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 influence 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 show that similarities in bacterial community structures when given identical microbiomes between different genetic backgrounds of *Ae. aegypti* was dependent on the source microbiome, 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 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.

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] and 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] and the ability of *Ae. aegypti* to be an efficient vector of arboviruses is a partially genetically controlled trait [9]. Variation in vector competence has been widely documented among different genotypes of *Ae. aegypti* [10-12]. In some cases, vector competence is dependent on the specific mosquito-virus interactions [13-15].

Additionally, abiotic (non-living) environmental factors [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 human 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 human artificial containers such as discarded buckets, cans, or tires 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 environment and is critical for establishing the nutritional status of the mosquito [38]. Importantly, interactions between *Ae. aegypti* larvae and different bacterial isolates 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 is remains unknown.

Here we expand on previous work [37] to determine if the impact of the larval microbiome on arboviruses susceptibility is dependent on mosquito genotype. Using single bacterial isolates, we demonstrate that the influence of specific bacterial isolates on adults ZIKV susceptibility 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 the larval microbiome and that ZIKV susceptibility is dependent on the specific pairing between *Ae. aegypti* genotype and complex microbiome. Our results provide empirical evidence that *Ae. aegypti* genotype by environment interactions drive variation in ZIKV susceptibility. As the earth becomes warmer and drier and de-forestation and urbanization increase, *Ae. aegypti* will 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 breeding sites will be forced to adapt and oviposit in urban artificial container types. Understanding how climate driven changes to the environment will increasingly shape the ability of *Ae. aegypti* to transmit important human pathogens is required to guide the development and deployment of control strategies.

Results

Single bacterial Isolates

Previously we observed that larval development in the presence of different single isolates alters DENV replication in *Ae. aegypti* [37], but it remains unknown if the influence of larval development in the presence of different single bacterial isolates on arbovirus infection is consistent across *Ae. aegypti* genotypes. To address this, we isolated bacteria colonies from larvae collected in Thiés, Senegal. A total of 64 bacteria were isolated from larvae homogenized in 1X PBS originating from a collection site in Thies. Three bacteria isolates were chosen (*Serratia spp.* (Bacterial Isolate A), *Chyrseobacterim spp.* (Bacterial Isolate B), and *Serratia spp.* (Bacterial Isolate C)) for further characterization (see Material and Methods for information on bacteria selection). To understand if larval development in the presence of 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 amounts of bacteria isolates in gnotobiotic system and the proportion of pupae was recorded each day. Pupation rates were measured in triplicate gnotobiotic flask daily from the onset of pupation (day five post hatching) until day 10. Overall, the THI line of *Ae. aegypti* pupated slower than the KED line (p. value < 0.0001). In both lines of *Ae. aegypti*, the time it took to reach 50% pupae (PD_{50}) was different between all three isolates (Figure 1A) (Supplemental Table 1). In both lines the bacterial isolate that had 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 isolated that resulted in quickest pupation rate was Bacterial Isolate B where it took 6.9 days 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

105 observed that the PD_{50} was dependent on the mosquito genotype, bacterial isolate, or
106 the interaction between the two variables (ANOVA on a general linearized model:
107 mosquito genotype: $p < 0.0001$, bacterial isolate: $p < 0.0001$, mosquito genotype x
108 bacterial isolate: $p < 0.0001$).
109
110 To determine if larval development in the presence of different single isolates influences
111 ZIKV infection rates in a mosquito genotype dependent manner, the THI and KED line
112 were exposed to ZIKV following larval development in Bacteria A, Bacteria B, or Bacteria
113 C. Seven days post exposure to ZIKV, infection rates were determined by detection of
114 viral RNA by RT-PCR. Infection rates ranged from 20-50% and was not dependent on
115 the bacteria isolate (p-value = 0.614) or the mosquito genotype (p-value = 0.372), but
116 was dependent on the interaction between the bacteria isolate and the mosquito
117 genotype (p-value = 0.010) (Figure 1B).
118
119 **Complex microbiomes**
120 To establish if the effect of the interaction between mosquito genotype and bacteria
121 isolate on Zika virus infection can be extended to complex microbiomes collected from
122 larvae in the field, whole microbiomes from the larvae pools collected in Thiés, Senegal
123 were introduced to both genotypes of *Ae. aegypti.* We first characterized the bacterial
124 communities by 16S amplicon sequencing. Out of 80 individual larvae sequenced, a total
125 of 87 OTUs were found which represent 30 genera after filtering for low abundance
126 OTUs and OTUs present in the negative controls. Rarefaction curves (Supplemental
127 Figure 1) show that sufficient sequencing depth was achieved. Principal component
128 analysis (PCA) was performed on a Bray-Curtis dissimilarity matrix on the larvae from
129 the same sites to test if the bacterial communities from each collection site that were
130 introduced to the larvae were in fact different. The community structure differed between

