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RESEARCH ARTICLE

The influence of the larval microbiome on susceptibility to Zika virus is mosquito genotype-dependent

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Abstract

The microbiome of the mosquito *Aedes aegypti* is largely determined by the environment and influences mosquito susceptibility for arthropod-borne viruses (arboviruses). Larval interactions with different bacteria can have carry-over effects on adult *Ae. aegypti* replication of arboviruses, but little is known about the role that mosquito host genetics play in determining how larval-bacterial interactions shape *Ae aegypti* susceptibility to arboviruses. To address this question, we isolated single bacterial isolates and complex microbiomes from *Ae. aegypti* larvae from various field sites in Senegal. Either single bacterial isolates or complex microbiomes were added to two different genetic backgrounds of *Ae. aegypti* in a gnotobiotic larval system. Using 16S amplicon sequencing we showed that the bacterial community structure differs between the two genotypes of *Ae. aegypti* when given identical microbiomes, and the abundance of single bacterial taxa differed between *Ae. aegypti* genotypes. Using single bacterial isolates or the entire preserved complex microbiome, we tested the ability of specific larval microbiomes to drive differences in infection rates for Zika virus in different genetic backgrounds of *Ae. aegypti*. We observed that the proportion of Zika virus-infected adults was dependent on the interaction between the larval microbiome and *Ae. aegypti* host genetics. By using the larval microbiome as a component of the environment, these results demonstrate that interactions between the *Ae. aegypti* genotype and its environment can influence Zika virus infection. As *Ae. aegypti* expands and adapts to new environments under climate change, an understanding of how different genotypes interact with the same environment will be crucial for implementing arbovirus transmission control strategies.

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Author summary

Adult mosquitoes transmit many viruses, including Zika virus, during the process of taking a bloodmeal from human hosts. An important parameter of how well a mosquito is at transmitting viruses is whether the mosquito can become infected and replicate the virus. Different mosquito populations can be genetically distinct from each other, and in some cases genetic differences are associated with the habitat they live in. An important factor of the mosquito habitat is the water source in which larvae develop into adults. These water sources harbor diverse bacterial communities and are the source of the larval microbiome, which is known to influence how well the mosquito can become infected with viruses as an adult. Here we show that the effect of the larval microbiome on adult susceptibility to Zika virus depends on the genotype of the mosquito. These results indicate that different genetic backgrounds of mosquitoes interact with their habitat differently and this has important consequences for how easy it is for a mosquito to become infected.

Introduction

Arthropod-borne viruses (arboviruses) transmitted by mosquitoes represent a major cause of morbidity and mortality worldwide [1,2]. The mosquito, *Aedes aegypti*, is the main vector for arboviruses worldwide including dengue (DENV), Zika (ZIKV), yellow fever (YFV), and chikungunya (CHIKV) viruses. Climate change and a warming world exacerbate this risk from vector borne diseases [3,4] by expanding the range of vectors. Additionally, arbovirus epidemics are poised to be a major threat in sub-Saharan Africa [5]. Given the ongoing and increasing risk of mosquito-borne viruses, especially in Africa, it is crucial to understand factors that contribute to their emergence and transmission.

Aedes aegypti demonstrates large phenotypic variability in its interactions with arboviruses, largely driven by genetic and environmental variation. *Aedes aegypti* is genetically diverse worldwide [6–8] where most of the genetic diversity is observed within Africa. In West Africa, genetic variation between populations of *Ae*. *aegypti* is largely driven by degree of urbanization and rainfall and linked to adaptation to human preference [7] and its ability to efficiently transmit arboviruses is a partially genetically controlled trait [9]. Specifically, different genotypes of *Ae*. *aegypti* [10–12] have been shown to result in different infection outcomes with arboviruses. In some cases, vector competence is dependent on the specific mosquito-virus interactions [13–15].

Additionally, abiotic (non-living) [16–29] and biotic (living) environmental factors [30–36] are known to contribute to the vector competence of *Ae*. *aegypti*. An important biotic ecological parameter influenced by the environment is the larval microbiome. Globally, *Ae*. *aegypti* occupies a variety of environments and diverse larval habitats. Outside Africa, domesticated *Ae*. *aegypti* oviposits in artificial containers such as discarded buckets or cans and tires around human habitats. In Africa, *Ae*. *aegypti* will oviposit and develop in a variety of container types ranging from artificial containers around human habitats, to tree holes and rockpools in forested habitats. Larval development sites represent different microbiomes [37]. The larval microbiome is largely determined by the aquatic environment and is critical for establishing the nutritional status of the mosquito [38]. Importantly, interactions between *Ae*. *aegypti* larvae and different bacterial strains have carry-over effects that drive variation in DENV susceptibility in adults [37,38]. However, whether different genotypes of *Ae*. *aegypti* interact differently with the same larval microbiome to drive variation in arbovirus susceptibility

remains unknown. As the earth becomes warmer and drier and de-forestation and urbanization increase, *Ae*. *aegypti* may expand into new environments [39,40] and exploit different oviposition container types. This is especially true in Africa where the larval development site is tied to the environment. *Aedes aegypti* genotypes accustomed to ovipositing in forest or natural larval sites may be forced to adapt and oviposit in urban artificial container types.

Here we expand on previous work [37] to determine if the carry-over effects of the larval microbiome on arbovirus susceptibility is dependent on mosquito genotype. Using single bacterial isolates, we demonstrate the influence of specific bacterial isolates on adult *Ae*. *aegypti* susceptibility to ZIKV is dependent on mosquito genotype. Using complex microbiomes isolated from larvae in Senegal, we demonstrate that different genotypes of *Ae*. *aegypti* retain different members of these larval microbiomes and that ZIKV susceptibility is dependent on the specific pairing between *Ae*. *aegypti* genotype and complex microbiome during larval development. Our results provide empirical evidence that *Ae*. *aegypti* genotype by microbiome interactions drive variation in ZIKV susceptibility.

