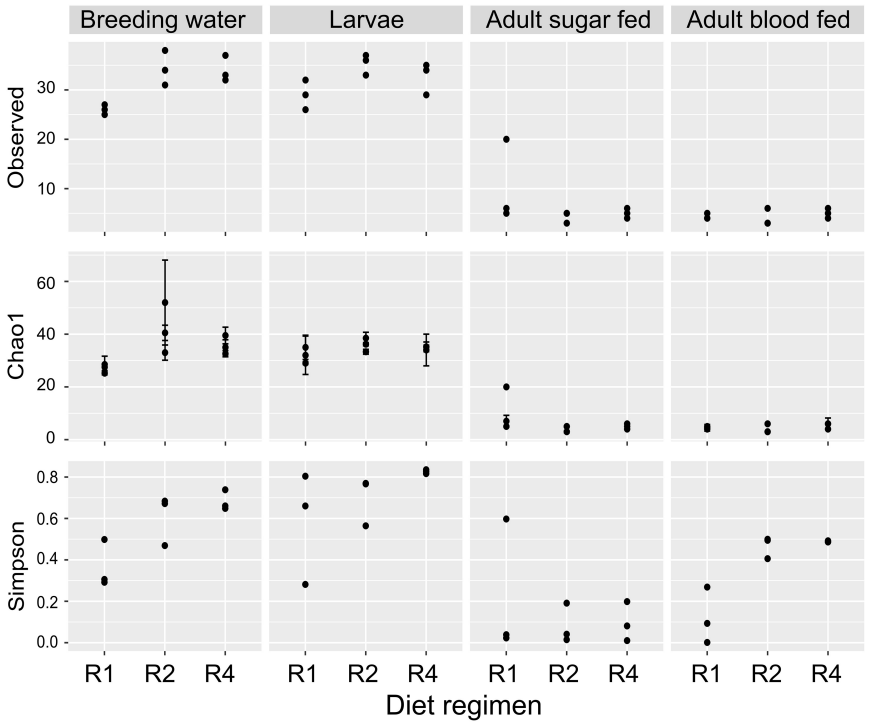

FIGURE 4 | Diet during larval breeding impacts alpha diversity. We measured alpha diversity across three larval diets (R1, R2, and R4) and four sample types (breeding water, larvae, adult sugar fed, adult blood fed). We performed a two-factor ANOVA including diet, sample type, and diet/sample type interaction as factors. For observed OTUs, the interaction of diet and sample type was not significant ($F = 2.193$; $p = 0.08$), nor was the main effect of diet ($F = 0.296$; $p = 0.7459$). Sample type, however, was significant ($F = 106.446$; $p = 4.38 \times 10^{-16}$). For Chao1 values, the interaction of diet and sample type was significant ($F = 3.847$, $p = 0.008$). For Simpson's index (1-D), the interaction of diet and sample type was not significant ($F = 2.174$; $p = 0.08$) but there was a significant effect of diet regimen ($F = 4.745$, $p = 0.016$) and sample type ($F = 20.193$; $p = 2.35 \times 10^{-7}$).

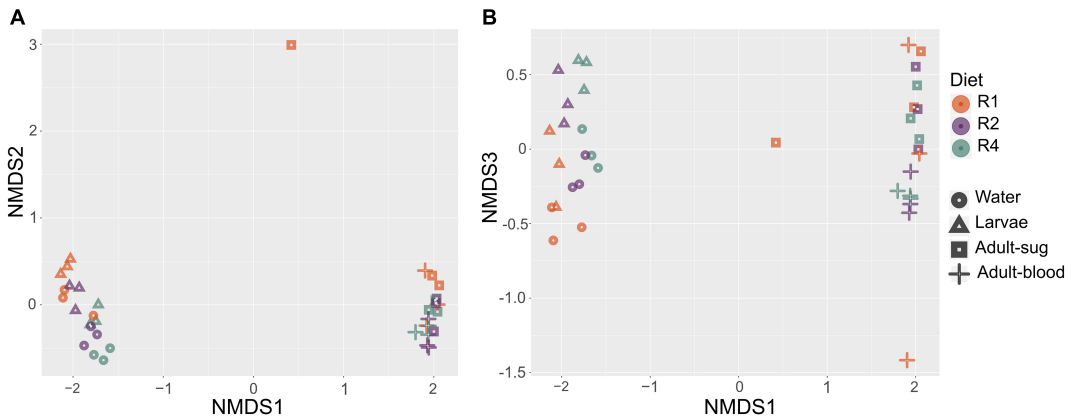

FIGURE 5 | Non-metric multidimensional scaling analysis reveals clustering by sample type and larval diet regimen. We performed NMDS analysis using Bray-Curtis dissimilarity values. Plots show axes 1 and 2 **(A)** and axes 1 and 3 **(B)**. Color is used to indicate diet regimen (orange = R1, purple = R2, green = R4) and shape is used to indicate sample type (circle = breeding water, triangle = larvae, square = adult sugar fed midguts, cross = adult blood fed midguts). Three biological replicate samples are plotted for each diet regimen-sample type combination.

This OTU was in relatively high abundance in the dataset (15.5% of total reads). A histogram of read counts from this single OTU showed its abundance was positively correlated with diet amount. It was much less abundant in individuals from the lowest "R1" diet compared to higher diets (R2 and R4, **Figure 6**). This was consistent across all sample types and replicates, with the exception of one replicate in the blood fed adult treatment (**Figure 6**).

Given that the microbiota composition of adults differed substantially from that of breeding water and larvae (**Figure 3**),

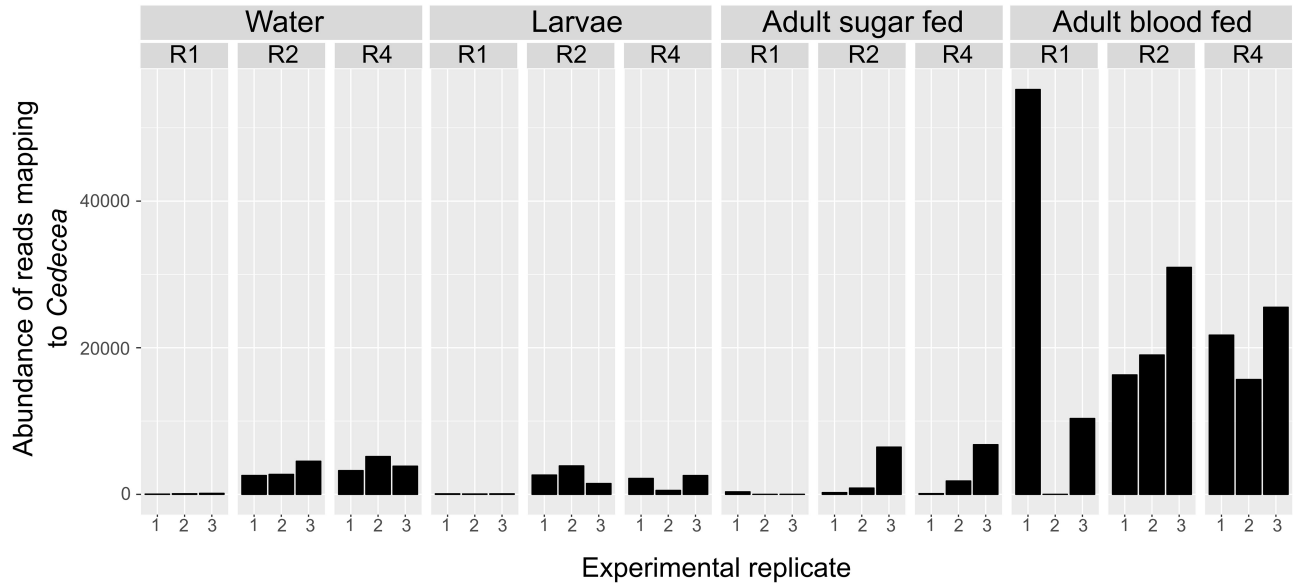

FIGURE 6 | Abundance of *Cedecea* is significantly affected by larval diet amount in all sample types. Abundance of one OTU that mapped to the genus *Cedecea* was significantly affected by larval diet amount (W = 58, significant at 0.8 cutoff threshold). Abundance is plotted for all sample types, diet treatments, and biological replicates. Abundance is lowest in R1 in general and higher in R2 and R4 treatments. This is consistent across all sample types and replicates with the singular exception of the first replicate of adult blood fed samples.

and given that the effect of diet on microbiota composition was primarily restricted to water and larval samples, we performed a subsequent ANCOM analysis on only breeding water and larval samples. Among breeding water and larval samples, we found six OTUs that differed significantly by diet (**Figure 7**). These OTUs mapped to the genera *Sphingomonas*, *Clostridium*, *Duganella*, *Pseudomonas*, and *Rhizobium*, and one could not be mapped below the family level *Comamonadaceae*. OTUs mapping to *Sphingomonas*, *Pseudomonas*, and *Comamonadaceae* were more abundant in R1, the lowest diet treatment compared to R2 and R4. The OTU mapping to *Clostridium* was much less abundant in R1 and R2 compared to R4, and that mapping to *Duganella* was absent in R1 but present in R2 and R4, albeit at low levels (**Figure 7**). Finally, the OTU mapping to *Rhizobium* was more abundant in R1 and R2 than in R4 where it was nearly absent.

DISCUSSION

In this work, we investigated how diet abundance in the larval breeding water affects bacterial community size and composition over the life history of *Aedes aegypti.* We found that the total amount of bacteria was lowest in samples from the lowest diet treatment (R1) and generally increased with higher larval diet abundance. We also found that diversity within samples increased with increasing diet abundance and that diet was a significant predictor of composition differences between samples. These effects were primarily observed in breeding water and larvae, however, we observed diet-induced changes in *Enterobacteriaceae* abundance that persisted into adulthood and even after blood feeding. Taken together, these findings suggest that the amount of diet available in the breeding water can influence the number of bacteria in the larval and adult midgut microbiota and these effects last long after adult eclosion. The data also suggest that nutrient availability can shift the composition of the microbiota during larval development, and that for certain OTUs, changes in composition persist into adulthood, when female mosquitoes transmit human pathogens.

The total amount of bacteria in our samples was, in general, positively correlated with larval diet abundance. This is not unexpected, as access to more nutrients in the breeding water would logically promote propagation of bacteria within the aquatic microbial community. Another non-mutually exclusive hypothesis is that shifts in the composition of the breeding water at higher larval diet abundance favor bacteria that grow to naturally higher numbers in the laboratory breeding environment or in the mosquito.

Bacterial load was lowest in R1 larvae and increased in R2 and R3 larvae. Bacterial load in R4 larvae was similar to R1 larvae despite this regimen having the highest bacterial load in the breeding water samples. Bacterial load in the larval digestive tract is logically expected to be directly related to the number of bacteria ingested. Larvae feed by sweeping water and detritus (and any accompanying bacteria) into their mouths using their brushes (Christophers, 1960), so it is probable that more bacteria in the breeding water would result in larvae ingesting more bacteria, and thus a larger microbial community in the gut. This could explain the differences we see between R1, R2, and R3, but not R4 larvae. One technical explanation for why bacterial load was low in R4 larvae is that the number of live bacteria in the gut is dynamic during development; live bacteria increase after a molt until larvae reach a critical size, at which point

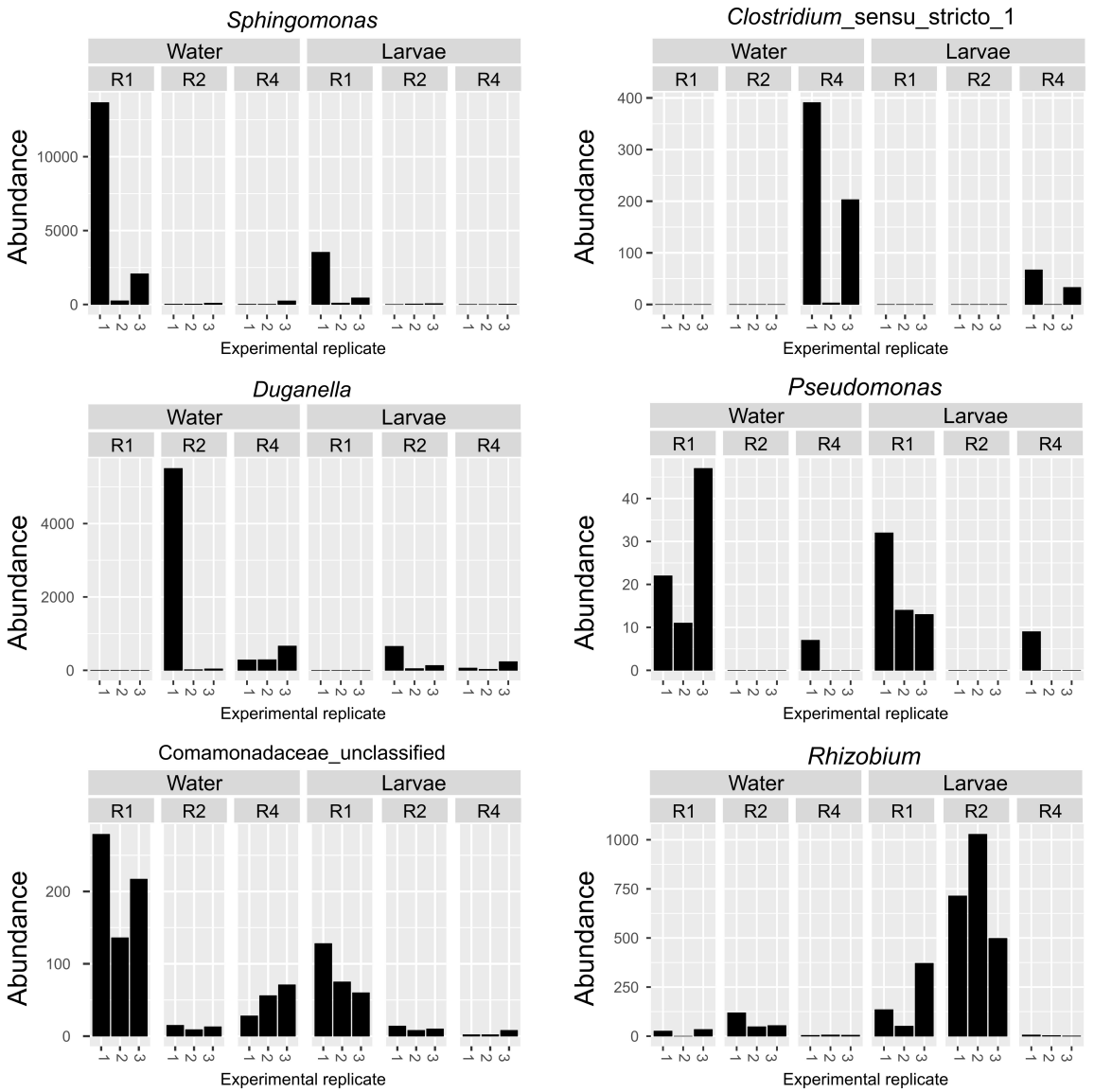

FIGURE 7 | Specific OTUs are significantly affected by larval diet amount in breeding water and larvae only. Abundance of six OTUs was significantly affected by diet amounts in breeding water and larval samples. For each OTU, abundance is plotted for each sample type, diet treatment and biological replicate.

bacteria in the gut start to die (Coon et al., 2017). We sampled larvae at day 5 post-hatching when we anticipated they would all be 4th instars but none would have pupated. It is possible that R4 larvae were hours more advanced in their developmental timing and that the decrease in bacterial load is a result of their being closer to pupation than the other treatments. Our sampling approach was not designed to capture fluctuations in bacterial load at such hour-level resolution, and the time scale of bacterial load fluctuation in developing mosquito larvae should be considered in future investigations of diet and mosquito microbiome formation.

From a biological perspective, there are many potential hypotheses for why we observed such low numbers of bacteria in R4 despite high bacterial load in the breeding water. For example, larvae show different rates of bacterial ingestion depending on the microbes in their breeding water (Souza et al., 2019). Bacterial composition significantly shifts with increased diet, and it is therefore possible that R4 larvae experience a behavioral shift toward reduced ingestion. However, additional work is necessary to test whether the impact of diet on microbial community affects larval feeding behavior. Bacterial load could also be influenced by the ability of different bacteria to persist in the mosquito, as well as immune system signaling or other physiological processes in the mosquito that influence bacterial survival, such as pH regulation or digestion. pH in the larval digestive tract has been well documented as highly alkaline (as high as pH 11 in parts of the midgut) (Dadd, 1975; Boudko et al., 2001; Corena et al., 2002). High pH has been shown to

negatively impact survival of bacteria commonly found in the mosquito breeding water and adult mosquito midgut (Lindh et al., 2008; Coon et al., 2017). It is therefore possible that shifts in composition in R4 breeding water may have favored bacteria more sensitive to alkalinity, resulting in lower overall bacterial loads. pH tolerance varies substantially even within a given bacterial genus (Barberán et al., 2017), therefore, species-level resolution would be required to determine if microbial alkalinity tolerance explains differences in bacterial load across diet treatments. Studies from other insect systems have shown that diet can influence systemic immune system signaling and susceptibility to infection in immature developmental stages, suggesting widespread connections between diet and the larval immune system (Ponton et al., 2013). It is possible that effects of diet on bacterial load in R4 larvae may be a result of differences in immune system activity. More research is needed to understand how different bacteria persist or are expelled from the larval mosquito and how mosquito larvae regulate immune signaling in response to diet and bacterial load in their environment.