131 all five of the bacterial communities (Figure 2) (PERMANOVA, p-value = 0.001). The
132 bacterial community from Site 1 was the most different from the other sites, and Sites 2-
133 5 showed greater similarity.
134
135 To assess if the two genotypes of *Ae. aegypti* acquire and maintain different bacterial
136 taxa after rearing in an identical bacterial community in a gnotobiotic system, the percent
137 abundance of different genera was compared between genotypes and collection sites.
138 The abundance of the top 20 most prevalent genera differed in both genotypes between
139 the collection sites (Figure 3A). Gnotobiotic larvae harboring the bacterial community
140 from Sites four or five are most similar between the genotypes. Gnotobiotic larvae
141 harboring the bacterial community from Sites one, two, and three are most different
142 between the genotypes. Abundance of specific genera was consistent between
143 individuals from each treatment (Supplemental Figure 2). To determine if there are
144 specific genera that are differentially abundant between genotypes across all the
145 collection sites in the gnotobiotic system, pairwise differential abundances of each genus
146 were compared between mosquito genotypes. Of the 30 genera identified in this study,
147 nine genera were more abundant in the genotype from THI, and seven genera were
148 more abundant in the genotype from KED (Figure 3B). Additionally, the overall bacterial
149 community structure differed between mosquito genotypes when fed identical
150 microbiomes. When all sites were analyzed together the overall bacterial community
151 structure differed between sites and genotypes (PERMANOVA p=0.001) (Supplemental
152 Figure 3). When each microbiome source was analyzed independently, the community
153 structure differed between microbiomes from Sites 1, 2, and 3, but not Sites 4, or 5
154 (Supplemental Figure 4).
155

156 To evaluate if the mosquito genotype by larval microbiome interactions on adult
157 susceptibility to arboviruses extends to complex microbiomes, the two different
158 genotypes of *Ae. aegypti* were reared in preserved complex microbiomes harvested
159 from larvae in natural breeding sites in Senegal and challenged with ZIKV. The
160 proportion of infected individuals was determined seven days post exposure by detecting
161 viral RNA by RT-PCR. The proportion of infected bodies varied based on the larval
162 microbiome in which it developed and the mosquito genotype. Specifically, the
163 proportion of the infected bodies was not dependent on the bacterial community (p-value
164 = 0.265) or the mosquito genotype (p-value = 0.392), but was dependent on the specific
165 pairing of larval microbiome and genotype (p-value = 0.017) (Figure 4A). To determine if
166 this phenotype extends to dissemination titers, the number of infectious particles was
167 enumerated in the heads seven days post infection by focus forming assay. The titer of
168 ZIKV in the heads was dependent on the bacteria community (p-value = 0.0415), but
169 was not dependent on the mosquito genotype (p-value = 0.057) or on the interaction
170 between mosquito genotype by larval microbiome interaction (p-value = 0.538) (Figure
171 4B).
172
173 **Discussion**
174 In this study, we explored the contribution of mosquito genotype and larval microbiome in
175 driving variation in ZIKV susceptibility. Having previously observed that adult replication of
176 DENV was dependent on the specific bacteria the mosquito was exposed to during larval
177 development [37], we sought to determine if the effect of larval gnotobiotic treatment on
178 arbovirus susceptibility was mosquito genotype dependent. We found that proportion of ZIKV
179 infected adults is dependent on the interaction between a single bacteria isolate during larval
180 development and mosquito genotype. When reared in identical complex microbiomes in a
181 gnotobiotic system, the mosquito genotypes differed in the abundance specific genera. Finally,