Results

Single bacterial isolates

Previously we observed that larval exposure to different individual bacterial isolates has carry-over effects which alter DENV replication in *Ae*. *aegypti* [37], but it remains unknown if this influence is consistent across vector genotypes. To address this, we measured whether a single bacterial isolate has the same carry-over effects on ZIKV infection between different genotypes of *Ae*. *aegypti* using a gnotobiotic assay. Of the 27 bacteria isolated from larvae collected from large metal drums in Thiés, Senegal, three bacterial isolates were chosen (*Serratia spp*. (Bacterial Isolate A), *Chyrseobacterium spp*. (Bacterial Isolate B), and *Serratia spp*. (Bacterial Isolate C)) for further characterization (see Material and Methods for information on selection). To understand if larval exposure to specific bacterial isolates alters pupation rates in an *Ae*. *aegypti* genotype-dependent manner, two lines of *Ae*. *aegypti* from Senegal with known genetic differences [7], Thiés (THI) and Kedougou (KED), were exposed to equal numbers of bacteria from each isolate in a gnotobiotic system, and the proportion of larvae that had pupated was recorded each day. Pupation rates were measured in triplicate gnotobiotic flasks daily from the onset of pupation (day five post-hatching) until day 10. Overall, the THI line of *Ae*. *aegypti* pupated more slowly than the KED line (p. value < 0.0001). In both lines, the time to reach 50% pupation (PD_{50}) was different among the three bacterial isolates (Fig 1A) (S1 Table). In both mosquito lines the bacterial isolate associated with the slowest pupation rate was Bacterial Isolate A with a PD_{50} of 8.5 days in the THI line and 7.4 days in KED line. Conversely, the bacterial isolate that resulted in quickest pupation was Bacterial Isolate B where a PD_{50} of 6.9 days occurred in the THI line and 6.1 days in the KED line. Bacteria C resulted in intermediate pupation rate of 7.4 days in the THI line and 6.7 days in the KED line. Interestingly, Bacteria A and C both belong to the genus *Serratia* but exert different effects on pupation rates. Overall, we observed that the PD_{50} was dependent on the mosquito genotype, bacterial isolate, or the interaction between the two variables (ANOVA on a general linearized model: mosquito genotype: $p < 0.0001$, bacterial isolate: $p < 0.0001$, mosquito genotype x bacterial isolate: $p < 0.0001$).

To determine if larval exposure to different single isolates influences ZIKV infection rates in a mosquito genotype dependent manner, the THI and KED line were challenged with ZIKV following larval exposure to Bacteria A, Bacteria B, or Bacteria C. Seven days post exposure to ZIKV, infection rates were determined by detection of viral RNA by RT-PCR. Infection rates ranged from 20–50% and was not dependent on the bacterial isolate (p-value = 0.614) or the

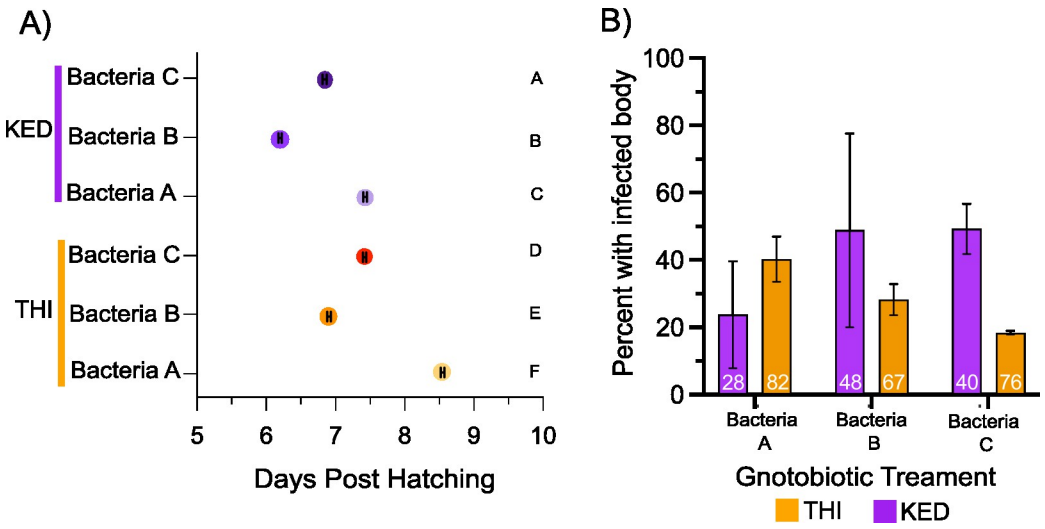

Fig 1. Larval exposure to different bacterial isolates alters pupation rate and ZIKV infection rates in mosquito genotype dependent manner. Variation in pupation rate (A) and ZIKV infection rates (B) are shown for *Ae. aegypti* reared in the presence of single bacterial isolates in a gnotobiotic system. (A) The PD_{50} (day where approximately 50% of the larvae have pupated) is shown for two distinct genotypes of *Ae. aegypti* [7] KED and THI reared in the presence of each bacterial isolate (Bacteria A, Bacteria B, and Bacteria C). Statistical significance of differences between PD_{50} was determined by multiple comparisons with two-way-ANOVA and Tukey's test (between bacterial treatments in each mosquito genotype) and Sidak's test (between bacterial treatments within the same mosquito genotype) and is designated by the letters. Error bars are shown within each point. Data represents two biological replicates with three gnotobiotic flasks per replicate. (B) The proportion of KED and THI lines of *Ae. aegypti* with a ZIKV positive body 7 days post-infection following larval rearing in single bacteria isolates, Bacteria A, Bacteria B, and Bacteria C. Error bars represent the standard deviation of the mean of two replicates. Data were analyzed by a two-way ANOVA on a binomial logistic regression (bacterial isolate: p-value = 0.614, mosquito genotype: p-value = 0.372, bacterial isolate x mosquito genotype: p-value = 0.010). Data represents two independent replicates with 8–62 individuals per treatment in each replicate.

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mosquito genotype (p-value = 0.372), but was dependent on the interaction between the bacterial isolate and the mosquito genotype (p-value = 0.010) (Fig 1B).

Complex microbiomes

Although using single bacterial isolates in a gnotobiotic system is more tractable, it is not representative of natural mosquito microbiomes. To establish if the effect of the interaction between mosquito genotype and bacterial isolate on susceptibility to Zika virus infection can be extended to complex microbiomes collected from larvae in the field, whole microbiomes from the larvae pools collected from five sites in Thiés, Senegal were introduced to both genotypes of *Ae. aegypti*. To establish if we were introducing different microbiomes to axenic larvae, we characterized the microbiomes of the larvae naturally developing in the collection sites at the same time of collection of larvae used for the gnotobiotic assay by 16S amplicon sequencing. Principal component analysis (PCA) on a Bray-Curtis dissimilarity matrices demonstrate that the initial bacterial community structure was different between sites (S1 Fig). We then characterized the bacterial communities of the gnotobiotic larvae following inoculation with the larval homogenates from each site to confirm the five microbial communities remained different. Out of 80 individual larvae sequenced, a total of 87 OTUs were found, which represent 30 genera after filtering for low abundance OTUs and OTUs present in the negative controls. Rarefaction curves (S2 Fig) showed that sufficient sequencing depth was achieved. Principal component analysis (PCA) was performed on a Bray-Curtis dissimilarity matrix on the larvae from the same sites to determine if the bacterial communities from each