Among adults, those reared with the lowest larval diet concentration had the lowest bacterial loads while those from all other treatments were significantly higher. This was consistent regardless of whether adults were sugar fed or blood fed, though bacterial loads were significantly higher overall among blood fed adults. Mosquitoes lose the vast majority of their enteric bacteria during eclosion from pupa to adult (Moll et al., 2001). Bacteria can be transstadially transmitted, though it is not clear if this happens internally or if the adult imbibes breeding water shortly after eclosion, thereby re-populating the gut with environmental bacteria (Lindh et al., 2008; Coon et al., 2014). In either case, we expect the number of bacteria in the adult midgut after eclosion to be quite low regardless of diet treatment. Differences in bacterial load after that point could potentially be influenced by ingestion of bacteria, and/or host-microbe or microbe-microbe interactions within the adult. Multiple studies have identified mechanisms that regulate the bacterial population in the adult mosquito midgut; some are driven by the bacteria themselves and some by the mosquito. For example, bacterial colonization is in some cases determined by ability of bacteria to form biofilms in the mosquito digestive tract (Hegde et al., 2019). Additionally, network analysis has shown that the presence of certain bacteria in the mosquito are significantly correlated with the presence or absence of other bacterial taxa, and the presence of certain bacteria in the digestive tract can act to reduce the numbers of other bacterial taxa (Hegde et al., 2018). This suggests that microbe-microbe interactions may play a substantial role in determining formation of the mosquito microbiota. The adult mosquito regulates the bacterial load in its gut in multiple ways, including immune system signaling (Meister et al., 2009; Clayton et al., 2013), regulating reactive oxygen species (Oliveira et al., 2011), and amino acid metabolic signaling (Short et al., 2017). Another important determinant of bacterial load is whether the mosquito is sugar fed or blood fed, as blood feeding causes rapid bacterial proliferation (Kumar et al., 2010; Oliveira et al., 2011). Low access to larval diet has been shown to affect transcript abundance of amino acid metabolism genes and many immune system genes (Telang et al., 2012; Price et al., 2015), and has also been shown to reduce melanization capacity and hemocyte number in adult mosquitoes (Suwanchaichinda and Paskewitz, 1998; Telang et al., 2012). These phenotypes were measured in whole adults, not the midgut, but they suggest multiple physiological changes that occur in response to low diet abundance that could impact bacterial load. Given our finding that low diet abundance resulted in reduced bacterial load in adult mosquitoes, further investigation is warranted to determine whether any of the potential mechanisms discussed above shape this relationship.

In addition to changes in total bacterial load, we found that diet amount significantly affected microbiota composition and diversity. As expected, alpha diversity was highest in breeding water and larvae and decreased dramatically in adults. Diet significantly affected species richness only in breeding water and higher diet correlated with significantly higher Simson's index values across all sample types. Since richness was not affected by diet in most sample types, we can reasonably infer that increased diet primarily affected evenness, and that higher larval diet resulted in a more even distribution of sequence reads between taxa. In investigating beta diversity, we found the strongest predictor of differences between samples to be sample type, with breeding water and larvae clustering together and away from all adult samples. This is consistent with other studies, which have also documented differences in microbiota composition between breeding water, larvae, and adults (Wang et al., 2011; Gimonneau et al., 2014). Most relevant to our study, we also found that diet was a significant predictor of differences between samples, especially among breeding water and larvae. Among these sample types, we observed clustering by diet, suggesting that access to different diet levels during larval development causes significant shifts in microbiota composition.

In light of the shifts in overall diversity, we found that specific taxa were especially affected by diet, in particular *Enterobacteriaceae*. As diet amount increased, so did relative abundance of *Enterobacteriaceae*, and this difference persisted into adulthood. Linenberg et al. (2016) demonstrated that rearing *Anopheles gambiae* larvae with fish food flakes vs. fish food pellets resulted in a decrease in total amounts of *Enterobacteriaceae* and an increase in relative levels of *Flavobacteriaceae* in larvae. As adults, those reared on pellets had less *Enterobacteriaceae* than those reared on flakes (Linenberg et al., 2016). Our findings, coupled with theirs, suggest that larval diet is a significant determinant of the degree to which *Enterobacteriaceae* colonize mosquitoes across diverse genera. Bacteria from the family *Enterobacteriaceae* are commonly found in the digestive tract of mosquitoes, including those from the genera *Serratia*, *Pantoea*, *Klebsiella*, and *Enterobacter* (Guégan et al., 2018), and bacteria from this family have been tied to a number of interesting phenotypes relevant to vector borne disease transmission. For example, *Serratia* has been shown to influence susceptibility to dengue and chikungunya viruses in *Aedes aegypti* (Apte-Deshpande et al., 2012, 2014; Wu et al., 2019), susceptibility to *Plasmodium* infection in *Anopheles* (Bahia et al., 2014;

Gendrin et al., 2015), and mosquito longevity (Bahia et al., 2014). Additionally, *Enterobacter* in *Anopheles* has been shown to be predictive of *Plasmodium* infection in the field and to have a significant impact on *Plasmodium* susceptibility in the laboratory (Cirimotich et al., 2011; Boissière et al., 2012). In our study, the increase in *Enterobacteriaceae* was primarily due to one OTU that mapped to the genus *Cedecea*. This OTU was at very low abundance in mosquitoes from the lowest diet (R1) but increased significantly in higher diet treatments (R2 and R4). Abundance was similar between R2 and R4, suggesting diet amount above a particular threshold does not cause additional *Cedecea* propagation. *Cedecea* has been shown to form biofilms in the digestive tract of adult mosquitoes, and biofilm formation is critical to colonization in adults (Hegde et al., 2019). It is possible that larval diet could impact abundance of these bacteria by influencing their ability to form biofilms, and future studies into this topic and other environmental drivers of bacterial colonization of mosquito midguts are warranted.

In addition to *Cedecea*, which was affected by diet across all sample types, we also identified multiple OTUs that were significantly affected by diet in only breeding water and larval samples. These included taxa identified as *Sphingomonas*, *Pseudomonas*, and an unidentified member of *Comamonadaceae*, which were all higher abundance in R1 individuals compared to R2 and R4. Interestingly, a study by Duguma et al. (2017) testing the effect of high vs. low organic matter (rabbit feed) in larval breeding water on microbiota composition in *Culex nigripalpus*, identified bacteria from the order Burkholderiales (which contains *Comamonadaceae*) as the primary indicator taxon for low organic matter. Additionally, we found that an OTU mapping to *Clostridium* was more highly abundant in R4 individuals relative to R1 or R2. In the same study, Duguma et al. found that Clostridiales (which contains *Clostridium*), was an indicator taxon for the high organic matter treatment. These findings are broadly consistent with the findings in our study. Both our study and that of Duguma et al. used rabbit chow as larval diet, however, we also supplemented our larval diet with liver powder and fish food. This suggests that enrichment of these taxa in low vs. high nutrient breeding water may be consistent across diverse genera of mosquitoes and a variety of diet types, though this requires further investigation. The consistent enrichment of these taxa in different studies of mosquito diet and microbiota formation also suggests that these relationships are reproducible under different experimental conditions (e.g., laboratory, semi-field).

Overall, we have shown that the amount of larval diet in the breeding water has a significant effect on microbiota size and composition in *Aedes aegypti*, and that these effects last into adulthood and persist after blood feeding. Nutrient levels in breeding water are variable in the field but may be predictable to some degree. For example, *A. aegypti* breed in man-made containers such as tires, but they also successfully breed in septic tanks. The nature of the nutrient content and dissolved organic material in these breeding sites is likely to be quite different and may drive predictable differences in microbiota composition. Continued investigation into the environmental drivers of variation in microbiota formation, both in controlled laboratory assays and in the field, is warranted and will provide a better understanding of how mosquito microbiomes are formed. Ultimately, these findings increase our understanding of how the microbiome may be used to understand human pathogen transmission and to develop and target interventions to reduce mosquito-borne disease transmission.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the NIH Sequence Read Archive, accession number PRJNA705151.

AUTHOR CONTRIBUTIONS

HM contributed to data collection and manuscript preparation. GD contributed to study design and manuscript preparation. SS contributed to study design, data collection, data analysis, and manuscript preparation. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.645362/full#supplementary-material

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The Effect of Secondary Metabolites Produced by *Serratia marcescens* on *Aedes aegypti* and Its Microbiota

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Serratia marcescens is a bacterial species widely found in the environment, which very efficiently colonizes mosquitoes. In this study, we isolated a red-pigmented *S. marcescens* strain from our mosquito colony (called *S. marcescens* VA). This red pigmentation is caused by the production of prodigiosin, a molecule with antibacterial properties. To investigate the role of prodigiosin on mosquito-*S. marcescens* interactions, we produced two white mutants of *S. marcescens* VA by random mutagenesis. Whole genome sequencing and chemical analyses suggest that one mutant has a nonsense mutation in the gene encoding prodigiosin synthase, while the other one is deficient in the production of several types of secondary metabolites including prodigiosin and serratamolide. We used our mutants to investigate how *S. marcescens* secondary metabolites affect the mosquito and its microbiota. Our *in vitro* tests indicated that *S. marcescens* VA inhibits the growth of several mosquito microbiota isolates using a combination of prodigiosin and other secondary metabolites, corroborating published data. This strain requires secondary metabolites other than prodigiosin for its proteolytic and hemolytic activities. In the mosquito, we observed that *S. marcescens* VA is highly virulent to larvae in a prodigiosin-dependent manner, while its virulence on adults is lower and largely depends on other metabolites.

Keywords: ***Serratia*, *Aedes aegypti*, secondary metabolite, prodigiosin, microbiota, serratamolide, serrawettin, Hfq**

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INTRODUCTION

Aedes aegypti mosquitoes are the main vectors of several arthropod-borne viruses of importance to human health, including dengue, Zika, and chikungunya viruses. The ability of mosquitoes to transmit diseases is influenced by several environmental and intrinsic factors, which affect mosquito population size, lifespan, and interactions with viruses. These factors notably include rainfall, temperature, mosquito antiviral immunity, as well as the microbial communities harbored by the mosquito epithelia (Lefèvre et al., 2013; Lee et al., 2019; Scolari et al., 2019). Interestingly, some studies have found that antibiotic treatment of *Ae. aegypti* increased permissiveness of mosquitoes to arboviral

development (vector competence), while others found that it did not affect viral infection, suggesting that this impact may depend on initial microbiota composition (Xi et al., 2008; Audsley et al., 2017).

Comparative studies of the influence of several bacterial strains further showed strain-specific impacts on vector competence, where some bacteria were found to protect mosquitoes against virus infection while others have no, or even a positive effect on arboviral infection (Ramirez et al., 2012; Wu et al., 2019). Increase in vector competence has notably been found in the case of several *Serratia* species, notably *Serratia marcescens* (Apte-Deshpande et al., 2012; Wu et al., 2019). This is due to the secretion of *SmEnhancin*, an enzyme which specifically degrades mucins in the gut of *Ae. aegypti* (but not in other mosquitoes) and limits the natural protection due to the gut mucus (Wu et al., 2019). This bacterial species, which has a very strong colonization ability in mosquitoes (Wang et al., 2017), is however also regarded with high interest as a potential malaria transmission blocker (Pike et al., 2017). Indeed, genetic manipulation of a *S. marcescens* strain to artificially produce arthropod antimicrobial peptides strongly reduces infection of *Anopheles* mosquitoes by malaria parasites, hence, may be used to block transmission *via* paratransgenesis (manipulation of symbionts to modify the host's phenotype). Some *S. marcescens* strains produce a red pigment called prodigiosin, which has some larvicidal and pupicidal activity in *Ae. aegypti* (Patil et al., 2011; Suryawanshi et al., 2015), yet its impact on mosquito physiology has not been investigated in detail. Moreover, prodigiosin has antimicrobial and antifungal properties (Williamson et al., 2006) which might impact other members of the mosquito microbiota. In this study, we characterized a prodigiosin-producing strain of *S. marcescens* which colonized our mosquito colony. We investigated whether secondary metabolite production by this bacterial strain affects microbe-microbe interactions and *Serratia* virulence in the mosquito.

RESULTS

Isolation of the *S. marcescens* VA Strain and Production of Prodigiosin-Deficient Mutants

When rearing our colony of *Ae. aegypti*, we observed a pink staining of the (non-autoclaved) sugar solution used to feed mosquitoes without any obvious loss in the colony. Concomitantly, we also observed that after rearing field-collected *Anopheles darlingi* (also called *Nyssorhynchus darlingi*) for one single generation in the same insectary using the same sugar source, 8/11 gut homogenates gave rise to red colonies after overnight incubation on LB (lysogeny broth) agar at 25°C. A chosen isolate of these colonies was identified as *S. marcescens via* biochemical analyses and sequencing of *16S* ribosomal DNA. We also noticed some mortality at the larval and pupal stage among *An. darlingi* mosquitoes, where dying individuals appeared pink and red colonies could be grown from their homogenates. As the pink sugar solution had been regularly observed in our insectary in the past and is not observed since its autoclaving has become a standard procedure, we hypothesize that the sugar meal may have been the source of this bacterial contamination in our colony. We called this strain *S. marcescens* VA, in reference to our entomology facility (Vectopole Amazonien). From this stage onward, all the experimental work in mosquitoes was performed on *Ae. aegypti*, as we did not have enough *An. darlingi* for further characterization.

Serratia marcescens owes its red color to prodigiosin, a bacteriostatic pigment with larvicidal activity in mosquitoes, which is produced at 30°C but not at 37°C. As the average temperature in French Guiana is close to 30°C, we hypothesized that prodigiosin may impact mosquito physiology, both directly and *via* bacteria-bacteria interactions in the mosquito gut. To test this, we generated prodigiosin-deficient mutants by random mutagenesis *via* UV treatment of a *S. marcescens* VA culture and isolation of white clones. Among the white colonies, we selected two clones, C1 and C3, which stay white even after 4 days of culture at 30°C and for at least two passages. We observed that colonies of both mutants had a slightly different morphology, C3 colonies having a granular appearance while C1 colonies looked more homogeneous (**Figure 1A**). Growth kinetics were similar in wild-type (wt) and mutant strains (**Supplementary Figure 1**).

Genome Sequencing of wt and Mutant *S. marcescens* VA

We sequenced the genomes of our three clones using MiSeq technology. We identified only five single-nucleotide polymorphisms (SNPs) in the C1 mutant, including two in non-coding regions and three non-synonymous substitutions in coding regions (**Figure 1B**). Two of these mutations resulted in amino acid substitutions while the third introduced a stop codon in the *pigC* gene, which encodes prodigiosin synthase, the last enzyme of prodigiosin biosynthesis. We therefore hypothesize that the white color is linked to the lack of prodigiosin synthase.

The genome of mutant C3 contains more mutations (**Figure 1B**): 1 SNP in non-coding regions, one single nucleotide deletion in a non-coding region, six synonymous substitutions and 10 non-synonymous substitutions (at eight locations) in coding regions, and a deletion followed with 19 SNPs in a prophage like-island. As the alignment was poorer at this specific locus, we cannot precisely determine the size of the deletion. We did not detect any mutation in genes encoding enzymes of the prodigiosin synthesis pathway, yet we found a mutation in the gene encoding RNA chaperone Hfq, known to control the production of several secondary metabolites including prodigiosin (Wilf et al., 2011).

Effect of *S. marcescens* VA on the Mosquito Microbiota

We first tested the impact of *S. marcescens* VA wt, CA and C3 on the mosquito microbiota. We quantified the antibacterial

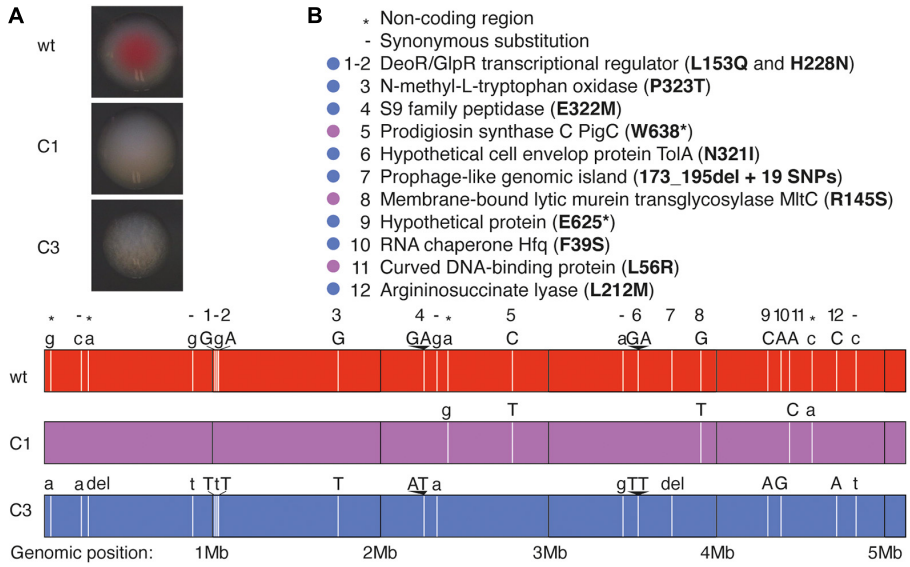

FIGURE 1 | Whole-genome sequencing analysis of *S. marcescens* VA prodigiosin-deficient mutants. **(A)** Pictures showing the aspect of colonies of wt *S. marcescens* VA and of its C1 and C3 mutants. **(B)** Description of the mutated loci in C1 and C3 mutants. *Lower panel*: Each vertical white bar represents the locus of a mutation, and the original (wt) and mutated (C1 or C3) nucleotides are indicated above the corresponding colored bars representing each genome. Uppercase letters indicate non-synonymous substitutions, while lower case letters and - indicate synonymous substitutions. The symbols * indicate mutations in non-coding regions. *Upper panel*: the legend indicates the gene where each mutation is located and the corresponding mutation in the amino acid chain. The color-code indicates the bacterial strains in which the mutations have been identified (purple: C1, blue: C3).

activity of *S. marcescens* VA and its white mutants by measuring their inhibition diameters on LB agar against several bacteria isolated from field-collected mosquitoes. *S. marcescens* VA inhibited the growth of 11/18 tested isolates belonging to the *Agrobacterium*, *Bacillus*, *Cupriavidus*, *Microbacterium*, and *Staphylococcus* genera (**Figure 2A**). The inhibition diameter of the mutant C1 was significantly reduced for eight of these 11 isolates, which belong to all of these five genera ($p_{adj} < 0.05$, *t*-test with Bonferroni–Dunn correction). We did not detect any growth inhibition by C3 except a minor inhibitory effect on the isolate of *Microbacterium* sp. (the inhibition zone was 98% smaller than that of wt, $p_{adj} < 0.001$). We then tested whether *S. marcescens* VA negatively impacts other bacteria within the mosquito gut, and thus affects microbiota composition. To this aim, we infected mosquitoes with bacteria by feeding them with a contaminated sugar solution [optical density $(OD)_{600\ nm} = 1$; estimated 10^8 CFU/mL]. We took advantage of the resistance of our strain to a penicillin-streptomycin cocktail to test the colonization success, which was high in wt and in mutants (**Figure 2B**; two-way ANOVA, day: $p = 0.30$; bacterial strain: $p = 0.63$; interaction: $p = 0.95$). We sequenced the V3–V4 region of the *16S* rRNA bacterial gene from pools of dissected midguts of our *Ae. aegypti* colony, 3 days after oral infection. However, we found that our colony was already dominated by penicillin-streptomycin susceptible *Serratia* sp., hence, this experiment did not allow us to conclude whether *S. marcescens* VA affects microbiota composition (**Figure 2C**). We also did not detect any impact of *S. marcescens* VA colonization on the microbiota alpha-diversity (**Supplementary Figure 2**). Together, our data indicate that *S. marcescens* VA is very efficient at colonizing mosquitoes at the tested concentration regardless of prodigiosin synthesis, and that the prodigiosin-producing wt strain is bacteriostatic on most of the tested bacterial isolates from field-collected mosquitoes.