182 we observed that the proportion of infected bodies, but not the head titers, was dependent on
183 the specific pairing between larval microbiome and mosquito genotype. Instead, the head titers
184 were dependent on the larval microbiome. Together these data demonstrate that different
185 genotypes of *Ae. aegypti* interact differently with their larval microbiome and these genotype
186 dependent interactions are important for ZIKV susceptibility.
187
188 In accordance with previously published work [37], we did not see an effect of larval
189 development in single bacterial isolates on the proportion of infected bodies. In Dickson et al.
190 2017 [37], the influence of larval development in different bacteria was only observed in the
191 amount of infectious DENV particles in the head. In the current study, we did not assay
192 dissemination titers. If done, we might also observe differences in the amount of infectious virus
193 outside the midgut dependent on which bacterial isolate the larvae developed in. None-the-less,
194 we still detect an interaction between mosquito genotype and larval microbiome on the
195 proportion of ZIKV infected bodies. Interestingly, we did observe that the amount of infectious
196 ZIKV in the head was dependent on complex bacterial community that the larvae were exposed
197 to in accordance with Dickson et al. 2017 [37], but no interaction between mosquito genotype
198 and larval microbiome was detected. This might suggest that mosquito genotype by larval
199 microbiome interactions are only important for infection rates, while the influence of the larval
200 microbiome on the amount of disseminated virus is largely driven by the larval microbiome
201 across mosquito genotypes.
202
203 The nutrition status and larval microbiome have previously been shown to influence mosquito
204 fitness [41, 42] and susceptibility to arboviruses [37, 38, 43, 44]. The microbiome is composed
205 of diverse microorganisms that colonize the mosquito's gut, interacting with the host's metabolic
206 processes [38, 45, 46] and modulating its immune response [36, 47-49]. Additionally, recent
207 research has shed light on the critical role of nutrition in determining the outcome of infection in

208 mosquitoes. In particular, lipid metabolism plays a crucial role in the replication and
209 dissemination of arboviruses within the mosquito's body [50-55], while amino acids are involved
210 in the mosquito's interaction with the microbiome [45, 56]. Given that the mosquito's nutritional
211 status is strongly influences by its microbiome, and that the nutritional status of the mosquito
212 can influence arbovirus infection, it is not surprising that we observe significant variation in the
213 rates of ZIKV infection when mosquitoes are reared in different bacterial communities.
214
215 Additionally, there have been several mosquito genes identified that impact the microbiome
216 composition and gut equilibrium [45, 57-60]. These genes can control the overall abundance of
217 the microbiome or specific taxa, through their involvement in bloodmeal digestion and immune
218 factors [57] [60]. Moreover, the mosquito microbiome has the potential to influence the
219 expression of particular genes, which can shape the mosquito's immune response and facilitate
220 efficient colonization of specific microbes [59]. Although it remains uncertain which mosquito
221 genes are responsible for the observed phenotypes, it is possible that genetic variation in genes
222 regulating specific bacterial taxa exist between the two genotypes of *Ae. aegypti* utilized in this
223 study.
224
225 An important finding of this study is that the different genotypes of *Ae. aegypti* larvae harbor a
226 different abundance of specific taxa when fed identical microbiomes. Multiple studies have
227 sought to determine if different genotypes or lines of mosquitoes have the same microbiome
228 when maintained in the same environment. While some studies observed no differences
229 between the microbiome between genotypes of *Ae. aegypt*i [61], other studies observe different
230 microbiomes between lines in the same environment [62] and these changes hold up across
231 microbially diverse environments [63]. While it is assumed that the bacteria the mosquitoes are
232 exposed to in the same insectary is the same across lines, this is not absolute and it is very
233 plausible that, during rearing, some larval trays could contain different microbes. By using a