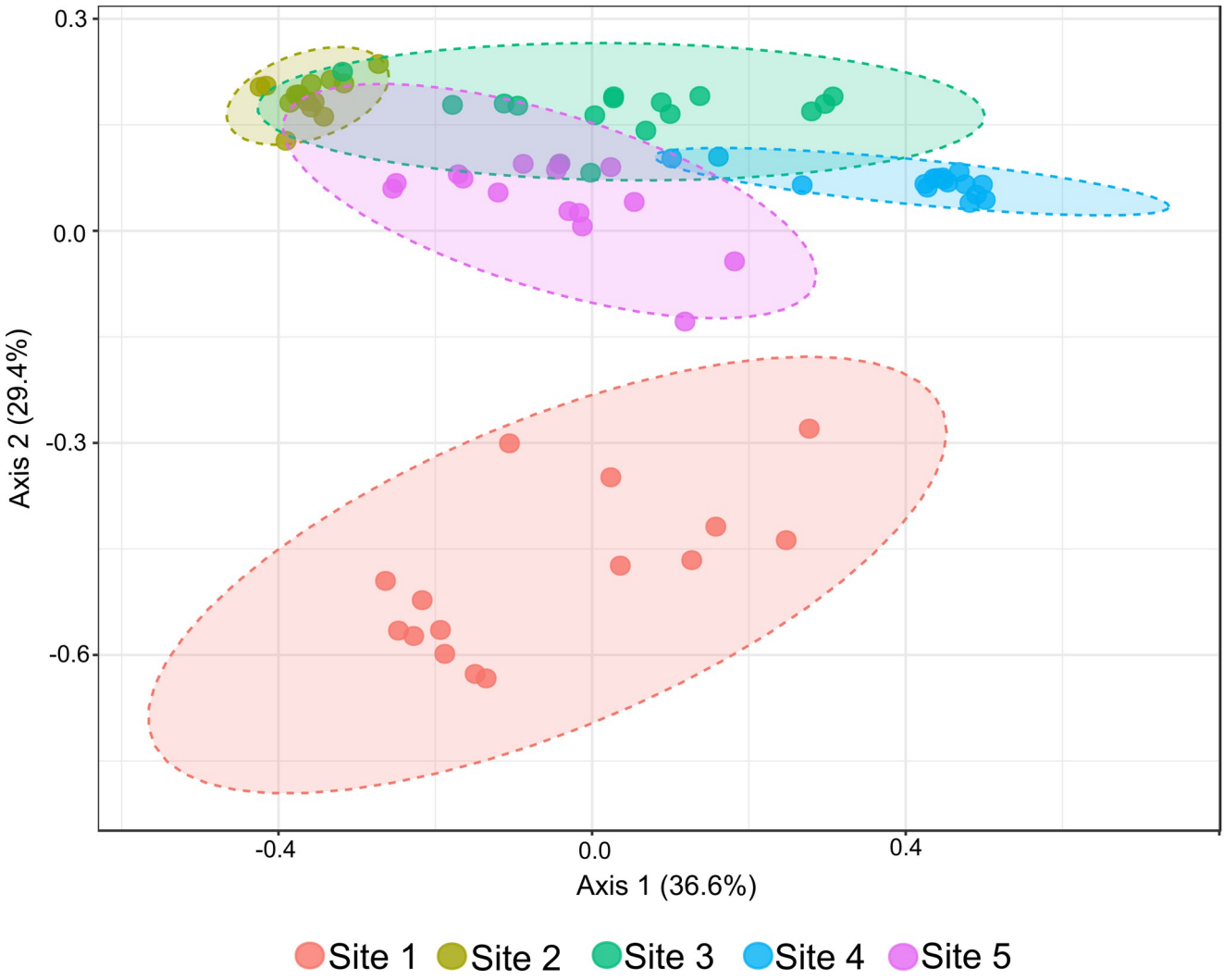

Fig 2. The bacterial community structure differs between the gnotobiotic larvae. Structure of bacterial communities of KED and THI larvae combined was determined by deep sequencing the V3-V4 region of the 16S gene in individual larvae reared in a gnotobiotic system inoculated with complex microbiomes generated from larvae homogenates collected in Senegal (Site1-Site5). Bacterial structure is represented by PCoA of a Bray-Curtis dissimilarity matrix based on mean genera abundance (PERMANOVA p = 0.001).

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collection site that were introduced to the larvae were in fact different. The community structure differed among all five of the bacterial communities (Fig 2) (PERMANOVA, p-value = 0.001). The bacterial community from Site 1 was the most different from the other sites, and Sites 2–5 showed greater similarity.

To assess whether the two genotypes of *Ae. aegypti* acquired and maintained different bacterial taxa after larval exposure to an identical bacterial community in a gnotobiotic system, the percent abundance of different genera was compared among genotypes and collection sites. The abundance of the top 20 most prevalent genera differed in both genotypes between the collection sites (Fig 3A). Gnotobiotic larvae harboring the bacterial community from Sites four or five were most similar between the genotypes. Gnotobiotic larvae harboring the bacterial community from Sites one, two, and three were most different between the genotypes. Abundance of specific genera was consistent among individuals from each treatment (S3 Fig). To determine if there are specific genera that are differentially abundant between genotypes in