Enzymatic Activity of *S. marcescens* VA Secreted Factors

Bacteria colonizing mosquitoes have been reported to have some proteolytic and/or hemolytic activity, which affect mosquito digestion dynamics and vector competence (de Gaio et al., 2011; Wu et al., 2019; Jupatanakul et al., 2020). Using an azocasein-based colorimetric assay, we observed that *S. marcescens* VA secretes a proteolytic factor in the supernatant during the first 24 h of the culture and that this factor is also produced in C1 mutant but not in C3 (**Figure 3A**; C1-wt – −12%, $p_{adj} > 0.99$; C3-wt – −92%, $p_{adj} < 0.0001$; two-way ANOVA + Bonferroni's multiple comparison tests). When testing cultures after 48 h, we observed that C3 did produce some proteolytic factor, but at a lower rate (**Figure 3A**; C1-wt – +10%, $p_{adj} > 0.99$; wt vs C3 – −58%, $p_{adj} < 0.0001$). Proteolytic activity in the bacterial lysate was significantly lower than in the supernatant at both time points (**Figure 3A**; all comparisons vs $wt_{supernatant-24\ h}$: $p_{adj} < 0.0001$). Considering hemolytic activity, we found that wt induces some hemolysis on blood agar, which was also present in C1 and lost in C3 (**Figure 3B**). This hemolytic activity was lost when wt bacteria were cultured at 37°C. However, when quantifying hemolysis in liquid culture, we did not find any difference between wt and either mutant; all of the strains had a similar hemolytic

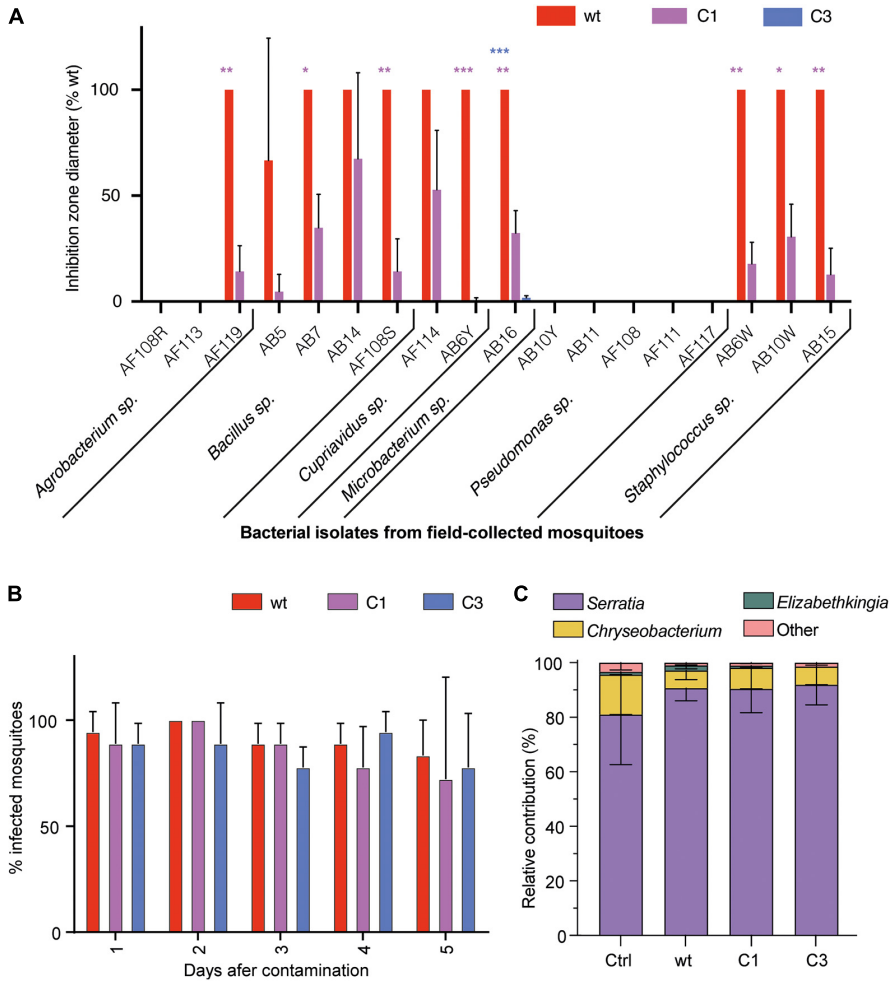

FIGURE 2 | Interaction of *S. marcescens* VA with the mosquito microbiota. **(A)** Inhibition zone diameter of the three strains on 18 bacterial strains isolated from the gut of field-collected mosquitoes. **(B)** Colonization success showed as the proportion of infected mosquitoes 1–5 days after feeding with a contaminated sugar solution ($OD_{600\ nm}$ = 1). **(C)** Relative contribution of the main bacterial genera found by high-throughput sequencing of 16S in mosquito guts 24 h after oral infection. Data show the average ± SEM of three independent replicates. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

activity to our SDS (sodium dodecyl sulfate)-based positive control (**Supplementary Figure 3**). Hence, our data indicate that *S. marcescens* VA exhibits a prodigiosin-independent proteolytic and hemolytic activity.

Characterization of Prodiginins and Serratamolides *via* HPLC and NMR Analyses

In line with our genome sequencing data, we hypothesized that the observed differences between both mutants may be linked to an additional deficiency of C3 in the production of serratamolide (also called serrawettin), a hemolytic secondary metabolite that is regulated in a temperature-dependent manner downstream the same pathway as prodigiosin under the control of the chaperone Hfq (Tanikawa et al., 2006; Shanks et al., 2012). To test this, we performed an acidified-ethanol extraction after culturing our three strains at 30°C and the wt strain at 37°C, as a negative control, and we qualified their metabolites by high-performance liquid chromatography (HPLC). As expected, we observed that the peak matching with purified prodigiosin, detected at 532 nm, was observed in extracts from *S. marcescens* VA cultured at 30°C, but neither after culture at 37°C nor in both mutants (**Figure 4A**). We observed several peaks around that of purified prodigiosin, which is consistent with a previous report indicating that seven different prodigiosin-related compounds (prodiginins) can be synthesized by *S. marcescens* (Eckelmann et al., 2018).

Considering serratamolide, we had no purified compound to formally identify its peak, but it is known to be detectable at 208 nm (Dwivedi et al., 2008). At this wavelength, we observed a family of peaks which is found in the wt at 30°C and not at 37°C, which makes them suitable candidates for serratamolide (**Figure 4B**). This family of peaks was not detected at 254, 280, and 532 nm (**Figure 4A** and **Supplementary Figure 4**). Such a family of multiple peaks is consistent with the fact that

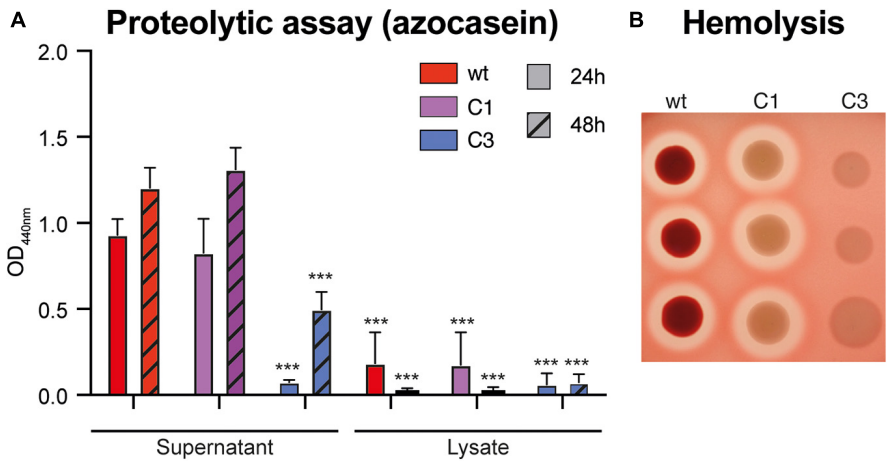

FIGURE 3 | Proteolytic and hemolytic activity of *S. marcescens* VA. **(A)** Proteolytic activity of wt, C1 and C3 *S. marcescens* VA extracts. Tests were performed using an azocasein colorimetric assay on extracts coming from cultured incubation for 24 or 48 h. Data show the average ± SEM of three independent replicates. ***$p < 0.001$. **(B)** Hemolytic activity on blood-agar containing human red-blood cells. The picture shows the result of one representative experiment with three technical replicates for each strain. Three independent replicates were performed.

26 different serratamolide metabolites have been reported to be produced by *S. marcescens* (Eckelmann et al., 2018). These peaks were also present in C1 extracts, but absent from C3 extracts (**Figure 4B**). To analyze whether they correspond to serratamolides, we purified these fractions from the C1 extract. We checked that the selected fractions showed a similar family of products, with an absorption maximum of 208 nm using HPLC analysis (**Figures 4A,B** and **Supplementary Figure 4**). We then submitted this fraction to nuclear magnetic resonance (NMR) analysis. NMR data indicated the presence of the characteristic serine and fatty acid moieties of serratamolides (**Figure 4C** and **Table 1**; Dwivedi et al., 2008). The two carbonyl signals were inferred from Heteronuclear Multiple Bond Correlation analysis (HMBC), at 171.8 and 171.0 ppm, and correspond to C1 and C′1, respectively. ^{1}H signals and 2D correlations are consistent with the presence of a C_{10} alkyl chain attached to the serine unit through its hydroxy group and we clearly identified the alkyl chain terminal methyl group (δ_H 0.90 ppm; t; J = 7.0 Hz; δ_C 14.2 ppm). These structural elements indicate the presence of serratamolide A or very similar compounds (Soto-Cerrato et al., 2005; Dwivedi et al., 2008; Zhu et al., 2018). Finally, we detected additional signals that are characteristic of an unsaturated branch unit (notably δ_H 5.35 ppm; t; J = 4.7 Hz; δ_C, 130.6 ppm, alongside with δ_C 136.3 ppm inferred from HMBC analysis, and COSY correlations between signals at δ_H 5.35, 2.04, and 1.31 ppm). A methoxy group (δ_H 3.65 ppm; s; δ_C 51.7 ppm) may also be part of the serratamolide structures, similar to previous reports on serratamolide G (Zhu et al., 2018) and overall consistent with the great structural diversity of this family of compounds (Eckelmann et al., 2018). Together, our data indicate that *S. marcescens* VA produces prodigiosin and serratamolides, probably dominated with serratamolide A. They further show that C1 produces serratamolides but no prodigiosin and that C3 is impaired in the production of both types of compounds.

Effect of *S. marcescens* VA on Mosquito Fitness

We performed further characterization of the virulence of *S. marcescens* VA in *Ae. aegypti*. A low infection dose (as used in **Figures 2B,C**; $OD_{600\ nm}$ = 1) caused a slight, yet significant reduction in survival, which was not affected in any mutant (**Supplementary Figure 5A**). We did not detect any significant difference between any strain on egg laying, hatching rate and sex ratio of the progeny (**Supplementary Figures 5B,C**). We then infected *Ae. aegypti* by providing a sugar solution mixed with a concentrated bacterial suspension (80-fold concentrated suspension compared to $OD_{600\ nm}$ = 1, referred to here as $OD_{600\ nm}$ = 80). *S. marcescens* VA wt infection was highly lethal to mosquitoes, causing 65 and 85% mortality by day 3 and day 4 post-infection respectively. C1 showed a slight reduction in virulence whereas C3 mutants were much less virulent (**Figure 5A**). We observed that infected mosquitoes survived longer when they had access to water in addition to the infectious bacteria (**Figure 5B**). This extension in lifespan was particularly marked with the case of C3 colonization, which even appeared to extend the lifespan of mosquitoes compared to controls provided a sugar solution and water.

Previous studies reported that purified prodigiosin caused some mortality to *Ae. aegypti* larvae, and that *S. marcescens* also secretes proteases and chitinases which have a larvicidal activity (Jupatanakul et al., 2020). On the other side, bacteria are required for normal larval development, and we have set up methods to test whether specific bacteria are able to support larval development (Coon et al., 2014; Romoli and Gendrin, 2020). Using our protocol, 100% sterile larvae are blocked at the first instar unless bacteria are added in the medium. We tested whether larval mono-colonization with *S. marcescens* VA was able to support development and/or whether it was killing larvae. When using our conventional concentration of the bacterial suspension (10^8 CFU/mL), we found that only C1

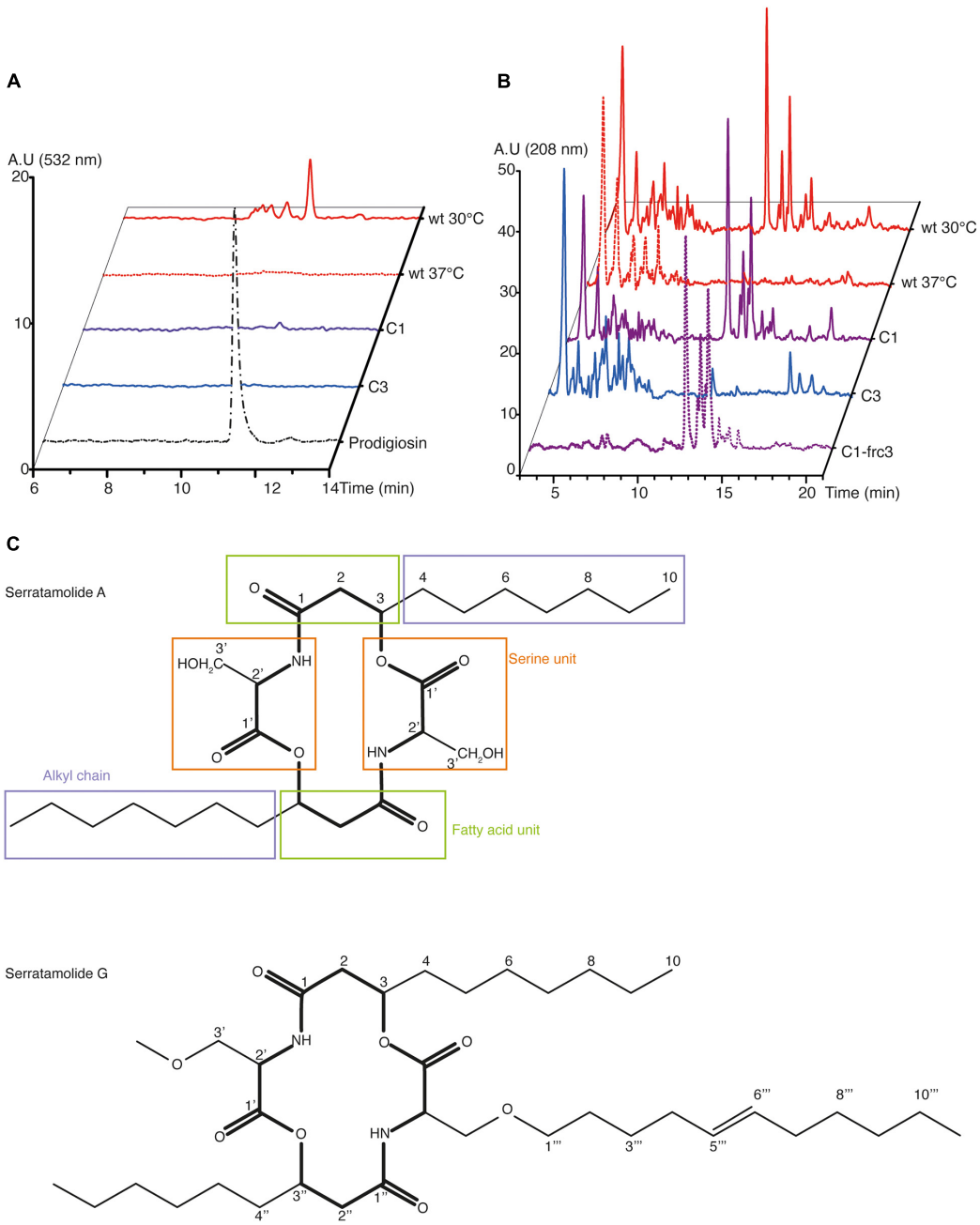

FIGURE 4 | HPLC and NMR analyses of the secondary metabolites produced by *S. marcescens* VA. **(A,B)** HPLC profile of *S. marcescens* VA extracts at the wavelengths reported to detect prodigiosin (532 nm, **A**) and serratamolide (208 nm, **B**). In **(A)**, the profile of purified prodigiosin was used as a control. In **(B)**, C1-frc3 shows data from the methanolic fraction, which has been analyzed *via* NMR. Data are representative of two independent experiments, and of one for C1-frc3. A. U, arbitrary units. **(C)** Structure of serratamolide A and G molecules, where the core indicated in bold. Serratamolide A encompasses a central symmetry and its different parts described in **Table 1** are indicated in colored boxes: fatty acid unit in green, alkyl chain in purple, and serine unit in orange.

and C3 efficiently support development, albeit to a lesser extent compared to the *Escherichia coli* positive control (**Figure 5C**, $p < 0.001$, mixed effect model; wt vs all: $p_{adj} < 0.001$; C1 vs *E. coli*: $p_{adj} = 0.02$; C3 vs *E. coli*: $p_{adj} = 0.01$; C1 vs C3: $p_{adj} = 0.08$, ns, Tukey's multiple comparisons test). *S. marcescens* VA wt supports the development of 12% of the larvae, but also killed over 80% of the individuals. This suggests that *S. marcescens* is metabolically able to support development, but also has a strong larvicidal activity, most likely due to prodiginins as it is strongly reduced in C1. We then tested whether this impact on development was different with a lower bacterial concentration (10^5 CFU/mL, **Figure 5C**). Again, wt was highly virulent, killing

TABLE 1 | NMR data (CD_3OD) obtained from fraction 3 spectrum and corresponding to serratamolides.