234 gnotobiotic system, we are ensuring that the different genotypes are receiving the same
235 microbiome in a highly controlled environment. We observed differences in the abundance of
236 specific genera between different genetic backgrounds when fed identical microbiomes. This
237 demonstrates that the mosquito genetic background plays a role in microbiome composition.
238 Other factors that contribute to microbiome composition are the environmentally available
239 bacteria [46], and competition between bacteria [64]. Given that the microbiome is remodeled
240 from larvae to adult development it is likely that that adult microbiomes are different than what is
241 reported here and we cannot conclude that the adult microbiome is different between the two
242 genotypes. Nevertheless, this work further highlights the importance of mosquito genotype or
243 mosquito line in shaping the microbiome.
244
245 The results observed in this study could be dependent on the ZIKV isolate used. The isolate of
246 ZIKV originated from Senegal and has higher infection and dissemination rates than epidemic
247 isolates of ZIKV [65]. Perhaps we could detect an interaction between mosquito genotype and
248 larval microbiome on dissemination titers if we had used a different isolate of ZIKV.
249
250 Even though these microbiomes originated from larvae collected in Senegal, we cannot make
251 any conclusions of their field relevance given that we likely partially altered the composition of
252 the microbiomes compared to larvae in the original habitat through preservation and transport.
253 How well the microbiomes recapitulate microbiomes in nature is not relevant for this study. We
254 simply aim to show that different complex bacterial communities can impact ZIKV infection in a
255 mosquito genotype dependent manner and using microbiomes harvested from larvae in the field
256 is more relevant than ad hoc mixing single bacterial isolates, even if our complex bacterial
257 communities are not identical to those in the field. Perhaps if we seeded our gnotobiotic flasks
258 with the microbiomes from more larvae in accordance with recent studies showing you can

259 transplant and preserve the microbiome [66], we could make conclusions based on the origin of
260 microbiomes.
261
262 Two of the single bacterial isolates used in this study (Bacteria A and Bacteria C) belong to the
263 same genus, yet we observed differences in the pupation rate and infection rates between these
264 two bacteria. The influence of these isolates on pupation rate is consistent across mosquito
265 genotypes, but the influence of these isolates on ZIKV infection is the opposite direction across
266 mosquito genotypes. In fact, the influence of these two isolates is likely driving the observed
267 interaction between bacterial isolate and mosquito genotype. Perhaps genetic differences
268 between these closely related bacteria species have variable interactions with different
269 mosquito genotypes which are important for ZIKV infection. This system could provide a highly
270 tractable system to investigate the mechanism of larval microbiome by mosquito interactions on
271 arboviruses susceptibility.
272
273 Taken together these results show that different genotypes of *Ae. aegypti* interact with their
274 environment differently to influence ZIKV infection. Future studies should expand on this work to
275 identify how different microbiomes influence the nutrition status of the mosquito in a mosquito
276 genotype dependent manner.
277
278 **Materials and Methods**
279 **Bacterial Isolation**
280 Mosquito larvae were collected in two sites (Dixième and Keur Dabo Ndione) in Thiès, Senegal
281 from plastic drums. A pool of 3 larvae from each site was rinsed in sterile 1X PBS (Phosphate-
282 buffered saline), incubated in 70% Ethanol for 5 minute (min), and then rinsed in sterile 1X PBS
283 three times. Next, they were homogenized in 500 µl sterile 1X PBS and had 30% glycerol
284 added.