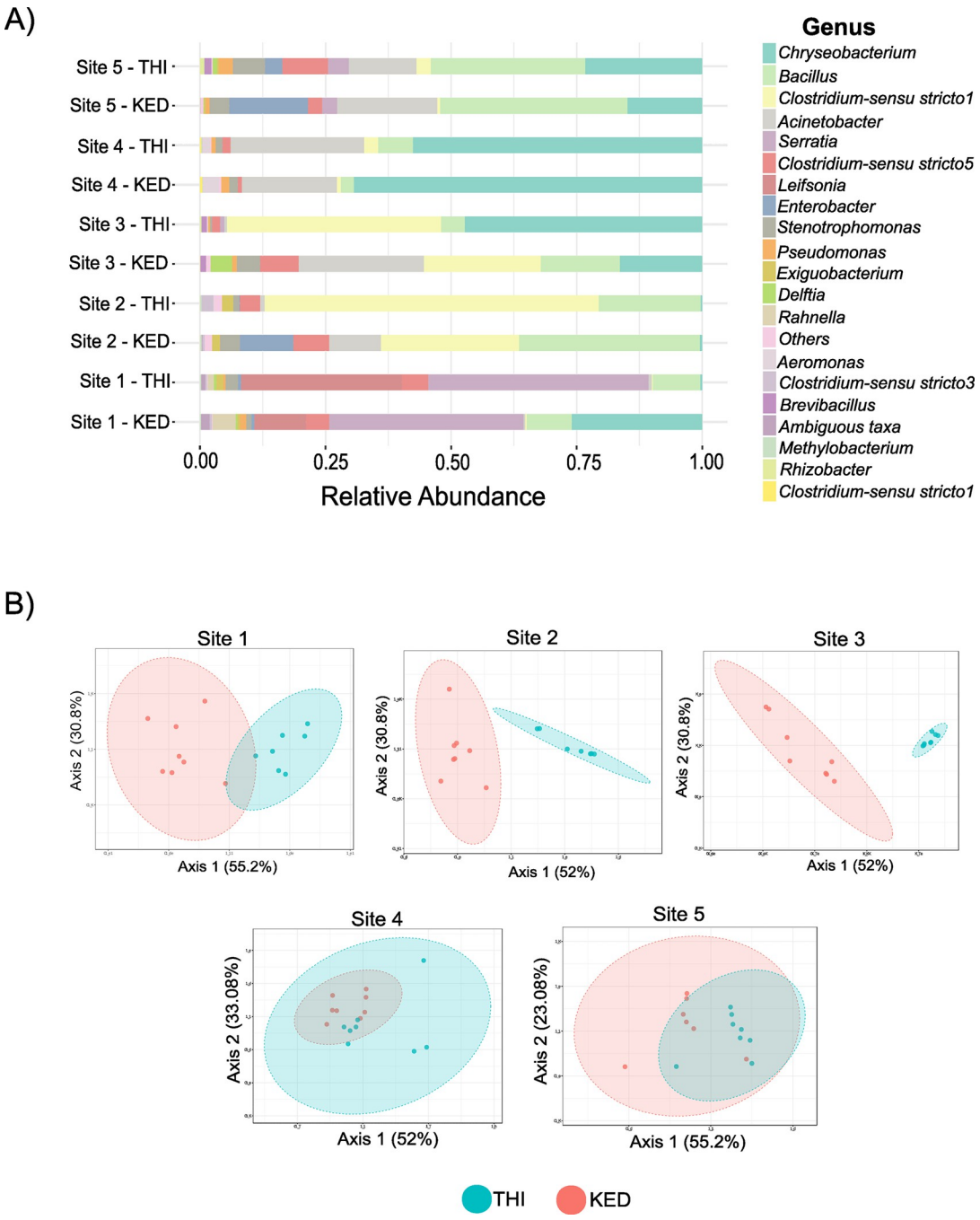

Fig 3. Abundance of specific taxa and community structure differs between lines of gnotobiotic larvae. (A) The percent abundance of the top 20 most abundant genera is plotted by larval treatment (Site1-5) and mosquito line (KED or THI). (B) Beta diversity metrics for the *Ae. aegypti* lines in each bacterial treatment analyzed separately. The dissimilarities between the two different lines of *Ae. aegypti* (KED and THI) in each of the five different larval microbiomes was analyzed by principal component analysis of Bray-Curtis dissimilarity matrixes. P-values from individual PERMANOVA analysis are as follows Site 1: p = 0.002, Site 2: p = 0.002, Site 3: p = 0.001, Site 4: p = 0.058, Site 5: p = 0.005).

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the gnotobiotic system, pairwise differential abundances of each genus were compared between mosquito genotypes after exposure to each of the five complex microbiomes. Of the 30 genera identified in this study, five to seventeen genera were differentially abundant

between genotypes. (S2 Table). Additionally, the overall bacterial community structure differed between mosquito genotypes when fed identical microbiomes. When all sites were analyzed together, the overall bacterial community structure differed between sites and genotypes (PERMANOVA p = 0.001) (S4 Fig). When each microbiome source was analyzed independently, the community structure differed between mosquito genotypes following exposure to microbiomes from Sites 1, 2, and 3, but not between Sites 4 or 5 (Fig 3B).

To evaluate if the mosquito genotype by larval microbiome interactions on adult *Ae*. *aegypti* susceptibility to arboviruses extends to complex microbiomes, the two different mosquito genotypes were each exposed to the same preserved complex microbiomes harvested from larvae from five natural Senegal habitats and challenged with ZIKV. Ingestion of the homogenized larvae from the five sites results in different bacterial community structures in the larvae (Fig 2), indicating that the mosquitoes were exposed to five different complex microbiomes during larval development prior to be being challenged with ZIKV as an adult. The proportion of infected individuals was determined seven days post oral exposure by detecting viral RNA by RT-PCR. The proportion of infected mosquito bodies varied based on the larval microbiome it was exposed to and the mosquito genotype. Specifically, the proportion of the infected bodies was not dependent on the bacterial community (p-value = 0.265) or the mosquito genotype (p-value = 0.392), but was dependent on the specific pairing of larval microbiome and genotype (p-value = 0.017) (Fig 4A). To determine if this infection phenotype extends to dissemination titers, the number of infectious particles was enumerated in the heads, a poxy for saliva [41], seven days post infection by focus forming assay. The titer of ZIKV in the heads was dependent on the bacteria community (p-value = 0.011), but not on the mosquito genotype (p-value = 0.159) or on the interaction between mosquito genotype by larval microbiome interaction (p-value = 0.772) (Fig 4B).

Discussion

In this study, we explored the contribution of mosquito genotype and larval microbiome in driving variation in ZIKV susceptibility. Having previously observed that adult replication of DENV is dependent on the specific bacteria that the mosquito was exposed to during larval development [37], we sought to determine if the effect of larval gnotobiotic treatment on arbovirus susceptibility was mosquito genotype-dependent. We found that the proportion of ZIKV-infected adults is dependent on larval exposure to individual bacterial isolates during larval development and *Ae*. *aegypti* genotype. When exposed to identical complex microbiomes in a gnotobiotic system, the different mosquito genotypes differed in the abundance specific genera and in the bacterial community structure retained. Finally, we observed that the proportion of ZIKV-infected bodies, but not the head titers, was dependent on the specific pairing between larval microbiome and mosquito genotype. Instead, the head titers were dependent only on the larval microbiome. Together these data demonstrate that different genotypes of *Ae*. *aegypti* interact differently with their larval microbiomes and these genotype-dependent interactions have carry-over effects important for ZIKV susceptibility.

In accordance with previously published work [37], we did not see an effect of larval exposure to a single bacterial isolates on the proportion of ZIKV-infected heads. Dickson et al. 2017 [37], only observed an influence of larval exposure to different bacteria on the DENV infectious load in the head, perhaps related to innate immune activation controlling viral replication and dissemination. Here, we did not assay dissemination titers. If done, we might also observe differences in the amount of infectious virus outside the midgut dependent on which bacterial isolate the larvae were exposed to. Nonetheless, we still detect an interaction between mosquito genotype and larval microbiome on the proportion of ZIKV-infected bodies.