Position	^1H: δ (ppm); multiplicity; *J* (Hz)	^{13}C: δ (ppm) according to HSQC and HMBC	HMBC ($^1H \rightarrow {}^{13}C$)	COSY ($^1H \rightarrow {}^1H$)
Serine unit				
1′		171.0; C		
2′	4.48; dd; *J* = 3.5; 3.1 Hz	56.0; CH		3′a; 3′b
3′	3′a: 4.08; dd; *J* = 10.6; 3.5 Hz, 3′b: 3.83; dd; *J* = 10.9; 3.1 Hz	62.9; CH_2		2′; 3′b
			C′1	2′; 3′a
Fatty acid unit				
1		171.8; C		
2	2a: 2.68; dd; *J* = 13.7; 5.1 Hz, 2b: 2.34; dd; *J* = 13.3; 2.4 Hz	40.9; CH_2	C1; C3	2a; 3
			C1	2b; 3
3	5.30; m	72.9; CH		4; 2a; 2b
C_{10} alkyl chain				
4	1.67; m	33.5; CH_2		3, -(CH2)n-
-(CH2)n-	1.29; m	23.6; 26.8; 30.2; 30.4; 32.8		-CH3
-CH3	0.90; t; *J* = 7.0 Hz	14.2; CH_3		-(CH2)n-
$-OCH_3$	3.65; s	51.7; CH_3		
-NH	7.90; d; *J* = 8.2 Hz			

Chemical shifts (δ) are in ppm downfield from tetramethylsilane (TMS), and coupling constants (J) are in Hz. s, singlet; d, doublet; t, triplet; m, multiplet; HSQC, Heteronuclear Single Quantum Coherence; HMBC, Heteronuclear Multiple Bond Correlation.

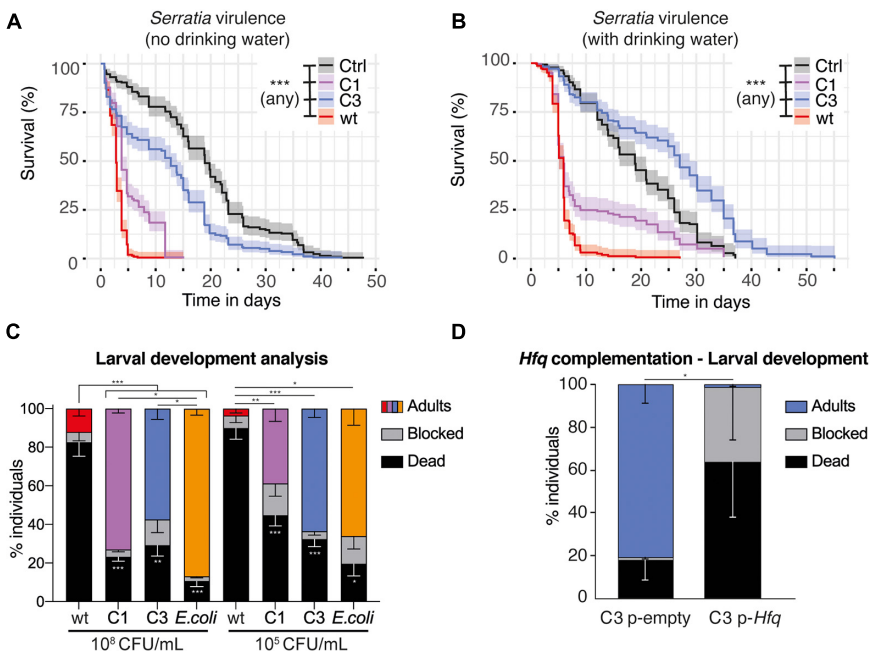

FIGURE 5 | Impact of *S. marcescens* VA on adult and larval *Ae. aegypti*. **(A,B)** Survival of adult females fed with a sugar solution contaminated with a concentrated suspension of *S. marcescens* ($OD_{600\ nm}$ = 80). In **(B)**, mosquitoes were provided with drinking water in addition to the infectious meal. **(C,D)**, Development success to adulthood of larvae contaminated with *S. marcescens* from the first larval instar. In **(C)**, the concentration of the bacterial suspension at the beginning of the experiment is indicated below. CFU, colony forming unit. In **(D)**, larvae are provided with C3 carrying an empty pBBR1MCS-2 plasmid or an *Hfq*-containing pBBR1MCS-2, at a 10^8 CFU/mL concentration. Data show the results of 2 **(A,B)**, 7 **(C)**, and 3 **(D)** independent replicates. Confidence intervals are shown in **(A,B)** and SEM in **(C,D)**. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

90% of the larvae. We observed that 63% of the larvae develop to adulthood with C3, similar to the *E. coli* positive control, and detected a slightly lower success with C1 (39%; $p < 0.001$, mixed effect model; wt vs C1: $p_{adj} = 0.01$; wt vs C3: $p_{adj} < 0.001$; wt vs *E. coli*: $p_{adj} = 0.02$; C1 vs C3: $p_{adj} = 0.07$; C1 vs *E. coli*: $p_{adj} = 0.28$; C3 vs *E. coli*: $p_{adj} = 1$, Tukey's multiple comparisons test). Intriguingly, development success was reproducibly higher when providing the high dose of C1 than the lower one, while this was not observed with C3 (C1 10^8 vs C1 10^5: $p_{adj} = 0.03$; C3 10^8 vs C3 10^5: $p_{adj} = 0.80$, Tukey's multiple comparisons test). Together, we found that *S. marcescens* VA has only a minor impact on mosquito fitness when adults are infected at an intermediate dose ($OD_{600\ nm} = 1$), while it is virulent in adults at a higher dose and in larvae at both concentrations tested (**Figures 5A–C** and **Supplementary Figure 5**). Our results also suggest that the prodiginins strongly participate in the virulence toward larvae, but have a minor impact in adults, where other secondary metabolites contribute to virulence.

To investigate potential larvicidal effects of *S. marcescens* VA metabolites in the absence of the bacterium itself, we tested whether a crude bacterial extract caused any lethality in third-to-fourth-instar larvae. Again, we used acidified-ethanol extracts from each strain after culture at 30°C, using as a negative control a wt crude extract after culture at 37°C, and quantified the effect of these extracts on third-to-fourth-instar larvae. As shown in **Supplementary Figure 6**, the crude extract of *S. marcescens* VA caused some significant lethality in larvae with an average LC_{50} (lethal concentration of 50% of the population) of 480 and 323 ppm if extracts were prepared 24 and 48 h after culture inoculation, respectively (wt 30°C vs wt 37°C, 24 h – $p = 0.023$, 48 h – $p = 0.047$, ANOVA with Bonferroni correction). When extracting from 24 h-old cultures, we found that C1 had a significant larvicidal activity, while C3 did not show any larvicidal activity compared to the negative control (C1 vs wt 37°C, $p = 0.029$; C3 vs wt 37°C, $p > 0.99$). Using extracts from 48 h-old cultures, data indicate some larvicidal activity of both C1 and C3, yet differences with the negative control are not significant (C1 vs wt 37°C, $p = 0.17$; C3 vs wt 37°C, $p = 0.22$). We also noted that the larvicidal activity was lost when extracts were kept, even frozen, for several weeks, indicating that the larvicidal compounds that we extracted are not sufficiently stable for any mosquitocidal application. Thus, in this setup we did not observe any impact of prodigiosin itself, but rather of other secondary metabolites produced under a temperature-dependent control.

Hfq-Complementation Restores C3 Virulence

Our sequencing data indicates that C3 carries several mutations. To investigate which mutation is responsible for the observed phenotypes in C3, we tested whether the complementation of the genes encompassing these mutations resulted in red colonies. We amplified the wt sequences of the seven corresponding genes (**Figure 1B**) and cloned them into the expression vector pBBR1MSC-2 in C3. The process was successful six genes, i.e., all except N-methyl-L-tryptophan oxidase, and among them the only complementation resulting in red colonies was with *Hfq* (**Supplementary Figure 7**). We thus focused on *Hfq* for further characterization of the impact of complementation. Indeed, these results were not surprising, as *Hfq* encodes a chaperone controlling the production of several secondary metabolites including prodigiosin and serratamolide (Wilf et al., 2011). When comparing the impact of *Hfq*-complemented C3 with C3 carrying an empty plasmid, we observed that Hfq restores the virulence of *S. marcescens* VA during larval development, significantly decreasing the percentage of fully developed adults (**Figure 5D**, mixed effect model: % adults, $p = 0.011$; % dead, $p = 0.16$; % blocked, $p = 0.25$).

DISCUSSION

Serratia marcescens is a bacterium that efficiently colonizes mosquitoes and its interactions with several species of mosquitoes have therefore received much interest in the recent years. In this study, we characterized a prodigiosin-producing strain, which efficiently colonizes *Anopheles* and *Aedes* mosquitoes and is virulent in mosquitoes, particularly at the larval stage.

We used a random mutagenesis approach and selected two strains that did not produce prodigiosin for phenotypic characterization. Among them, C1 was found to only carry three non-synonymous mutations including a single nonsense mutation. The latter affects prodigiosin-synthase, hence phenotypes observed in C1 are likely consequences of the lack of prodigiosin. These phenotypes include firstly a reduced antimicrobial activity against eight bacterial strains belonging to four different classes (Alphaproteobacteria, Betaproteobacteria, Bacilli, and Actinobacteria) and secondly, an attenuated virulence in mosquitoes, particularly at the larval stage. This is consistent with a previous report of a larvicidal and pupicidal effect of purified prodigiosin in *Ae. aegypti* (Suryawanshi et al., 2015).

The genome of C3 includes a larger amount of mutations, including the RNA chaperone Hfq, which is involved in the regulation of the production of secondary metabolites including prodigiosin, serratamolide, and a carbapenem antibiotic (Wilf et al., 2011). We observed that C3 is highly affected in antimicrobial activity and has impaired production of prodigiosin and serratamolides. These phenotypes are consistent with a deficient regulation of secondary-metabolite production downstream to Hfq. We validated that the complementation of *Hfq* in C3 restored the bacterial pigmentation and larvicidal effect. The loss of these other metabolites affected proteolysis and hemolysis activity of *S. marcescens* VA, its antimicrobial activity as well as its virulence in adults. Whilst we did not identify the specific metabolites responsible for such phenotypes, serratamolide is a known hemolytic factor, previously found to contribute *in vitro* to the virulence of *S. marcescens* by increasing its resistance against phagocytosis by human polymorphonuclear leukocytes (Miyazaki et al., 1993; Shanks et al., 2012). Contrary to adults, bacterial virulence toward larvae seem to rely on prodiginins, but not on the other secondary metabolites, as we did not detect any significant difference between both mutants in larval development success. When using 10^8 CFU/mL,

we detect some residual virulence of C1 and C3 on larvae compared to *E. coli*. This may be due to the production of proteases and chitinases, previously found to participate to the larvicidal activity of *S. marcescens* in *Anopheles dirus* (Jupatanakul et al., 2020).

We observed that *S. marcescens* VA is a very efficient colonizer in adult mosquitoes regardless of its ability to synthesize secondary metabolites. This is consistent with previous observations of a very high colonization efficiency by a white strain of *S. marcescens* in *Anopheles* mosquitoes (Wang et al., 2017). Alternatively, a colonization phenotype may appear in a colony with less *Serratia* in its microbiota. Indeed, colonization resistance assays using gnotobiotic mosquitoes (i.e., mosquitoes with a known initial microbiota composition) showed that the initial microbiota impacts the colonization efficiency of other *S. marcescens* strains in *Ae. aegypti* (Kozlova et al., 2020).

Our survival assays using concentrated bacteria were based on two alternative set ups, with or without provision of an additional source of water separately to the sugar solution containing bacteria. We were intrigued to see the extent of which lifespan is prolonged in the presence of water. Median survival was prolonged by 3 days after infection with *S. marcescens* VA. This impact was even stronger in the case of the C3 mutant, where a 12-day extension in median survival was observed. In *Drosophila*, *S. marcescens* oral infection was found to cause thinning of the gut epithelium as a purge mechanism allowing recovery (Lee et al., 2016). We hypothesize that such a defense response may increase the risk of dehydration and/or the need of water for tissue reconstruction. Moreover, mosquitoes may avoid the sugar solution that is contaminated with *S. marcescens* and therefore become dehydrated. Such avoidance has been observed in *Caenorhabditis elegans*, where it is elicited by a serratamolide, serrawettin W2 (Pradel et al., 2007). The observed strong difference between C3 infection in the presence or absence of drinking water may be consistent with such a lack of avoidance, where thirsty mosquitoes would maintain a chronic C3 infection by drinking this contaminated-sugar meal as an alternative to water while C3 infection would be rapidly cleared out in thirsty mosquitoes provided water.

The observed virulence of wt *S. marcescens* VA was reduced in both C1 and C3 when using live bacteria throughout larval development, while the crude extract of C1 had a similar larvicidal activity to wt during late larval development. This difference may be linked with the time of the experiment, as deaths were often observed at least 3 days after the beginning of the experiment during larval development analyses, while impact of the crude extract was only tested over 48 h. Alternatively, it may be explained by differences in the larval stage, where first instar larvae are more sensitive to prodigiosin than third and fourth-instar larvae, as suggested by Suryawanshi et al. (2015) after treating larvae with purified prodigiosin. Indeed, we observed that if larvae do not die during the first instars, they were generally stalled in larval development and able to survive until the end of the 2-week experiment.

Together, our results characterize a new strain of *S. marcescens* bacteria which is virulent in mosquitoes and allowed us to investigate the impact of secondary metabolites produced by this strain on mosquito fitness and development.

MATERIALS AND METHODS

Isolation of *S. marcescens* VA and Generation of Mutants

The wt *S. marcescens* VA strain was isolated from *An. darlingi* mosquitoes reared in the laboratory from field-collected adults. To produce mutant strains, a diluted overnight culture was plated on LB and exposed for 30 s to UV light under a microbiological safety cabinet (Herasafe KS, Thermo Fisher Scientific). Three non-pigmented mutants were selected, C1 to C3. After three passages on LB plates, only mutants C1 and C3 were kept as C2 turned red. Resistance of the wt, C1 and C3 to penicillin-streptomycin (Sigma-Aldrich), gentamycin (Sigma-Aldrich), and kanamycin (Sigma-Aldrich) was tested by depositing an 8 μL droplet of antibiotics at diverse concentrations after plating 1/100 dilution of a *S. marcescens* VA overnight culture on LB-agar (Sigma-Aldrich) and detecting halos in the bacterial lawn after a further overnight incubation at 30°C (**Supplementary Table 1**). Using this method, we determined that our strains were resistant to a cocktail of 200 U/mL penicillin and 200 μg/mL streptomycin, and we used this condition to select and quantify our *S. marcescens* isolate compared to the rest of bacteria composing the mosquito microbiota in subsequent colonization and infection experiments. An experiment was not considered valid if the non-infected control showed any growth in these conditions.

Growth curves were produced by growing bacteria in liquid LB for 22 h at 30°C and quantifying medium absorbance at 600 nm every 30 min in a FLUOStar Omega plate reader (BMG Labtech).

Whole-Genome Sequencing of *S. marcescens* VA wt, C1 and C3 Strains

DNA was extracted from overnight cultures using the MagJET genomic DNA kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Paired-end libraries (150 bp) were prepared using the Nextera XT DNA library preparation kit (Illumina) and sequenced on a MiSeq system by Biofidal (Vaulx-en-Velin, France). Raw reads were demultiplexed and adaptors sequences were trimmed by the sequencing facility. In total, 1.74, 1.63, and 1.73 million reads were obtained for *S. marcescens* VA wt, C1 and C3, respectively. Sequences were quality trimmed using Trimmomatic version 0.39 (Bolger et al., 2014) surviving reads wt: 95.4%, C1: 95.3%, C3: 98.0%) and genomes were assembled *de novo* using SPAdes version 3.14.1 (Nurk et al., 2013). VarScan version 2.3.9 (Koboldt et al., 2012) was used to identify SNPs and indels in C1 and C3 genomes compared to wt.