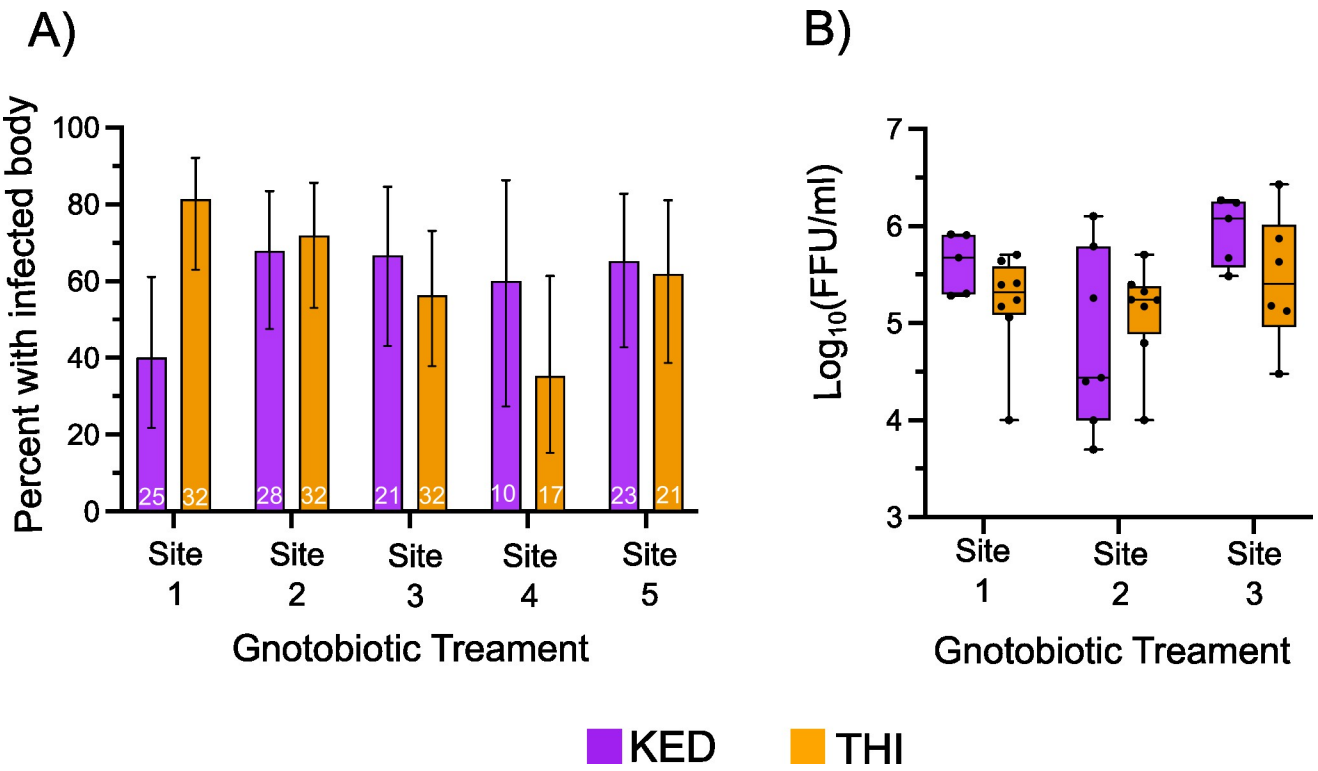

Fig 4. Larval development in different complex microbiomes alters ZIKV infection rates in a mosquito genotype dependent manner. (A) The proportion of blood-fed *Aedes aegypti* females from the KED and THI lines with a ZIKV-positive body 7 days post-infectious blood meal by RT-PCR following larval rearing in complex microbiomes, Site1-5. The y-axis indicates the proportion of ZIKV-infected female bodies and error bars represent the 95% confidence intervals of the proportions. Data were analyzed by binomial logistic regression as a function of bacterial treatment, mosquito genotype, and their interaction (bacterial treatment: p-value = 0.265, mosquito genotype: p-value = 0.392, but was dependent on the specific pairing of bacterial treatment x mosquito genotype: p-value = 0.017. The number of individual mosquitoes is shown within each bar. (B) Boxplot showing the dissemination titers of infectious ZIKV particles expressed as the Log10-transformed number of focus-forming units (FFU) per ml detected in the *Ae. aegypti* head seven days post-infectious blood meal. The points represent individuals and the mean is represented by a horizontal line. The error bars represent the 95% confidence interval. Data were analyzed by Two-way ANOVA as a function of bacterial treatment, mosquito genotype, and their interaction (bacterial treatment: p-value = 0.011, mosquito genotype: p-value = 0.159, bacterial treatment x mosquito genotype: p-value = 0.772). The number of individual mosquitoes assayed is Site 1 Ked: 5, Site 1 THI: 8, Site 2 Ked: 7, Site 2 THI: 8, Site 3 Ked: 5, Site 3 THI: 6, Site 5 Ked: 3, Site 5 THI: 3.

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Interestingly, we did observe that ZIKV infectious loads in the mosquito head was dependent on complex bacterial community that the larvae were exposed to in accordance with Dickson et al. 2017 [37], but no interaction between mosquito genotype and larval microbiome was detected. This suggests that mosquito genotype by larval microbiome interactions only have carry-over effects on infection rates, while the influence of the larval microbiome on the amount of disseminated virus is largely driven by the larval microbiome across mosquito genotypes, perhaps due to conserved differences in specific immune responses across genotypes. The mosquito immune system is known to respond to different microbiomes [32,42]. Furthermore, the results observed in this study could be dependent on the ZIKV isolate used, which originated from Senegal and produces higher infection and dissemination rates than epidemic isolates of ZIKV [43]. Perhaps we could detect an interaction between mosquito genotype and larval microbiome on dissemination titers if we had used a different isolate of ZIKV.

The nutrition status of the mosquito and larval microbiome have previously been shown to influence mosquito fitness [44,45] and susceptibility to arboviruses [37,46–48]. The microbiome is composed of diverse microorganisms that colonize the mosquito's gut, interacting

with the host's metabolic processes [46,49,50] and modulating its innate immune response [36,51–53]. Additionally, recent research has shed light on the critical role of nutrition in determining the outcome of infection in mosquitoes. In particular, lipid metabolism plays a crucial role in the replication and dissemination of arboviruses within the mosquito's body [54–59], while amino acids are involved in the mosquito's interaction with the microbiome [49,60]. Given that the mosquito's nutritional status is strongly influences by its microbiome, and that the nutritional status of the mosquito can influence arbovirus infection, it is not surprising that we observed significant variation in the rates of ZIKV infection when mosquitoes were reared in different bacterial communities.

Additionally, there have been several mosquito genes identified that impact the microbiome composition and gut equilibrium [49,61–64]. These genes can control the overall abundance of the microbiome or specific taxa, through their involvement in bloodmeal digestion and immune factors [61,64]. Moreover, the mosquito microbiome has the potential to influence the expression of particular genes, which can shape the mosquito's immune response and facilitate efficient colonization of specific microbes [63]. Although it remains uncertain which mosquito genes are responsible for the observed phenotypes in this study, it is possible that genetic variation in genes regulating specific bacterial taxa exist between the two genotypes of *Ae*. *aegypti* utilized in this study.

While numerous studies have identified mosquito genes that interact with the microbiome [49,61–64] or have identified bacteria that influence mosquito interactions with arboviruses [32,36,65–71], to the best of our knowledge there are no studies which directly test the interaction between the mosquito larval microbiome and mosquito genotype on arboviruses infection outcome. The importance of host genotype by microbiome interactions on various phenotypes has been demonstrated in other organisms such as *Drosophila* [72,73] and bumble bees (*Bombus terrestris*) [74]. Given the importance of mosquito genotype [75–78] and the importance of the mosquito microbiome arboviruses [32,36,65–71] in driving interactions with pathogens, it is relevant to investigate how these two variable interact to drive variation in mosquito-pathogen interactions.

An important finding of this study is that the different genotypes of *Ae*. *aegypti* larvae harbor different abundances of specific taxa after being fed identical microbiomes. Multiple studies have sought to determine if different genotypes or lines of mosquitoes have the same microbiome when maintained in the same environment. While some studies observed no differences in the microbiome between genotypes of *Ae*. *aegypt*i [79], others observed different microbiomes between lines in the same environment [80] and these changes held up across microbially diverse environments [81]. While it is assumed that the bacteria the mosquitoes are exposed to in the same insectary is the same across lines, this is not absolute and it is very plausible that, during rearing, some larval trays could contain different microbes. By using a gnotobiotic system, we ensured that the different genotypes received the same microbiome in a highly controlled environment. We observed differences in the abundance of specific genera between different genetic backgrounds when fed identical microbiomes. This demonstrates that the mosquito genetic background plays a role in microbiome composition. Other factors that contribute to microbiome composition are the environmentally available bacteria [50], and competition between bacteria [82]. Given that the microbiome is remodeled during the transition from larvae to adult development, it is likely that that adult microbiomes are different than what was provided to larvae, so we cannot conclude that the adult microbiome was different between the two genotypes. Nevertheless, our work further highlights the importance of mosquito genotype or mosquito line in shaping the microbiome.

Even though these microbiomes originated from larvae collected in Senegal, we cannot make any conclusions regarding their field relevance given that we likely partially altered their

composition through preservation and transport compared to larvae in the original habitat. However, how well these microbiomes recapitulated those in nature is not relevant for this study. We simply aimed to show that different complex bacterial communities can impact ZIKV infection in a mosquito genotype-dependent manner, and using microbiomes harvested from larvae in the field is more relevant than ad hoc mixing single bacterial isolates, even if our complex bacterial communities are not identical to those in the field. Perhaps if we seeded our gnotobiotic flasks with the microbiomes from more larvae in accordance with recent studies showing you can transplant and preserve the microbiome [83], we could make conclusions based on the origin of microbiomes.

Two of the single bacterial isolates used in this study (Bacteria A and Bacteria C) belong to the same genus, yet we observed differences in the pupation rate and infection rates between these two bacteria in a mosquito population dependent manner. The influence of these isolates on pupation rate was consistent across mosquito genotypes, but the influence of these isolates on ZIKV infection was the opposite direction across mosquito genotypes. In fact, the influence of these two isolates is likely driving the observed interaction between bacterial isolate and mosquito genotype. Perhaps genetic differences between these closely related bacteria species have variable interactions with different mosquito genotypes which are important for ZIKV infection. This system could provide a highly tractable system to investigate the mechanism of larval microbiome by mosquito interactions on arboviruses susceptibility.

Taken together our results show that different genotypes of *Ae*. *aegypti* interact with their larval microbiome differently to influence ZIKV infection. Future studies should expand on this work to mechanistically identify how different microbiomes influence infection outcomes of the mosquito in a mosquito genotype-dependent manner.

Materials and methods

Bacterial isolation

Mosquito larvae were collected from five sites in two locations (Dixième and Keur Dabo Ndione) in Thiès, Senegal. Thiès is a city in the Northwestern part of Senegal. All larvae were collected from large plastic drums during the dry season of 2021. A pool of 3 larvae from each site was rinsed in sterile 1X PBS (Phosphate-buffered saline), incubated in 70% Ethanol for 5 minutes (min), and then rinsed in sterile 1X PBS three times. Next, they were homogenized in 500 μl sterile 1X PBS and 30% glycerol was added.

A portion of the glycerol stock from Site one containing homogenized larvae was plated on Trypticase Soy Agar (rich media) and incubated for 3 days at 30˚C. Individual colonies were picked from the plates and used to inoculate 3 ml of LB media, which were shaken at 30˚C until bacterial growth occurred (1 OD) and used to create new glycerol stocks of the individual isolates. DNA was extracted from each colony with the QIAGEN Dnaesy blood and tissue kit following the manufacture's protocol. The bacterial DNA was used to amplify the entire 16*S* region by PCR [5′-AGAGTTTGATCCTGGCTCAG-3′ (forward) and 5′-AAGGAGGTGATC-CAGCCGCA-3′ (reverse)] using Expand High-Fidelity Polymerase (Sigma-Aldrich). The PCR products were purified using the QIAquick PCR Purification kit (Qiagen), quantified by NanoDrop (NanoDrop Technologies Inc.), and sequenced by Sanger sequencing (Molecular Genetics Facility at University of Texas Medical Branch). The sequences were aligned and classified at the genus level using the SILVA database (www.arb-silva.de/). A total of 27 isolates were isolated from Site one and 22 were identified taxonomically from (S3 Table). This list does not represent the entire population of bacteria that was isolated. The intended purpose of isolating bacteria from these samples was to have isolates from *Ae*. *aegypti* that colonize the larvae in the field for use in our gnotobiotic assay. We were not intending to fully characterize

the culturable bacteria from each site. Three isolates *Serratia spp*. (Bacterial Isolate A), *Chryseobacterium spp*. (Bacterial Isolate B), and *Serratia spp*. (Bacterial Isolate C). *Chryseobacterium* was chosen for the gnotobiotic assay based off their presence in the larvae after larvae development in the bacterial community from Site One (Fig 3), indicating it was good at colonizing the larvae. The *Serratia* isolates were chosen given previous associations of blocking pathogens in mosquitoes [84,85]. These isolates were not chosen to test any specific hypothesis about the isolates, draw conclusions about the isolates themselves, or to mirror any field relevance.

To standardize the amount of bacteria that would be introduced into the gnotobiotic system, aliquots of equal amounts of bacteria were made. The amount of aliquoted bacteria was then quantified by enumerating colony forming unit (CFU) for each bacterial isolate. To make the aliquots, 200 μl of each bacterial glycerol was added to 200 ml of LB and shaken at 30˚C until bacterial growth occurred, then 50 ml was pelleted by 3000 rpm centrifugation for 15 min. The pellet was washed two times with 50 ml fresh LB broth. After the second wash, the pellet was resuspended in 50 ml of LB and aliquots were made by mixing 500 μl of resuspended bacteria and 500 μl of 50% glycerol to make 1 ml aliquots. To quantify the amount of bacteria in each stock, 10 μl was taken from an aliquot and serially diluted and plated on LB plates. The number of colonies were counted and used to calculate CFU/ml.