Determination of the Inhibition Zone Diameter

Serratia marcescens antimicrobial activity was assessed by diffusion in LB-agar. The LB agar plate surface was

inoculated by spreading a volume of the test bacteria inoculum over the entire agar surface (OD_{600} = 0.02). Then, 10 µL of overnight culture of *S. marcescens* (OD_{600} = 2) was inoculated as a drop on the same plate. Plates were incubated at 30°C for 24 h. The antimicrobial compounds produced by *S. marcescens* diffuse in the agar medium and inhibit the growth of the microbial strain tested. Then, the diameters of inhibition growth zones were measured using ImageJ[1].

Mosquito Colony and Maintenance

Aedes aegypti New Orleans mosquitoes were reared in standard insectary conditions, at 28–30°C on a natural 12:12 h light/dark cycle. Larvae were reared on a yeast-based diet, while adults were fed with a sterile 10% (w/v) sucrose solution. Female mosquitoes were blood-fed on anesthetized mice.

Experimental Mosquito Infection

Bacteria were inoculated from single fresh colonies in LB and incubated at 30°C, shaking at 200 rpm overnight. Bacterial cultures were centrifuged and diluted in a 10% sterile sucrose solution to a final $OD_{600\ nm}$ = 1 or 80 (in the latter case, $OD_{600\ nm}$ was determined on a 1000-fold diluted sample). After 24 h of starvation (without any sugar solution nor water), five to 7-day-old female mosquitoes were fed with a sterile or bacteria-containing 10% sucrose solution on a sterile cotton ball.

For colonization efficiency assay, six mosquitoes per condition were dissected every 24 h after infection to quantify bacterial contamination.

For survival assays at $OD_{600\ nm}$ = 1, bacteria-containing sucrose was replaced every day, while for $OD_{600\ nm}$ = 80, a contaminated sucrose solution was provided on day 0 and day 2. Infection was verified 24 and/or 48 h later by culture of six dissected gut homogenates on LB-agar supplemented with 200 U/mL penicillin and 200 µg/mL streptomycin and considered valid if at least 4/6 mosquitoes were infected and if 0/6 non-infected controls (**Figures 2B**, **4A,B** and **Supplementary Figures 2**, **4**) gave rise to any colony or colonies with a clearly distinct morphology (generally no colonies were observed). Two to three biological replicates were performed, based on 150 mosquitoes/condition/replicate.

For microbiota sequencing, 30 mosquitoes/condition were sampled 24 h after infection and mosquitoes fed on a sterile sugar solution were used as a control. Infection was verified by culture of dissected gut homogenates as above.

Midgut Dissection and CFU Quantification

Female mosquitoes were surface-sterilized in 70% ethanol for 3 min and then rinsed three times in sterile phosphate-buffered saline (PBS). Midguts were dissected, individually transferred in 100 µL sterile LB and homogenized with a sterile plastic grinder. Homogenates were serially diluted and plated on LB agar plates supplemented with 200 U/mL penicillin and 200 µg/mL streptomycin to quantify bacterial loads.

16S rRNA Sequencing

DNA was extracted from pools of 30 midguts using the ZymoBIOMICS MagBead DNA Kit following manufacturer's instructions. DNA was shipped to Biofidal (Vaulx-en-Velin, France) where it was quantified using the Quantifluor® dsDNA kit (Promega) on a Safire microplate reader (Tecan). The V3–V4 region of the *16S* rRNA gene was amplified using the couple of primers 341F and 805R (**Supplementary Table 2**), both containing overhang Illumina adapter sequences. PCR reactions consisted of 20 ng of gDNA, 1X HOTBIOAmp® Blend Master Mix with 12.5 mM $MgCl_2$ (Biofidal), 1X GC-rich Enhancer (Biofidal), 0.2 mg/mL BSA, 400 nM of each primer. PCR cycle consisted of an initial denaturation step at 96°C for 10 min, 35 cycles of 20 s at 96°C, 30 s at 56°C, 1 min at 72°C and a final elongations step at 72°C for 10 min. PCR products were purified with SPRIselect magnetic beads (Beckman Coulter), quantified using the Quantifluor® dsDNA kit (Promega) on a Qubit® 2.0 fluorometer (Thermo Fisher Scientific) and visualized on a QIAxcel apparatus (QIAGEN). A second PCR (15 cycles) was performed on amplicons to add indexes and P5/P7 adapters. Indexed PCR amplicons were purified, quantified and visualized as described previously. Libraries were pooled after equimolar normalization and sequenced using Illumina MiSeq in paired-end (read length: 300 bp). On average, 34,071 reads were obtained for each sample (median: 35,533, min: 14,558, max: 43,660). After raw reads demultiplexing and adapters trimming from the sequencing facility, qiime2 (Bolyen et al., 2019) was used to inspect read quality. The dada2 plugin (Callahan et al., 2016) was used to trim low-quality portions, denoise reads, remove chimera, and merge FOR and REV reads. Read taxonomy assignation was performed in qiime2 using the SILVA database (Glöckner et al., 2017). Alpha and beta-diversity analyses were performed in qiime2.

Proteolytic Activity Assay

The culture was incubated for 24 or 48 h, as indicated **Figure 3A**. It was then centrifuged at 20,000 g for 10 min and the supernatant was filtered through a 0.2 µm pore size membrane. The pellet was washed with PBS and sonicated in a final volume of 300 µL with amplitude 60% for 4 min and then for 30:30 s ON/OFF cycles until the OD reached 0. The suspension was centrifuged for 10 min at max speed to eliminate cell debris. Proteolytic activity was assayed with azocasein as substrate (Sigma-Aldrich) as described previously (Caldas et al., 2002). The sample was mixed 1:1 with a 2% azocasein solution and incubated at 37°C for 1 h in triplicates. Non-digested azocasein was precipitated using 1.3 volumes of 10% TCA (w/v in water) and spun down at 10,000 g for 10 min. The supernatant was then mixed with 2 volumes of 1 M NaOH and absorbance at 440 nm was measured using a microplate reader FLUOstar Omega (BMG Labtech). The blank was obtained by precipitating the sample with the substrate in TCA without any prior incubation. The positive control consisted of 10 mg/mL trypsin (Sigma-Aldrich).

[1]http://imagej.nih.gov/ij/

Hemolytic Activity

Blood Agar Plate Test

Bacteria were inoculated from single fresh colonies in LB and incubated at 30°C shaking at 200 rpm overnight. 10 µl of each bacterial culture was spotted on blood agar plates (BD Biosciences) and incubated overnight at 30°C.

In Liquid Medium

Bacteria were inoculated from single fresh colonies in 3 mL of LB and incubated at room temperature overnight. Human red blood cells (RBCs, Etablissement Français du Sang de Guadeloupe-Guyane) were washed three times with sterile PBS, centrifuged at 1500 g and diluted in PBS to obtain a 1% RBC solution. A total of 200 µL of bacterial cultures was incubated with 200 µL of 1% RBC solution for 24 h at 30°C without shaking. As negative controls, RBCs were incubated with sterile LB and with an overnight *E. coli* HS culture. As positive control, RBCs were incubated with a 0.1% SDS solution. After incubation, RBC suspensions were centrifuged at 12,000 *g* for 5 min and the absorbance of the supernatant at 540 nm was measured. The experiment was performed three times and, for each replicate, three technical replicates were performed.

Larvicidal Assay in Tubes

Larvicidal assay in tubes was performed as described previously (Falkowski et al., 2016). The crude extract was solubilized in 100% ethanol, adjusted to pH = 6 and a 1.5-fold dilution series of this extract was prepared. This series was further diluted 1% (v/v) in water in 5 mL glass tubes, so that final concentrations ranged from 132 to 1000 ppm. The assay was performed in 20 tubes/concentration, with 5 third or fourth instar larvae in each tube. Larval mortality was recorded 24 and 48 h after exposure. Absolute ethanol was used as a negative control and led to an average 0.75 and 1.8% mortality after 24 and 48 h, respectively.

Egg Laying

Mosquitoes were fed with a 10% (w/v) sucrose solution contaminated with *S. marcescens* at an OD_{600} = 1 and then with sterile sucrose changed daily. On day 4 post-infection, 10 female mosquitoes were transferred to a cup for blood feeding with bovine blood (kind gift from the Abattoir Régional de Guyane) using a membrane feeding system (Hemotek®). Females were kept individually in 30 mL tubes closed with a mesh for individual egg laying, providing sterile sucrose. The number of eggs laid by each female was counted 5 days later.

Hatching

Individual egg clutches were transferred in water for hatching. For each clutch, 24 larvae were transferred in a 24-well plate to follow larval development and the other larvae were left to develop in a cup for sex ratio assessment.

Larval Development

Larval development assays were performed as described previously (Romoli and Gendrin, 2020). Eggs were sterilized to obtain axenic larvae. Larvae were transferred individually into 24-well plates (Greiner Bio-One). In each well, 2 mL of bacteria suspension (10^8 or 10^5 CFU/mL) and 100 µL of sterile 5% fish food (Tetramin baby) were added. Larval development was followed for 21 days to quantify development success. *E. coli* HS was used as positive control.

Culture Extraction and HPLC Profiling

The crude extract was obtained as described previously (Patil et al., 2011). Briefly, 2 × 500 mL of culture were incubated for 24 or 48 h at 30 and 37°C (only for wt), shaking at 180 rpm. Cells were harvested by centrifugation at 3900 rpm for 60 min. Pellets were washed four times with 40 mL acidified ethanol (1% v/v HCl 37% in absolute ethanol). The supernatants were dried under low pressure with a rotatory evaporator (Heidolph Laborota 4000) below 40°C.

Prodigiosin hydrochloride (HPLC purity ≥90%, CAS N°: 56144-17-3; Sigma-Aldrich) was dissolved to 0.2 mg/mL in methanol and used without further purification. HPLC samples were prepared from extracts diluted to 10 mg/mL in methanol and 0.22 µm-filtered. For each sample, 10 µL was injected in a Varian 920-LC system equipped with a UV-VIS detector and a photodiode array detector (C_{18} Hypersil Gold column, Thermo Fisher Scientific, 3 µm, 2.1 × 150 mm, flow rate 0.7 mL/min). All samples were analyzed using a linear gradient of H_2O/CH_3CN/formic acid (98:2:0.1 to 2:98:0.1) and detection was performed at 208, 254, 280, and 532 nm.

NMR Analysis

For NMR analysis, fractions were purified using a hydrophobic C18 reverse phase cartridge, which allows the extraction of non-polar to moderately polar compounds. This purification led to the separation of the C1 extract into three fractions. Only the methanolic fraction (frc3) presents an HPLC profile with compounds showing an absorption maximum at 208 nm, characteristic wavelength of serratamolides (**Figures 4A,B** and **Supplementary Figures 4A,B**). Methanolic fraction was dissolved in deuterated methanol (CD_3OD) prior to introduction into the NMR sample tube. Nuclear magnetic resonance (NMR) spectra (^1H and 2D sequences) were recorded on a Varian 400 NMR spectrometer equipped with a 5 mm inverse probe (Auto X PGF $^1H/^{15}N$-^{13}C). ^1H NMR spectra were recorded at 400 MHz and ^{13}C NMR spectra at 100.6 MHz. Chemical shifts are in ppm and coupling constants (*J*) are in Hz (s for singlet, d for doublet, t for triplet, m for multiplet).

Gene Complementation in C3

Candidate genes were amplified from wt *S. marcescens* VA using primers indicated **Supplementary Table 2** and cloned into pBBR1MCS-2 (kind gift from Kenneth Peterson – Addgene plasmid #85168; Kovach et al., 1995) in *E. coli*.

Plasmid sequences were verified by Sanger sequencing. Each recombinant plasmid was then electroporated with a GenePulser (Bio-Rad) in electrocompetent *S. marcescens* C3 cells, prepared with the GenePulser according to the manufacturer's instructions. For each gene, bacteria were plated on LB supplemented with kanamycin (50 μg/mL) to select those carrying the plasmid.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The link to the repository and accession number are as follows: https://www.ebi.ac.uk/ena, PRJEB45268.

ETHICS STATEMENT

The animal study was reviewed and approved by the French Direction Générale de la Recherche et de l'Innovation, ethical board # 089, under the agreement # 973021.

AUTHOR CONTRIBUTIONS

KH, OR, JS, YEp, EH, YEs, and MG designed the experiments. KH, OR, JS, RA, YEp, VK, EH, and YEs performed the experiments. KH, OR, JS, EH, YEs, and MG analyzed the data. MG drafted the manuscript. KH, OR, EH, and YEs edited the manuscript. All authors accepted the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.645701/full#supplementary-material

Supplementary Figure 1 | Growth kinetics of *S. marcescens* VA at 30°C in LB. The average ± SEM of three independent replicates is shown.

Supplementary Figure 2 | Chao1 index in mosquitoes contaminated with *S. marcescens* VA. Data were generated from the same experiments as those to produce **Figure 2B**, and show the average ± SEM of three independent replicates.

Supplementary Figure 3 | Quantification of hemolysis activity of *S. marcescens* VA in liquid. *E. coli* and sterile LB (-) were used as a negative control and SDS as a positive control. Data show the average ± SEM of three independent replicates.

Supplementary Figure 4 | Peaks detected by HPLC at 208 nm are not found at other wavelengths. HPLC profile of *S. marcescens* crude extracts at 254 nm **(A)** and 280 nm **(B)**. C1-frc3 shows data from the methanolic fraction, which has been analyzed *via* NMR. A. U., arbitrary units.

Supplementary Figure 5 | Effect of a low-dose oral infection of *S. marcescens* VA on *Ae. aegypti*. Adult females were fed with a sugar solution contaminated with bacterial strains at $OD_{600\ nm} = 1$. **(A)** Survival analysis. ns, non-significant; $^{***}p < 0.001$. **(B)** Number of eggs laid per female and proportion of eggs hatching to larvae after contamination of the mother. **(C)** Proportion of males and females in the progeny at the adult stage. Data show the average ± CI **(A)** and ± SEM **(B,C)** of three independent replicates.

Supplementary Figure 6 | Larvicidal activity of a crude extract of *S. marcescens* VA. Lethal concentration of bacterial extracts killing 50% of the larvae (LC_{50}) in 48 h. Bacterial extracts were prepared after culturing bacteria at 30 or 37°C for 24 or 48 h. The larvicidal assay was performed on 100 third-to-fourth instar larvae per condition and per replicate. Data show the average of three independent replicates and error bars show SEM.

Supplementary Figure 7 | Coloration of *Hfq*-complemented *S. marcescens* C3. Bacteria were grown overnight at 30°C on LB-agar + Kanamycin. *Left*: *Hfq*-complemented C3; *Right*: C3 electroporated with an empty plasmid.

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Horizontal Transmission of the Symbiont *Microsporidia MB* in *Anopheles arabiensis*

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The recently discovered *Anopheles* symbiont, *Microsporidia MB*, has a strong malaria transmission-blocking phenotype in *Anopheles arabiensis,* the predominant *Anopheles gambiae* species complex member in many active transmission areas in eastern Africa. The ability of *Microsporidia MB* to block *Plasmodium* transmission together with vertical transmission and avirulence makes it a candidate for the development of a symbiont-based malaria transmission blocking strategy. We investigate the characteristics and efficiencies of *Microsporidia MB* transmission between *An. arabiensis* mosquitoes. We show that *Microsporidia MB* is not transmitted between larvae but is effectively transmitted horizontally between adult mosquitoes. Notably, *Microsporidia MB* was only found to be transmitted between male and female *An. arabiensis*, suggesting sexual horizontal transmission. In addition, *Microsporidia MB* cells were observed infecting the *An. arabiensis* ejaculatory duct. Female *An. arabiensis* that acquire *Microsporidia MB* horizontally are able to transmit the symbiont vertically to their offspring. We also investigate the possibility that *Microsporidia MB* can infect alternate hosts that live in the same habitats as their *An. arabiensis* hosts, but find no other non-anopheline hosts. Notably, *Microsporidia MB* infections were found in another primary malaria African vector, *Anopheles funestus s.s*. The finding that *Microsporidia MB* can be transmitted horizontally is relevant for the development of dissemination strategies to control malaria that are based on the targeted release of *Microsporidia MB* infected *Anopheles* mosquitoes.

Keywords: symbiosis, *Anopheles*, malaria, vector, *Microsporidia*

IMPORTANCE STATEMENT

The malaria disease burden remains a major impediment to good health and economic development in many regions of sub-Saharan Africa. We have recently reported that a microsporidian symbiont (*Microsporidia MB*) naturally blocks *Plasmodium* transmission in *Anopheles arabiensis*, a major vector of malaria in Africa. *Microsporidia MB* could form the basis of a novel transmission blocking intervention for malaria control. However, the development of *Microsporidia MB* as an intervention a strategy will require a better understanding of the symbiont's biology. Of particular relevance are the natural mosquito to mosquito transmission routes that enable *Microsporidia MB* to spread within *Anopheles* mosquito populations and which could potentially be used to disseminate *Microsporidia MB* as part of a malaria transmission blocking strategy. We investigate the natural routes of *Microsporidia MB's* mosquito to mosquito transmission and find that it can be transmitted horizontally between adult *An. arabiensis* of opposite sexes. This finding will aid the development of a *Microsporidia MB* dissemination strategy, potentially involving targeted release of *Microsporidia MB* infected *Anopheles* mosquitoes.