Gnotobiotic larvae

To create axenic larvae, *Aedes aegypti* eggs were collected from seventh-generation and eighth-generation laboratory colonies of Thiés (THI) and Kédougou (KED), respectively, derived from natural populations from Thiés, Senegal, and Kédougou, Senegal [7]. Colonies were made by collecting eggs from each colony using ovitraps as described in [7]. Thiés is located in the Northwest part of Senegal and Kedougou is located in the Southeast part of the Senegal. Mosquitoes from these two locations differ in preference for humans and these two locations differ in the degree of urbanization, and the amount of rainfall and genomic data for these lines exist and they represent different genotypes of *Ae. aegypti* [7]. Eggs were gently scrapped off the paper into a 50 ml falcon tube. The eggs were sterilized by incubation in 70% ethanol for 5 min, 3% bleach for 3 min, and 70% ethanol for 5 min. The eggs were then rinsed in distillated (d) sterile water three times and then they were allowed to hatch in 30 ml of d-water in a 50 ml falcon tube with a 0.2 μM filter lid. Upon hatching, as a control, 10–15 axenic larvae were transferred to a sterile 25 cm^2 tissue-culture flask containing 15 ml of d-water and 50 μl of sterile fish food (1 gram ground fish food flakes per 10 ml d water autoclaved for 20 minutes at 121˚C). These axenic larvae were used as an egg-sterilization control and did not develop past the 1st instar larval stage in accordance with previously published work [86]. Gnotobiotic larvae were made by distributing 50 ± 5 (T-75 cm^2 tissue-culture flasks) or 80 ± 20 (T-150 cm^2 tissue-culture flasks) axenic larvae to sterile 75 or 150 cm^2 tissue-culture flasks in duplicate or triplicate containing either 45 or 120 ml of d-sterile water and 1 ml of sterile fish food. For the single isolate, 5 x10 ^{5} CFUs/ml of washed-bacteria (for details go to bacteria growth section) was added to each flask. For each bacterial isolate tested, three replicate flasks were used. A total of two independent experiments was performed. To make gnotobiotic larvae with complex microbiomes, equal amounts of a single glycerol stocks from each collection site (Sites 1–5) were added to each of two duplicate T-150 flasks. By adding a homogeneous mixture from a single tube, each flask and mosquito genotype is receiving the same bacterial inoculum. Data represents one experimental replicate due to availability of field material. Control and gnotobiotic larvae were maintained on 50 (T-75 flask) and 500 (T-150 flask) μl sterile fish food every other day, respectively. Bacterial treatments were added immediately following hatching at the L1 stage. Following pupation and eclosion, adults were maintained under standard

insectary conditions and allowed to be colonized by environmental bacteria. This was done because we are measuring the carry-over effects of the larval microbiome and wanted the adult microbiome to be seeded under standard insectary conditions.

16S and metagenomic analysis

To characterize the microbiome of the larvae developing in the field sites prior to use in the gnotobiotic assay, larvae were collected and surface sterilized in 70% ethanol for 5 min and rinsed 3 times in sterile dwater before placing in RNALater (Qiagen) and frozen at -80˚C and transported to UTMB. DNA was extracted by placing individual larvae into 2 ml tube containing a 5mm grinding bead and homogenized for 3 minutes at a 30Hz/s frequency in a TissueLyser II grinder (Qiagen). DNA extraction of individual larvae was carried out using the QIAamp DNA Kit (Qiagen, Germany) following the manufacturer's protocol. Larvae for initial 16S characterization and for use in the gnotobiotic assay were collected and processed at the same time.

To characterize the microbiome in the gnotobiotic larvae seeded with the complex microbiomes, eight individual L3 larvae were collected from each treatment flask and transferred to a 96-well cell culture plate. The larvae were surface sterilized in 70% ethanol for 5 min and rinsed 3 times in sterile dwater. Next, individual larvae were transferred to 2 ml tubes containing a 5mm grinding bead and placed in the -80˚C freezer until DNA was extracted. Individual mosquitoes were homogenized for 3 minutes at a 30Hz/s frequency in a TissueLyser II grinder (Qiagen). DNA extraction of individual larvae was carried out using the QIAamp DNA Kit (Qiagen, Germany) following the manufacturer's protocol. No-mosquito controls were used for each extraction batch and included in the sequencing run.

Sequencing libraries for each isolate were generated using universal 16S rRNA V3-V4 region primers [87] in accordance with Illumina 16S rRNA metagenomic sequencing library protocols. DNA concentrations of each library were determined by Qubit and equal amounts of DNA from each barcoded library were pooled prior to sequencing. The samples were barcoded for multiplexing using Nextera XT Index Kit v2. The pooled libraries were diluted to 4 pM and run on the Illumina Miseq using a MiSeq Reagent Kit v2 (500-cycles).

To identify known bacteria, sequences were analyzed using the CLC Genomics Workbench 21.0.5 Microbial Genomics Module (CLC MGM). Reads containing nucleotides below the quality threshold of 0.05 (using the modified Richard Mott algorithm) and those with two or more unknown nucleotides or sequencing adapters were trimmed out. Reference-based Operational Taxonomic Unit (OTU) picking was performed using the SILVA SSU v132 97% database [88]. Sequences present in more than one copy but not clustered to the database were placed into de novo OTUs (97% similarity) and aligned against the reference database with an 80% similarity threshold to assign the "closest" taxonomical name where possible. Chimeras were removed from the dataset if the absolute crossover cost was three using a k-mer size of six. OTUs with a combined abundance of less than two were removed from the analysis. Low abundance OTUs were removed from the analysis if their combined abundance was below 10 or 0.1% of reads. The number of reads per sample used in the analysis ranged from 13,214–111,218. Only reads that mapped to bacteria were kept. Taxa classified as "Ambiguous Taxa" are reads mapping to bacterial DNA, but that cannot be identified at the taxonomic level.

Pairwise differential abundance of specific genera was done in MicrobiomeAnalyst. Statistical significance between groups was determined by T-test/ANOVA and corrected for multiple testing. Presented p-values reflect correction for multiple testing. (S2 Table).

Abundance profiling was performed using MicrobiomeAnalyst [89,90]. The analysis parameters were set so that OTUs had to have a count of at least 10 in 20% of the samples and

above 10% inter-quantile range. Analysis was performed using actual and total sum scale abundances. Alpha diversity was measured using the observed features to identify the community richness using Chao1. Statistical significance was calculated using T-test/ANOVA. Beta diversity was calculated using the Bray-Curtis dissimilarity measure (genus level). Permutational Multivariate Analysis of Variance (PERMANOVA) analysis was used to measure effect size and significance on beta diversity for grouping variables [91]. Relative abundance analysis was done in MicrobiomeAnalyst at the level of genera.

Out of 80 individual larvae sequenced, a total of 1768 OTUs were identified. After filtering, 92 OTUs remain which represent 30 genera. Sequences from three individuals were removed from the analysis because they did not achieve enough reads, one from Site 1, 4, and 5. After removing OTUs belonging to the negative control, 87 OTUs remained. These final 87 OTUs were used for the analysis in the MicrobiomeAnalyst.

Pupation rate

Pupae were counted from the onset of pupation (Day 5) until a majority of the larvae pupated (Day 10) in the same triplicate flasks used for the adult viral challenge assays. Larvae that did not pupate were counted and considered as total amount of individuals. To determine the rate of pupation, the percent pupae was determined at each day by dividing the number of pupae by the total number of individuals. Data from two independent experiments was used, each with three internal replicates (three replicate flasks). Graphpad (Version 8) was used to generate a simple logistic regression that computed the day that 50% of larvae pupated (PD_{50}). An ANOVA was run on the summary statistics of the PD_{50} generated from the logistic regression to determine if the PD_{50} was dependent on the bacterial isolate, the mosquito genotype, or an interaction between the two. Multiple comparison by-two-way-ANOVAs were performed to compare the mean PD_{50} between populations (Sidak's test), and between each of three different bacterial treatments within population (Tukey's test) and between each bacterium in the two populations (Sidak's test). Data are a summary of two biological experiments done each time in triplicates. The number of larvae used per experiment along with statistical information associated with each comparison are listed in S1 Table.