INTRODUCTION

Malaria continues to be a major health threat across sub-Saharan Africa, with this region accounting for 93% of the global malaria deaths (World Health Organization, 2020). The major preventive strategies for malaria control remain the use of long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS). In conjunction with improvements in case detection and management, these strategies have reduced malaria cases by up to 40% between 2000 and 2015 (Bhatt et al., 2015). However, progress has plateaued and possibly reversed, with case levels remaining the same between 2014 and 2016 and increasing between 2016 and 2017 (D'Alessandro, 2018; World Health Organization, 2020). It is apparent that current malaria control strategies have their limitations and there is a vital need for complementary tools (Huijben and Paaijmans, 2018).

The malaria transmission cycle relies on female *Anopheles* mosquitoes becoming infected by feeding on human blood that contains the *Plasmodium* gametocyte stage. *Plasmodium* gametocytes undergo a series of developmental changes before traversing the mosquito midgut to form a sporogonic oocyst, which produces sporozoites that are released into the mosquito hemocoel. Sporozoites in the hemocoel travel to the mosquito salivary glands to enter the mosquito's saliva, which results in an infected mosquito, usually 8–14 days after the bloodmeal (Baton and Ranford-Cartwright, 2005). This transmission cycle can be impeded by inhibitory interactions with mosquito-associated microbes (Romoli and Gendrin, 2018). One of the most promising new management strategies involves the use of vertically (mother to offspring) transmitted symbiotic microbes that prevent the establishment of disease-causing viruses in mosquito vectors. This strategy is currently used as a control mechanism against the arboviral disease, Dengue, through the bacterial symbiont, *Wolbachia* (Moreira et al., 2009; Bian et al., 2010; Hoffmann et al., 2011; Walker et al., 2011; Frentiu et al., 2014; Ant et al., 2018; Nazni et al., 2019).

The *Anopheles*-associated symbiont *Microsporidia MB* colonizes mosquito ovaries and is vertically transmitted. This microsporidian can also block the transmission of malaria by *Anopheles* mosquitoes (Herren et al., 2020), and therefore could potentially contribute to the control of malaria. The successful deployment of symbiont-based vector-borne disease control strategies requires the ability to spread symbionts through host insect populations and the maintenance of a high prevalence of infection. In *Wolbachia*-based strategies, cytoplasmic incompatibility can effectively drive symbionts through mosquito populations. In the absence of cytoplasmic incompatibility, other driving mechanisms would be required to spread *Microsporidia MB* through *Anopheles* populations. *Microsporidia MB* is naturally found in populations of *Anopheles* mosquitoes in Kenya, ranging in prevalence from 0 to 25% (Herren et al., 2020). From the standpoint of symbiont-based control strategies, the different *Microsporidia MB* transmission routes could be relevant for interventions that could generate a higher prevalence of the transmission-blocking symbiont in *Anopheles* mosquito populations, leading to reductions in malaria transmission.

Microsporidia are a diverse clade of obligate, intracellular organisms that infect an array of hosts, including vertebrates and invertebrates and are found in both terrestrial and aquatic environments (Vossbrinck and Debrunner-Vossbrinck, 2005). The morphology of Microsporidia can be simplified into the meront phase, which is present during proliferation, and the spore, which is resistant to environmental degradation and transmission-specialized. Microsporidian spores are characterized by a chitinous wall and a polar filament involved in host cell penetration (Stentiford et al., 2013). In arthropods, Microsporidian transmission can occur vertically (mother to offspring) and horizontally (from one individual to another of the same generation, Stentiford et al., 2013). There are also many reported incidences of microsporidians using a combination of vertical and horizontal transmission. Vertical transmission generally occurs via the transovarial route with spores germinating on the periphery or inside of ovaries to colonize developing eggs. Vertical transmission is associated with greater host specificity and lower Microsporidia burden and virulence (Vávra and Lukeš, 2013). There are different forms of horizontal transmission in arthropod-associated Microsporidia, however the most widespread is oral and involves the ingestion of spores, which subsequently germinate and inject their sporoplasm into the host intestinal cells through a polar filament. Microsporidia that predominately rely on oral horizontal transmission tend to be associated with lower levels of host specificity and high virulence as microsporidian spores will usually be released en masse from deceased hosts to infect other hosts (Han and Weiss, 2017). Other forms of horizontal transmission that are not associated with high virulence, for example sexual transmission, have also been demonstrated in several microsporidian species. *Nosema plodiae* is a microsporidian pathogen of the Indian meal moth, *Plodia*

interpunctella, which invades the reproductive organs of its host and is transmitted from male to female moths during mating (Kellen and Lindegren, 1971).

The Microsporidia transmission mode influences host specificity and life-cycle complexity (Stentiford et al., 2013). Microsporidians can be generalists, infecting a variety of different hosts or exhibit high levels of host specialization. Microsporidians can have specialization toward a single (simple lifecycle) or several intermediate hosts (complex lifecycle). Vertical and sexual transmission result in limited opportunities for Microsporidia to infect hosts of a different species and are therefore likely to lead to higher levels of host specificity. In contrast, horizontal transmission by spore ingestion is likely to be associated with lower levels of host specificity. Microsporidians with simple and complex lifecycles can use both vertical and horizontal transmission. In most cases, different spores types become specialized for different transmission routes (Stentiford et al., 2013).

We investigated a number of possible horizontal transmission routes for *Microsporidia MB* in *An. arabiensis*. We established that transmission was only found to occur between adult mosquitoes. In addition, transmission was only observed between different sexes, which indicates that *Microsporidia MB* is sexually transmitted in *An. arabiensis*.

RESULTS

Horizontal Transmission of *Microsporidia MB* Occurs Between Adult *An. arabiensis*

To determine if *Microsporidia MB* is horizontally transmitted at the adult or larval stages, *Microsporidia MB* infected and uninfected larvae and mosquitoes were housed together in larval rearing troughs or cages. Since it is difficult to reliably mark or determine the sex of larvae, we placed infected and uninfected larvae in two adjacent sections of rearing trough that was separated by a screen mesh. For larval experiments a roughly equal number of infected donor and uninfected recipient L1 larvae (N = 16–35) were placed in mesh separated compartments and allowed to develop into adults. After adults eclosed both donor and recipient specimens were screened for the presence of *Microsporidia MB*. Under these conditions horizontal transmission of *Microsporidia MB* was not observed (**Figure 1A** and **Table 1**). The addition of homogenized infected larvae to the rearing water of uninfected larvae and to sugar sources given to uninfected adult *An. arabiensis* also did not result in horizontal transmission of *Microsporidia MB* (**Table 2**). Altogether these findings indicate that intact, alive *An. arabiensis* larvae or the homogenates of *Microsporidia MB*-infected larvae and adults are not able to transmit *Microsporidia MB* horizontally to other *An. arabiensis* individuals (larval or adult).

To investigate horizontal transmission of *Microsporidia MB* between live adults, we established cages with *Microsporidia MB* infected and uninfected mosquitoes. Adult mosquitoes were maintained in these cages for a period of 2 days before they were screened for the presence of *Microsporidia MB*. Additionally, to determine if horizontal transmission between mosquitoes could involve sugar sources, these were screened; *Microsporidia MB* was not detected in sugar sources (**Table 3**). In cages that had *Microsporidia MB* infected and uninfected mosquitoes of the same sex, the mosquitoes were marked with dye to indicate *Microsporidia MB* "donors" and "recipients" prior to exposure. In general, 2–6 infected *An. arabiensis* were kept together with 10–25 uninfected mosquitoes in standard 30 cm × 30 cm × 30 cm cages. At the end of the experiment all mosquitoes were screened to confirm infection status and determine if horizontal transmission had occurred. Out of 47 cage experiments, horizontal transmission was observed in 15 cage experiments (**Figure 1B** and **Table 4**). Notably, horizontal transmission was only observed in cages that had opposite sexes of *Microsporidia MB* infected and uninfected adult *An. arabiensis*. Out of 16 cages that had *Microsporidia MB* infected males and uninfected females, transmission was confirmed in 9 cages (56%). Amongst 15 cages that had *Microsporidia MB* infected females and uninfected males, transmission was confirmed in 5 cages (33%). In 15 cages that had the same sex *Microsporidia MB* infected and uninfected adult *An. arabiensis,* horizontal transmission was not observed. To investigate the link between *Microsporidia MB* transmission from male *An. arabiensis* to females and insemination, and to approximate the mating frequency in cage experiments, female *An. arabiensis* spermatheca were dissected and checked for the presence of sperm (**Table 5**). The mating frequency in cage experiments where spermatheca were checked (N = 3) was found to range from 0 to 8%. Notably, *Microsporidia MB* transmission was only recorded in females that had sperm in their spermatheca.

The Success of *Microsporidia MB* Horizontal Transmission Is Not Linked to Male Infection Intensity

To investigate the factors that influence the rate of *Microsporidia MB* male to female transmission in *An. arabiensis,* we established cages with a single *Microsporidia MB* infected male and 11–48 *Microsporidia MB* uninfected females. Adult mosquitoes were maintained in these cages for a period of 2 days prior to being screened for the presence and intensity of *Microsporidia MB* by quantitative PCR. Out of a total of 33 individual *An. arabiensis* males, 17 were able to infect at least one *An. arabiensis* female in their cage (51.5%). The highest number of females infected by a single male was 3 females. There was no significant link between male intensity of *Microsporidia MB* infection and odds of successfully infecting of one or more uninfected females [exp(b) = 0.982, P = 0.715 df = 31]. The number of females per cage did not affect the odds of *Microsporidia MB* transmission to one or more females [exp(b) = 0.968, P = 0.421 df = 31]. The correlation between the *Microsporidia MB* infection intensity in donor males and in the female "recipients" was not significantly correlated (R^2 = 0, P = 0.34 df = 31) and **Supplementary Figure 1**). Notably, the average infection intensity in recipient females (10.33) was twice as high as the average infection intensity in male "donors" (5.01).

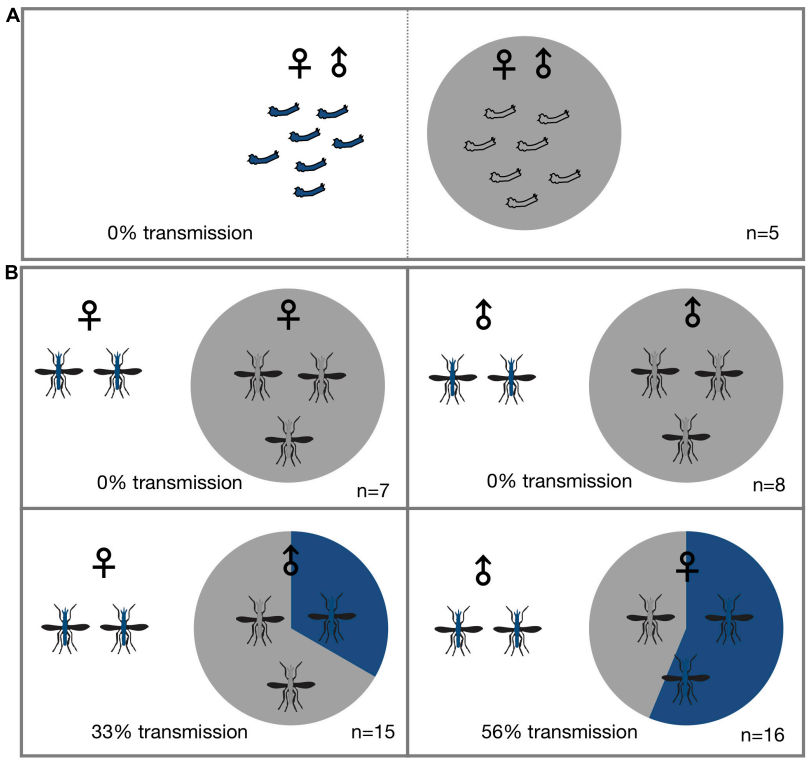

FIGURE 1 | Horizontal transmission of *Microsporidia MB*. Mosquitoes carrying *Microsporidia MB* are represented with blue shading in pie charts and n = number of independent experiments. **(A)** No transmission of *Microsporidia MB* was observed between *An. arabiensis* larvae reared in the same larval trough but separated by a screen mesh. **(B)** Horizontal transmission of *Microsporidia MB* was observed when adults were kept together in cages, and specifically when either infected males or females were housed with uninfected *An. arabiensis* of the opposite sex. Top row, no transmission was observed between infected and uninfected individuals of the same sex. Bottom left, transmission between *Microsporidia MB* infected *An. arabiensis* females and uninfected males was observed in 5 out of 15 cages (33%). Bottom right, out of a total of 16 experiments that had *Microsporidia MB* infected males and uninfected females and horizontal transmission was confirmed in 10 of these cages (56% transmission).

TABLE 1 | Horizontal transmission is not observed when *An. arabiensis* larvae are reared in the same larval trough but separated by a screen mesh.

Expt # (Sheet labels)	Number of donor larvae	Microsporidia MB positives in donor larvae	Number of recipient larvae	Infection prevalence in donor larvae	Transmission rate
LL1	10	8	14	0	0
LL3	9	9	20	0	0
LL4	14	4	31	0	0
LL5	20	13	10	0	0
LL6	7	7	9	0	0

Microsporidia MB Is Localized to Male *An. arabiensis* Midgut, Gonads, and Seminal Fluid

To determine if *Microsporidia MB* organ distribution in *An. arabiensis* could be linked to transmission routes, adult males were dissected and *Microsporidia MB* intensity was quantified in the midgut, male gonads and carcass (**Figure 2A**). In the majority of male *An. arabiensis* specimens, *Microsporidia MB* was detected in the midgut (11/22) or male gonads (7/22). In 2/22 specimens, *Microsporidia MB* was detected in both the midgut and the male gonads, whereas in only 3/22 specimens could *Microsporidia MB* be detected in the carcass. In line with these findings, the intensity of *Microsporidia MB* infections were found to be highest in the *An. arabiensis* midgut and male gonads and was found to be lower in carcasses (**Figure 2B**). The collection of seminal fluid from *Microsporidia MB* infected male *An. arabiensis* revealed that high intensities of *Microsporidia MB* could be detected in seminal fluid collected from 4/10 *An. arabiensis* males (**Figures 2C,D**).

Microsporidia MB Cells Are Present in the *An. arabiensis* Male Ejaculatory Duct

Fluorescence microscopy of male gonads revealed that *Microsporidia MB* cells were present in the male ejaculatory duct (**Figure 3**). Only in *Microsporidia MB*

TABLE 2 | Homogenates from larval and adult *Microsporidia MB* infected mosquitoes are not able to establish infections after being ingested by *An. arabiensis*.

Source of *Microsporidia MB* inoculum	Target *Anopheles* stage	Number of experimental repeats	Number of samples per experiment	*Microsporidia MB* Transmission
Homogenized larvae	Larvae (in rearing water)	3	20,14,17	0/20, 0/14, and 0/17
Homogenized larvae	Adults (in sugar source)	4	13,16,16	0/13, 0/16, and 0/16
Homogenized adults	Adults (in sugar source)	3	31,36,40	0/31, 0/36, and 0/40
Homogenized adults	Larvae (in rearing water)	3	28,31,28	0/28, 0/31, and 0/28

Homogenized infected larvae and adults were fed to adult (in sugar source) and larval (in rearing water) An. arabiensis, to determine if Microsporidia MB could be transmitted horizontally by ingestion. None of the An. arabiensis that fed on Microsporidia MB infected homogenates became infected with Microsporidia MB.

infected male *An. arabiensis* were the multinucleated cells corresponding to *Microsporidia MB* observed. Syto-9 nucleic acid staining revealed that the *Microsporidia MB* cells generally had either 4 or 8 nuclei, which likely corresponds to the progression on of 4-nuclei sporogonial plasmodia into an 8-nuclei stage (3rd sporogonic nuclear division) and ultimately becoming sporophorous vesicles (Sokolova and Fuxa, 2008).

Microsporidia MB Can Be Transmitted Vertically After Horizontal Transmission

To determine whether *An. arabiensis* females that horizontally acquired *Microsporidia MB* from the infected males could vertically transfer the infection to their offspring, we gave the recipient *An. arabiensis* females from all single male transmission cages a blood meal and collected eggs from them. Notably, only 4 out of 22 (18%) females successfully acquired a blood meal. Two out of the 4 female *An. arabiensis* that successfully acquired a blood meal laid eggs. Eggs were then allowed to develop into adults prior to being screened. *Microsporidia MB* was detected in 37% of the progeny of recipient female *An. arabiensis* mosquitoes, indicating that *Microsporidia MB* that is horizontally acquired can be subsequently vertically transmitted in the next gonotrophic cycle (**Figure 4**).

TABLE 3 | Sugar sources fed on by *Microsporidia MB* infected mosquitoes do not contain detectable levels of *Microsporidia MB*.

Experiment ID	Experiment type	Infection status
MF4	Male + /Female −	Not infected
MF5	Male + /Female −	Not infected
MF6	Male + /Female −	Not infected
MF7	Male + /Female −	Not infected
MF8	Male + /Female −	Not infected
MF9	Male + /Female −	Not infected
MF10	Male + /Female −	Not infected
FM2	Female + /Male −	Not infected
FM3	Female + /Male −	Not infected
FM4	Female + /Male −	Not infected
MM1	Male + /Male −	Not infected
MM2	Male + /Male −	Not infected
MM3	Male + /Male −	Not infected
MM4	Male + /Male −	Not infected
FF1	Female + /Female −	Not infected
FF2	Female + /Female −	Not infected
FF3	Female + /Female −	Not infected

Microsporidia MB Was Not Detected in Potential Secondary Hosts

Since microsporidians can have complex life cycles that involve secondary hosts, we screened a number of other mosquito species and aquatic organisms that inhabit the same habitats as *An. arabiensis* in Western Kenya. *Microsporidia MB* was not detected in mosquitoes in the genus *Aedes* and *Culex* as well as *Culicoides* midges (**Table 6**). In addition, no *Microsporidia MB* infections were found in crustaceans in the genera *Mesocyclops*, *Macrocyclops* and *Daphnia*. *Microsporidia MB* was detected in *Anopheles funestus s.s.* but not *Anopheles coustanii*. While this survey of potential secondary hosts was not exhaustive, these findings suggest that *Microsporidia MB* is likely to be an *Anopheles*-specific symbiont.

DISCUSSION

The results clearly demonstrate that *Microsporidia MB* is transmitted horizontally between adult *An. arabiensis*. Transmission was only observed in cages that had opposite sexes of *Microsporidia MB* infected and uninfected adult *An. arabiensis* suggesting that *Microsporidia MB* is transmitted sexually. *Anopheles gambiae s.l.* males package seminal fluid that is produced in the male accessory glands into a coagulated mating plug that is digested in the female atrium several days after mating (Giglioli and Mason, 1966). Sperm received by mated female *Anopheles* are stored in a dedicated organ called the spermatheca, which is relied upon by *Anopheles gambiae s.l.* females for a lifetime of offspring production (Tripet et al., 2003). We observed that *Microsporidia MB* intensity was much higher in the midgut and male gonads than in the carcass of male *An. arabiensis*. This suggests that *Microsporidia MB* either migrates to or proliferates in the male gonad. We observed multinucleate Microsporidia cells only in specimens that were infected with *Microsporidia MB*, indicating that these cells are developmental stages of *Microsporidia MB*. *Microsporidia MB* cells were specifically localized to the *An. arabiensis* male ejaculatory duct. The *Microsporidia MB* cells observed had either 4 or 8 nuclei, which likely indicates that *Microsporidia MB* sporogenesis is occurring in the *An. arabiensis* male ejaculatory duct as 4-nuclei sporogonial plasmodia develop into an 8-nuclei stage and finally to become sporophorous vesicles. This developmental

TABLE 4 | Horizontal transmission of *Microsporidia MB* between adults housed together in cages.

	Expt # (Sheet labels)	Number of donor mates in the cage	Number of confirmed MB+ donor mates in the cage	Total exposed screened	Donor 1 intensity	Donor 2 intensity	Donor 3 intensity	Donor 4 intensity	Donor 5 intensity	Donor 6 intensity	# Recipients acquired Infection	# Recipients didn't acquire Infection	Recipient 1 intensity	Recipient 2 Intensity	Recipient 3 Intensity
Male to Female															
	MF4	2	1	14	6.468956	NA	NA	NA	NA	NA	1	13	15.67		
	MF5	3	1	14	3.950098	NA	NA	NA	NA	NA	1	13	0.659		
	MF6	2	2	14	1.308167	8.4389	2.8418	NA	NA	NA	0	14			
	MF7	2	2	15	12.48482	41.056	6.1403	NA	NA	NA	3	12	2.041	0.8229	0.496
	MF8	2	2	19	4.897402	94.392	8.5958	NA	NA	NA	3	16	0.624	36.948	0.254
	MF9	2	1	14	14.75445	NA	NA	NA	NA	NA	1	13	173.6		
	MF10	2	1	21	0.489231	NA	NA	NA	NA	NA	2	19	14.54	6.3849	
	MF11	2	1	25	1.320645	NA	NA	NA	NA	NA	1	24	4.996		
	MF16	2	1	17	1.329321	NA	NA	NA	NA	NA	0	17			
	MF18	2	1	17	0.224129	NA	NA	NA	NA	NA	0	17			
	MF24	2	1	25	0.148271	NA	NA	NA	NA	NA	0	25			
	MF3	3	1	13	0.476983	NA	NA	NA	NA	NA	0	13			
	SPM 212	7	3	33	2990.757	3.4633	449.23	7.5334	207.08	NA	1	32	354.4		
	SPM 297	7	1	31	11.42972	NA	NA	NA	NA	NA	0	31			
	SPM 304	11	3	37	27.86255	1.1878	16.141	1E-05	3660	NA	2	35	5.132	25.646	
	SPM 315	3	2	17	4.047635	5.3183	2.1773	N/A	N/A	NA	0	17			
Female to Male															
	FM2	6	2	20	20.15738	1.6845	NA	NA	NA	NA	0	20			
	FM3	5	1	25	5.352259	NA	NA	NA	NA	NA	0	25			
	FM4	4	1	25	1.467548	NA	NA	NA	NA	NA	0	30			
	BDF03	11	1	16	13.74596	NA	NA	NA	NA	NA	0	16			
	BDF47	13	1	14	7.607281	NA	NA	NA	NA	NA	1	13	0.337		
	BDF53	15	5	13	0.45705	42.397	1.3176	4.1668	44.417	NA	3	10	0.397	1.8002	4.485
	BDF64	9	2	19	2.01744	0.2731	NA	NA	NA	NA	2	17	9.998	1.1225	
	BDF 77	13	2	21	10.08619	0.1461	NA	NA	NA	NA	0	21			
	318 B	15	6	26	25.97944	14.504	9.4598	5.8088	2.2449	7.2807	0	26			
	319A	10	1	43	173.9875	NA	NA	NA	NA	NA	0	43			
	338D	11	4	39	2.583147	4.761	2.2513	1.1604	NA	NA	0	39			
	339A	5	2	19	1305.295	2.6964	NA	NA	NA	NA	1	18	20.43		
	BDF345	4	1	23	7.598649	N/A	NA	NA	NA	NA	0	22			
	BDF346	4	3	11	0.249339	11.79	6.4213	NA	NA	NA	1	10	2.269		
	BDF349	7	6	12	1.095105	1.2174	7.1288	27.826	50.479	1.2548	0	12			
Male to Male															
	MM1	2	1	17	0.99955	NA	NA	NA	NA	NA	0	17			
	MM2	3	1	12	2.348149	NA	NA	NA	NA	NA	0	12			
	MM3	1	1	15	6.259642	NA	NA	NA	NA	NA	0	15			
	MM4	2	1	20	3.925768	NA	NA	NA	NA	NA	0	20			
	PPM02	2	1	24	2.349105	NA	NA	NA	NA	NA	0	24			
	PPM08	3	2	31	0.516775	11.651	NA	NA	NA	NA	0	31			
	PPM25	9	1	28	11.96361	NA	NA	NA	NA	NA	0	28			
	PPM31	13	1	13	9.222971	NA	NA	NA	NA	NA	0	13			
Female to Female															
	FF1	2	1	13	1.62354	NA	NA	NA	NA	NA	0	13			
	FF2	3	1	25	0.21161	NA	NA	NA	NA	NA	0	25			
	FF3	5	2	16	30.35195	14.257	NA	NA	NA	NA	0	16			
	PPF01	6	5	19	26.56585	21.535	57.306	302.93	51.256	NA	0	19			
	PPF07	4	1	21	1.339635	NA	NA	NA	NA	NA	0	21			
	PPF22	5	1	23	2.30285	NA	NA	NA	NA	NA	0	23			
	PPF32	7	3	19	0.257489	11.032	54.376	NA	NA	NA	0	19			

Horizontal transmission of Microsporidia MB was observed when either infected males or females were housed with uninfected An. arabiensis of the opposite sex.

TABLE 5 | *Microsporidia MB* transmission is linked to the presence of sperm in female *An. arabiensis* spermatheca.

	# of male donors	# of male donors MB+	# of female recipients	# of female recipients with sperm in spermatheca	# of female recipients sperm+ and MB+	# of female recipients sperm– and MB+
SPC12	3	1	25	2	1	0
SPC09	1	1	40	0	0	0
SPC15	3	2	59	2	1	0

sequence has been reported in greater detail in Microsporidians associated with fire ants (Sokolova and Fuxa, 2008) and Daphnia (Refardt et al., 2008). It is therefore likely that the sporogenesis of *Microsporidia MB* in the male ejaculatory duct produces infectious spores that are released with seminal secretions and therefore transferred to females upon mating. Transmission from female to male *An. arabiensis* was also observed, but further investigation will be required to establish the basis of this transmission route.

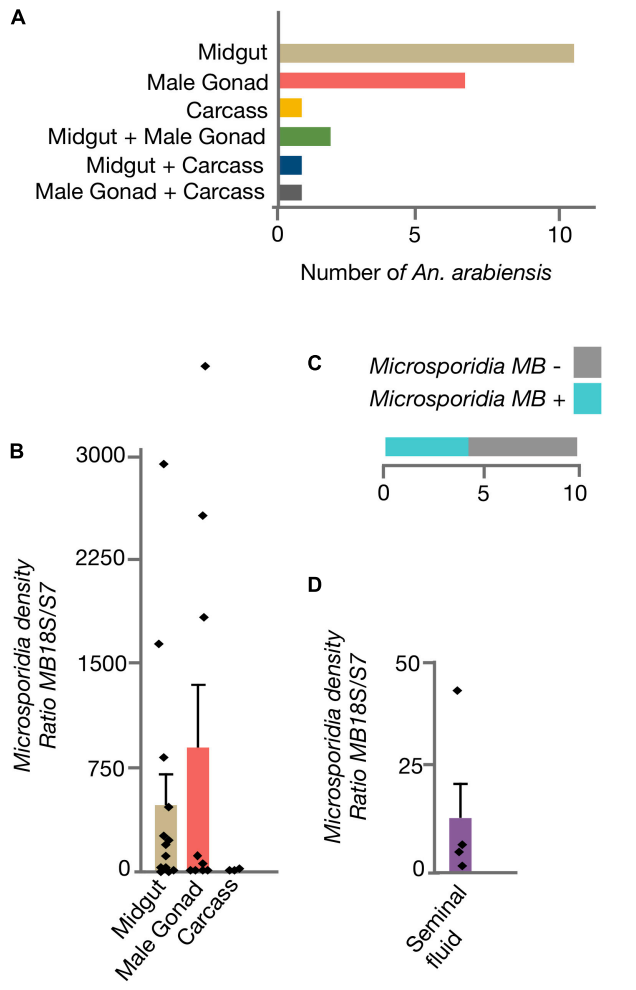

FIGURE 2 | Distribution of *Microsporidia MB* across male *An. arabiensis* organs. **(A)** The screening dissected organs from 22 male *An. arabiensis* specimens, reveals that *Microsporidia MB* is detected primarily in the midguts and male gonads. **(B)** The intensity of *Microsporidia MB* infection is highest in the midgut and male gonads. **(C)** The screening male *An. arabiensis* seminal fluid revealed that *Microsporidia MB* was detected in 4/10 specimens. **(D)** The intensity of *Microsporidia MB* infection in *An. arabiensis* seminal fluid ranges from a ratio of 0.87 to 41.8 MB18S/S7. Error bars reflect SEM.

Two findings indicate that mating is required for *Microsporidia MB* transmission. Firstly, the absence of *Microsporidia MB* transmission in same sex transmission cage experiments, and secondly the finding that in the three cage experiments where female *An. arabiensis* spermatheca were checked for the presence of sperm, only inseminated females acquired *Microsporidia MB*. The experimental design precluded the quantification of precise transmission rates, since in the majority of experiments female insemination events were not confirmed. However, in light of the low rate of female insemination in cages where spermatheca were checked, it can be expected that the rate of *Microsporidia MB* transmission from males to female *An. arabiensis* per successful mating is likely to be high.

The number of females infected and the intensity of *Microsporidia MB* infections in recipient females was not dependent on the intensity of *Microsporidia MB* in donor males. A possible explanation for this finding is that *Microsporidia MB* are localized to midguts and gonads. It is possible that localization to the male gonad is a pre-requisite for sexual transmission and that only the intensity of *Microsporidia MB* infection in gonads is correlated with transmission capacity. The finding that *Microsporidia MB* intensity was high in the midgut and that some male *An. arabiensis* had high intensity of *Microsporidia MB* only in the midgut suggests that this organ may play a yet to be determined role in transmission or alternatively that the midgut is a reservoir of *Microsporidia MB*. Notably, since the majority of gonadal tissue development occurs during metamorphosis, localization to the midgut could be required for maintenance of *Microsporidia MB* infection in *An. arabiensis* larval stages.

From the perspective of symbionts that are strictly maternally inherited, males are a dead end. In many cases, including for maternally inherited microsporidians, this can lead to the evolution of feminization or male-killing (Ironside and Alexander, 2015). Another possible outcome is that maternally inherited infections evolve to become sexually transmitted. The sexual transmission of beneficial heritable microbes has been reported in aphids (Moran and Dunbar, 2006). It is probable that in aphids sexual transmission enabled decreased pathogenicity of symbionts and co-evolution toward obligate mutualism.

Sexual horizontal transmission has been reported in a variety of insect-associated microsporidians (Knell and Mary Webberley, 2004) and in most cases it is associated with other complementary forms of transmission. Sexual transmission is likely to be more

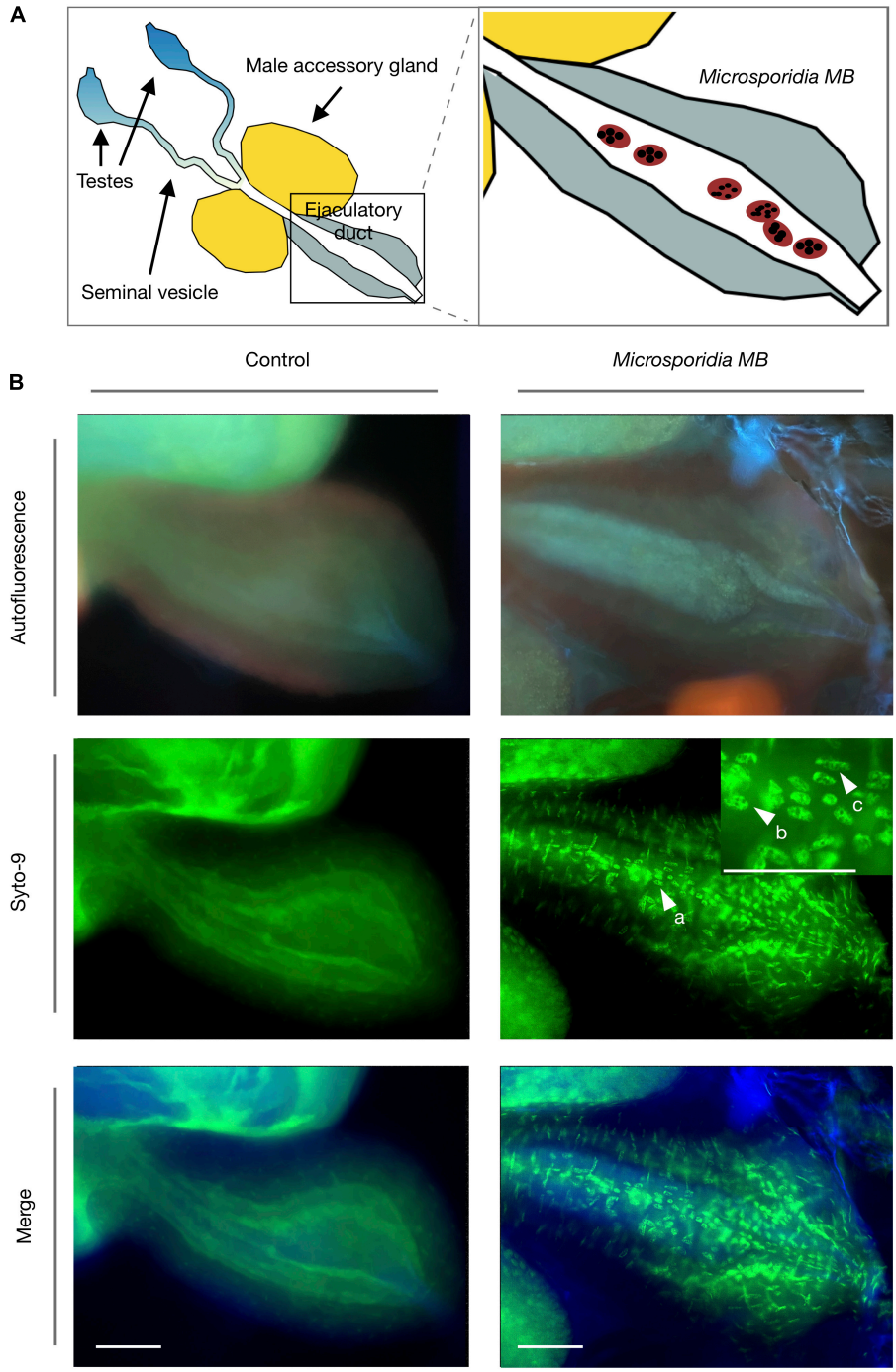

FIGURE 3 | Fluorescence microscopy of *Microsporidia MB* in *An. arabiensis* male ejaculatory ducts. **(A)** Schematic diagram of the male *Anopheles* gonad shows the position of the ejaculatory duct in relation to seminal vesicle, male accessory gland and testes. **(B)** Fluorescence microscopy images indicate that *Microsporidia MB* meronts (a) are found in the male *An. arabiensis* ejaculatory duct. Multinucleate *Microsporidia MB* cells can be observed containing 4 and 8 distinct nuclei (b,c), which likely corresponds to the progression on of 4-nuclei sporogonial plasmodia into the 8-nuclei sporogonial plasmodia and ultimately into sporophorous vesicles. Scale bar = 50 μm.

effective in insect species that have overlapping generations and higher levels of promiscuity. In *Anopheles*, the bacterial symbionts *Asaia* (Favia et al., 2007) and *Serratia* AS1 (Wang et al., 2017) have been shown to be sexually transmitted. It is notable that *Anopheles gambiae s.l.* is largely monandrous and therefore it is unlikely that symbionts could rely solely on sexual horizontal transmission. Indeed, both *Asaia* and *Serratia* AS1 are also transmitted vertically and by other horizontal transmission