Mosquito infections

Mosquito infection assays was conducted using the ZIKV DAKAR 41524 isolate received from the World Reference Center for Emerging Viruses and Arboviruses at UTMB. After pupation, pupae were transferred to a 1-pint carton box with netting and 10% sucrose solution until adult emergence. After adult emergence, 4 to 5-day-old females were sorted and transferred into a new cup with netting and deprived of sucrose solution for 24 hours and transferred to an Arthropod Containment level 2 facility (ACL-2). Females were offered an artificial blood meal for 15 minutes using the Hemotek system with de-salted pig intestine as the membrane. The infectious blood meal consisted of a 2:1 mixture of defibrinated sheep blood (Colorado Serum Company) and virus at a final concentration of 1.49×10^7 focus-forming units (FFU)/ml. The blood meal was supplemented with 10 mM adenosine triphosphate (ATP). Prior to addition to the blood, sodium bicarbonate was mixed with the virus stock at 1% final concentration. Following exposure to an infectious bloodmeal, fully engorged females were sorted into 1-pint carton boxes with *ad libidum* access to 10% sucrose solution and kept in an incubator under controlled conditions (28˚C, 12h:12h light: dark cycle). After 7 days of incubation, the head and body of ZIKV-exposed mosquitoes were separated to determine infection rate (the proportion of blood-fed mosquitoes with ZIKV-positive body) and dissemination titer (the amount of virus in the head tissues of ZIKV-infected mosquitoes). To determine the infection rate, female

bodies were homogenized in 200 μl of a crude RNA extraction buffer (10 mM Tris HCl, 50 mM NaCl, 1.25 mM EDTA, fresh 0.35 g/L proteinase K) during two rounds of 3 minutes at a 30Hz/s frequency in a TissueLyser II grinder (Qiagen). Total RNA was converted into complementary DNA (cDNA) using M-MLV reverse transcriptase (Invitrogen) and random hexamers, the reaction was carried out as follows: 10 min at 25˚C, 50 min at 37˚C, and 15 min 70˚C. The cDNA was amplified by PCR carried out in a 25μl reaction containing 12.5μl of 1x DreamTaq DNA polymerase (Thermo Fisher Scientific) and 10 μM of each ZIKV primer (forward: 5'-GTATGGAATGGAGATAAGGCCCA-3', and reverse: 5'-ACCAGCACTGCCATTGATG TGC-3'). Cycling conditions were as follow, 2 min at 95˚C, followed by 35 cycles of 30s at 95˚C, 30s at 60˚C, and 30s at 72˚C with a final extension step of 7 min at 72˚C. Amplicons were visualized on a 2% agarose gel. The proportion of ZIKV-infected females was analyzed by binomial logistic regression as a function of treatment, colony, and their interaction in R.

To determine the dissemination titer, the heads of females with positive ZIKV-infected bodies were titrated by focus-forming assay in Vero cells. Only three sites were used due to low sample sizes in the other treatments. Heads were homogenized individually in 200 μl of Vero cell media (DMEM 1X) supplemented with 2% heat inactivated fetal bovine serum (FBS) and 1X Antibiotic-Antimycotic (Life Technologies) for 3 minutes at a 30Hz/s frequency in a TissueLyser II grinder (Qiagen). Vero cells were seeded in 24-well plates and incubated for 24 hours to reach confluency. Each well was inoculated with 200 μl of head homogenate in 10-fold dilutions (from 10^1 to 10^6) and incubated at 37˚C (5% CO^2) for 1 hour, rocking every 15 minutes. Infected cells were overlaid with α-MEM media supplemented with 1.25% carboxymethyl cellulose, 5% FBS, and 1% Pen-Strep. After three days of incubation at 37˚C, infected cells were fixed with 10% formalin for at least 1 hour and cells were washed three times in 1X PBS. Approximately 500 μl of blocking solution (5% w/v non-fat powdered milk in 1X PBS) was added to each well and the plates were placed on the plate rocker for 30 minutes. The blocking solution was discarded and 200 μl of primary antibody (obtained from the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA) at UTMB) (ZIKV antibody diluted 1:1000 in blocking solution) was added to each well and plates were placed on plate rocker overnight. The primary antibody solution was discarded, and plates were washed three times with 1X PBS again, and 200 μl of secondary antibody (peroxidase-labeled goat anti-mouse IgG human serum KPL-474-1806) solution (secondary antibody diluted 1:2000 in blocking solution) was added to each well. Plates were placed on plate rocker for 1 hour. The secondary antibody solution was discarded, and plates were washed three times with 1X PBS. To develop visible foci, 100μl of TrueBlue peroxidase substrate (KPL 5510–0050) was added to each well, and plates were placed on the plate rocker until foci could be seen, around 10 min. Plates were washed with deionized water and FFU was counted with the help of a light. Focus-forming units were Log_{10} transformed to represent the concentration of infectious ZIKV particles detected in *Ae*. *aegypti* heads. Head titer data were analyzed by two-way ANOVA as a function of bacterial treatment, mosquito genotype, and their interaction in R. Infection rate data was analyzed by a two-way ANOVA on a binomial logistic regress in R.

Supporting information

S1 Fig. The bacterial community structure differs between the larvae in the field collection sites. Structure of bacterial communities was determined by deep sequencing the V3-V4 region of the 16S gene in individual larvae collected from large metal drums at five sites in Senegal (Site1-Site5). Bacterial structure is represented by PCoA of a Bray-Curtis dissimilarity matrix based on mean genera abundance (PERMANOVA p = 0.001).
(TIF)

S2 Fig. Rarefaction curves showing the sequencing depth of each library. The number of species is shown on the Y axis, and the number of sequencing reads is shown on the X axis.
(TIF)

S3 Fig. The percent abundance of the top 20 most abundant genera is plotted by larval treatment (Site1-5) and mosquito line (KED or THI) and separated out by individual.
(TIF)

S4 Fig. Beta diversity metrics for the *Ae. aegypti* lines in each bacterial treatment. The dissimilarities between the two different lines of *Ae. aegypti* (KED and THI) in each of the five different larval microbiomes was analyzed by principal component analysis of Bray-Curtis dissimilarity index (PERMANOVA, p = 0.001).
(TIF)

S1 Table. Summary of statistics for pupation rate.
(XLSX)

S2 Table. The log_2fold change of bacteria genera with significant pairwise differences between KED and THI is plotted. Pairwise differential analysis was performed between larvae from the KED or THI after receiving identical complex bacterial communities.
(XLSX)

S3 Table. ID of the 22 bacterial isolates sequenced for use in the gnotobiotic assay.
(XLSX)

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