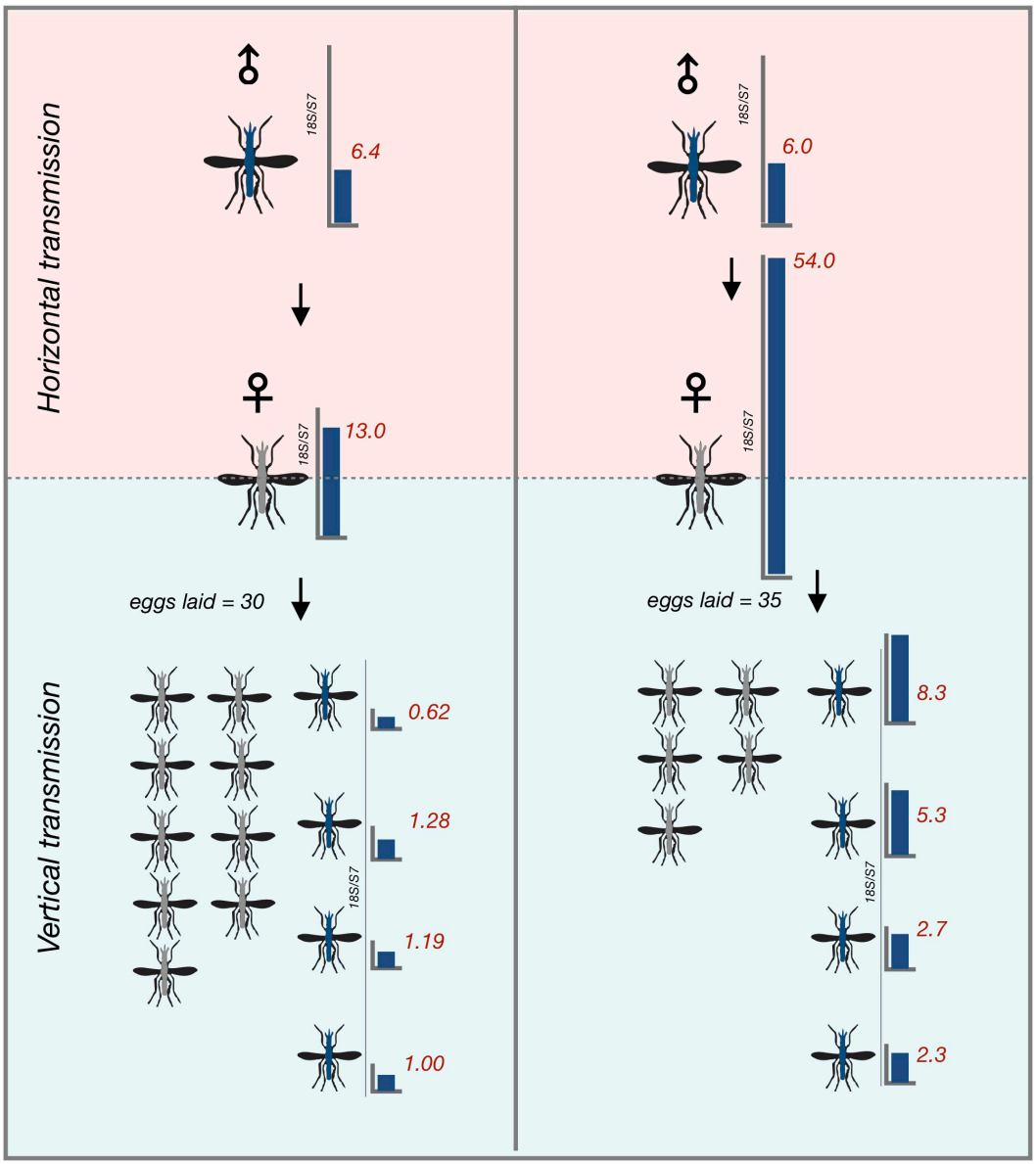

FIGURE 4 | Vertical transmission of horizontally acquired *Microsporidia MB* infections in *An. arabiensis*. Vertical transmission of *Microsporidia MB* is observed in recipient females that have become infected with *Microsporidia MB* after being kept with *Microsporidia MB* infected donor males in the first gonotrophic cycle after horizontal acquisition. Red numbers indicate *Microsporidia MB* infection intensity in individual *An. arabiensis* adults as determined by qPCR.

routes. It is notable that sexually transmitted infections of insects tend to reach much higher prevalence levels than infections with other forms of horizontal transmission (Knell and Mary Webberley, 2004). An example is the Microsporidian *Nosema calcarati*, which is sexually and vertically transmitted in its host *Pitogenes calcaratus* and found at a prevalence of 50% (Purrini and Halperin, 1982). High prevalence may be in part due to the fact that sexual transmission selects for lower levels of virulence toward the hosts. Sexually transmitted infections can manipulate insect host physiology or behavior to favor higher levels of transmission, for example sexually transmitted mites were shown to increase the mating success of male midge hosts (McLachlan, 1999). Whether any sexually transmitted pathogens of *Anopheles* affect mating behavior has not been established.

Microsporidia MB was not found in non-anopheline arthropods that are found in the same habitats as *An. arabiensis* larvae. Since *Microsporidia MB* is transmitted vertically and by sexual horizontal transmission, a high level of host specificity could be expected since neither vertical (Herren et al., 2020), nor sexual horizontal transmission would be effective across species. It is noteworthy that *Microsporidia MB* was found in another species of anopheline mosquito, *An. funestus s.s.*, which is a primary vector of malaria in Sub-Saharan Africa. If the *Microsporidia MB* found in *An. funestus s.s* have similar

TABLE 6 | *Microsporidia MB* was not observed in non-anopheline arthropods from the same habitats as *An. arabiensis*.

Genus of organism	Total number of individuals screened	Collection sites (n)	Presence of *Microsporidia MB*
Aedes aegypti	215	Kilifi/Malindi	No infection
Aedes sp.	10	Mbita	No infection
Culex quinquefaciatus	82	Kilifi/Malindi (15), Nairobi (37), and Mbita (30)	No infection
Culex sp.	61	Kilifi/Malindi	No infection
Culicoides sp.	42	Ahero (20) and Mwea (22)	No infection
Anopheles coustanii	42	Ahero (20) and Mwea (22)	No infection
Anopheles funestus s.s.	73	Ahero (73)	*Microsporidia MB* in 5 specimens
Mesocyclops sp.	34	Ahero (5) and Mwea (29)	No infection
Macrocyclops sp.	51	Ahero (20) and Mwea (31)	No infection
Daphnia sp.	20	Ahero (15) and Mwea (5)	No infection

characteristics to *Microsporidia MB* found in *An. arabiensis*, including *Plasmodium* transmission blocking, then *Microsporidia MB* could be developed as a tool for malaria control in several primary vector species.

To be successfully developed into a strategy to control malaria, an effective method of disseminating *Microsporidia MB* into *Anopheles* populations will need to be established. Our results show that *Microsporidia MB* infected male mosquitoes can infect their female counterparts and that horizontally infected females can transmit *Microsporidia MB* to their offspring. We previously showed that *Microsporidia MB* is vertically transmitted in *An. arabiensis* (Herren et al., 2020) and therefore *Microsporidia MB* infected males for releases could be produced by sorting the offspring of *Microsporidia MB* infected *An. arabiensis* colonies. These findings could be the basis for a dissemination strategy that involves targeted release of *Microsporidia MB* infected male *Anopheles* mosquitoes, potentially avoiding the need to release biting females, which would be advantageous in terms of community engagement and acceptance of the intervention. In principle, such a strategy would be similar to the mass-release of sterile males (Bouyer et al., 2020), except that instead of sterilizing females *Microsporidia MB* infected males would decrease the capacity of infected females and their offspring to transmit malaria for multiple generations. The capacity of *Microsporidia MB* to be vertically transmitted after infecting females would potentially make this approach more sustainable and cost-effective than SIT.

MATERIALS AND METHODS

Field Collections

Resting gravid and engorged female mosquitoes were collected indoors through manual aspiration. Collections were undertaken in Ahero (–34.9190W, –0.1661N) and Mwea (–37.3538W, –0.6577N) between Feb and June 2020 between 0630 h and 0930 h using electric torches/lights and aspirators. Collected females were placed in large cages supplied with 6% glucose and transported to *icipe*-Thomas Odhiambo Campus (*iTOC*) from Ahero and *icipe* Duduville campus from Mwea for processing. Mosquito larvae and other organisms were collected from larval habitats in Mwea and Ahero using larval collection dippers between March and July 2019. *Anopheles funestus* and *coustanii* were collected in November 2018 in Ahero as adults in houses (for *Anopheles funestus*) and cattle-baited traps (for *Anopheles coustanii). Aedes aegypti* and *Culex* sp. larval stages were collected in March 2018 from old discarded wheel tires in Kilifi and Malindi and transported to the rearing facility at Pwani University for emergence.

Mosquito Identification, Processing, and Rearing

All transmission experiments were carried out on wild-collected *Anopheles gambiae sl.*, which were identified morphologically. In all of the collection sites, *An. arabiensis* is the most common member of the *An. gambiae* species complex, with >97% of complex members being identified as *An. arabiensis*. The high percentage of *An. arabiensis* in field collections from both sites was re-confirmed using PCR (Santolamazza et al., 2008). *An. funestus* species were identified by PCR (Koekemoer et al., 2002). Wild collected mosquitoes were maintained in an insectary at 27 ± 2.5°C, humidity 60–80% and 12-h day and 12-h night cycles and induced to oviposit in individual microcentrifuge tubes containing a wet 1 cm × 1 cm Whatman filter paper. Eggs from each female were counted under a compound microscope using a paint brush and then dispensed into water tubs for larval development at 30.5°C and 30–40% humidity. Tetramin^{TM} baby fish food was used to feed developing larvae. Upon laying eggs, the G_0 females were screened for presence of *Microsporidia MB* by PCR. The larval offspring of *Microsporidia MB* positive field-caught female mosquitoes were pooled into larval rearing troughs for experimentation. *Microsporidia MB* uninfected controls were obtained from the *An. arabiensis* colonies at *icipe iTOC* Mbita and Duduville campuses.

Inoculation of *Microsporidia MB* Homogenate by Feeding

Five infected *An. arabiensis* larvae or adults were placed in 1.5 ml microcentrifuge tubes containing 500 µl 1 × PBS. *An. arabiensis* larvae and adults were homogenized using a pestle and then transferred directly into larval rearing water or sugar sources. For larval rearing water, 500 µl homogenate was

added to 500 ml of distilled water at the L2 larval stage. For adults, 250 μl homogenate was added to 20 ml of 10% sucrose solution. Recipient larvae that developed in rearing water with homogenate were screened as 1–2 day old adults. Recipient adults were screened 2 days after initial homogenate exposure. Aliquots of the homogenate were kept at −20°C and screened by PCR, all homogenates used were *Microsporidia MB* positive.

Transmission Between Live *An. arabiensis* Larvae

Microsporidia MB infected donor and uninfected recipient *An. arabiensis* larvae (donor $N = 7–20$ and recipient $N = 9–31$) were transferred into a 15 cm x 30 cm larval rearing trough that had a 70 μm mesh divider between two sections. Donor and recipient larvae were placed in separated sections and maintained until they emerged as adults. Both donor and recipient *An. arabiensis* were screened as 1–2 day old adults to determine the percentage of donors that were infected and if recipients had horizontally acquired *Microsporidia MB*.

Transmission Between Live *An. arabiensis* Adults

Microsporidia MB infected and uninfected *An. arabiensis* virgin adults were transferred into 30 cm × 30 cm × 30 cm cages. Virgin mosquitoes were obtained by separating the sexes at the pupal stage after visual examination of the terminalia. To increase the chances of observing transmission, several (2–6) *Microsporidia MB* infected *An. arabiensis* donors were kept with 12–25 virgin uninfected recipient mosquitoes for 2 days. In the experiments where sex could not be used to differentiate male and female mosquitoes, dyes (red and blue) were used to mark mosquito wings and indicate donors and recipients. Upon completion of the transmission experiment all *An. arabiensis* mosquitoes were screened to determine the percentage of donors that were infected and if recipients had horizontally acquired *Microsporidia MB*. To investigate the efficiency of horizontal transmission and the importance of *Microsporidia MB* intensity, additional cages with single *Microsporidia MB* infected donor males and 10–50 virgin *Microsporidia MB* uninfected recipient females were established and maintained for 2 days. In the single infected donor male cage experiments, post exposure, female recipients were allowed to feed on a human arm for 15 min at 19:00 h. Mosquitoes that fed were placed in individual micro centrifuge tubes with wet filter papers to induce oviposition Upon completion of the transmission experiment all *An. arabiensis* mosquitoes were screened by qPCR to determine the infection status and intensity of donor and recipient *An. arabiensis*. The offspring from *Microsporidia MB* infected recipient females from single male cage experiments were reared until they were 1–2 day old adults and then screened for *Microsporidia MB* to determine if vertical transmission had occurred. To investigate mating rates and the link between acquiring *Microsporidia MB* and female insemination status, the presence of sperm in *An. arabiensis* females maintained in some of the cages with *Microsporidia MB* infected males were examined by the dissection of spermathecae and scoring sperm presence.

Quantification of *Microsporidia MB* Distribution Across Male *An. arabiensis* Organs

Quantification of *Microsporidia MB* was conducted on dissected organs from G_1 *Microsporidia MB*-infected *An. arabiensis* adult males, 3–5 days post emergence. Midguts and gonads were separated from the remainder of the mosquito which was designated as the carcass. Each organ and the carcass was individually screened for *Microsporidia MB* presence and intensity by qPCR. Quantification of *Microsporidia MB* in the male seminal fluid was carried out on different *An. arabiensis* specimens. Briefly, 10–12 day old males were decapitated and used immediately in forced mating experiments with virgin females (full method given at www.mr4.org). Upon successful copulation, seminal secretions produced by the male were collected with a pulled capillary tube and transferred to a 10uL 1 × PBS and placed under ice. Genomic DNA was collected as previously described prior to *Microsporidia MB* quantification by qPCR.

Microscopy of *An. arabiensis* Male Gonad

Microscopy was conducted on dissected G_1 *Microsporidia MB*-infected and uninfected (control) *An. arabiensis* adult male gonads, 3–5 days post emergence. Gonads were fixed in 4% Paraformaldehyde (PFA) solution for 30 min. After three quick washes with PBS-T, samples were stained in 0.1mM Syto-9 in PBS for 1 h. After two quick washes and one 10 min wash, the gonads were placed on a slide and were visualized immediately using a Leica SP5 confocal microscope (Leica Microsystems, United States). Images were analyzed with the ImageJ 1.50i software package (Schneider et al., 2012).

Specimen Storage and DNA Extraction

All *An. arabiensis* specimens were dry frozen at –20°C in individual microcentrifuge tubes prior to DNA extraction. DNA was extracted from each section individually using the protein precipitation method (Puregene, Qiagen, Netherlands).

Molecular Detection of Presence and Intensity of *Microsporidia MB*

Microsporidia MB specific primers (MB18SF: CGCCGG CCGTGAAAAATTTA and MB18SR: CCTTGGACGTG GGAGCTATC) were used to detect *Microsporidia MB* in *An. arabiensis* larvae and adults (Herren et al., 2020). A 10 μl PCR reaction consisted of 2 μl HOTFirepol® Blend Master mix Ready-To-Load (Solis Biodyne, Estonia, mix composition: 7.5 mM Magnesium chloride, 2 mM of each dNTPs, HOT FIREPol® DNA polymerase), 0.5 μl of 5 pmol μl^{-1} of forward and reverse primers, 2 μl of the template and 5 μl nuclease-free PCR water was undertaken. Conditions used were initial denaturation at 95°C for 15 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 62°C for 90 s and extension at 72°C for a further 60 s. Final elongation was done at 72°C for 5 min. The intensity of *Microsporidia MB* infection was determined by a qPCR assay using MB18SF/MB18SR primers. These were normalized against the *Anopheles* ribosomal S7 host gene

primers (S7F: $^{5'}$TCCTGGAGCTGGAGATGAAC$^{3'}$ and S7R $^{5'}$GACGGGTCTGTACCTTCTGG$^{3'}$, Dimopoulos et al., 1998).

Statistical Analysis

We carried out statistical analyses using the two-tailed paired spearman's rank test to compare paired donor and recipient *Microsporidia MB* intensity data values which had a non-normal distribution. To analyze if donor *Microsporidia MB* intensity or number of available mates affected the odds of *Microsporidia MB* transmission, a logistic regression analysis was carried out. All statistical analyses were undertaken using GraphPad Prism version 6.0c software and R (version 3.5.3). *P*-values of $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ were deemed to be statistically significant.

DATA AVAILABILITY STATEMENT

All the datasets presented in this study can be found an online repository: https://doi.org/10.6084/m9.figshare.14846925.v2.

AUTHOR CONTRIBUTIONS

JH and GN conceived and designed the majority of the experiments. GN, TM, TB, and EEM performed the majority of the experiments. GN, EEM, TM, DM, and EO collected mosquitoes and screened them for symbionts. JH, SS, EM, EEM, LM, ET, JP, JB, GN, TB, TO, FO, and GM analyzed the data. JH, TO, and FO carried out the microscopy. JH and GN wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.647183/full#supplementary-material

Supplementary Figure 1 | The intensity of *Microsporidia MB* in recipient females is not correlated to donor male intensity, with a regression slope that does not significantly differ from zero ($P = 0.34$, $r = 0.177$, and $n = 31$).

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