285

286 A portion of the glycerol stock containing homogenized larvae was plated on Trypticase Soy
287 Agar (rich media) and incubated for 3 days at 30 °C. Individual colonies were picked from the
288 plates and used to inoculate 3 ml of LB media, which were shaken at 30°C until bacterial growth
289 occurred (1 OD) and used to create new glycerol stocks of the individual isolates. DNA was
290 extracted from each colony with the QIAGEN Dnaesy blood and tissue kit following the
291 manufacture's protocol. The bacterial DNA was used to amplify the entire 16*S* region by PCR
292 [5′-AGAGTTTGATCCTGGCTCAG-3′ (forward) and 5′-AAGGAGGTGATCCAGCCGCA-3′
293 (reverse)] using Expand High-Fidelity Polymerase (Sigma-Aldrich). The PCR products were
294 purified using the QIAquick PCR Purification kit (Qiagen), quantified by NanoDrop (NanoDrop
295 Technologies Inc.), and sequenced by Sanger sequencing (Molecular Genetics Facility at
296 University of Texas Medical Branch). The sequences were aligned and classified at the genus
297 level using the SILVA database (www.arb-silva.de/). To standardize the amount of bacteria that
298 would be introduced into the gnotobiotic system, aliquots of equal amounts of bacteria were
299 made. The amount of aliquoted bacteria was then quantified by enumerating colony forming unit
300 (CFU) for each bacterial isolate. To make the aliquots, 200 μl of each bacterial glycerol was
301 disposed in 200 ml of LB and shaken at 30°C until bacterial growth occurred, then 50 ml was
302 pelleted by 3000 rpm centrifugation for 15 min. The pellet was washed two times with 50 ml
303 fresh LB broth. After the second wash, the pellet was resuspended in 50 ml of LB and aliquots
304 were made by mixing 500 μl of resuspended bacteria an 500 μl of 50% glycerol to make 1ml
305 aliquots. To quantify the amount of bacteria in each stock, 10 μl was taken from an aliquot and
306 serially diluted and plated on LB plates. The number of colonies were counted and the number
307 of CFU/ml were calculated.

308

309 **Gnotobiotic larvae**

310 To create axenic larvae, *Aedes aegypti* eggs were collected from seventh-generation and
311 eighth-generation laboratory colonies of Thiés (THI) and Kédougou (KED), respectively, derived
312 from natural populations from Thiés, Senegal, and Kédougou, Senegal [7]. Genomic data for
313 these lines exist and they represent different genotypes of *Ae. aegypti* [7]. Eggs were gently
314 scrapped off the paper into a 50 ml falcon tube. The eggs were sterilized by incubation in 70%
315 ethanol for 5 min, 3% bleach for 3 min, and 70% ethanol for 5 min. The eggs were then rinsed in
316 distillated (d) sterile water three times and then they were allowed to hatch in 30 ml of d-water in
317 a 50ml falcon tube with a 0.2 μM filter lid. Upon hatching, as a control, 10-15 axenic larvae
318 were transferred to a sterile 25 cm^2 tissue-culture flask containing 15ml of d-water and 50 μl of
319 sterile fish food (1 g ground fish food flakes per 10 ml d water autoclaved for 20 minutes at 121
320 °C). These axenic larvae were used as an egg-sterilization control and did not develop past the
321 1st instar larval stage in accordance with previously published work [67]. Gnotobiotic larvae were
322 made by distributing 50 $\pm$ 5 (T-75 cm^2 tissue-culture flasks) or 80 $\pm$ 20 (T-150 cm^2 tissue-
323 culture flasks) axenic larvae to sterile 75 or 150 cm^2 tissue-culture flasks in duplicate or
324 triplicate containing either 45 or 120 ml of d-sterile water and 1 ml of sterile fish food. For the
325 single isolate, 5 x10^5 CFUs/ml of washed-bacteria (for details go to bacteria growth section)
326 was added to each flask. For each bacterial isolate tested, three replicate flasks were used. A
327 total of three independent experiments was performed. To make gnotobiotic larvae with
328 complex microbiomes, equal amounts of the glycerol stocks from each collection site (Sites 1-5)
329 were added to each of two duplicate T-150 flasks. Data represents one experimental replicate
330 due to availability of field material. Control and gnotobiotic larvae were maintained on 50 (T-75
331 flask) and 500 (T-150 flask) μl sterile fish food every other day, respectively. Bacterial isolates
332 were chosen based on the presence of the corresponding genera in the 16S amplicon
333 sequencing dataset.
334

335 **16S and metagenomic analysis**

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

345

346 Sequencing libraries for each isolate were generated using universal 16S rRNA V3-V4 region
347 primers [68] in accordance with Illumina 16S rRNA metagenomic sequencing library protocols.
348 DNA concentrations of each library were determined by Qubit and equal amounts of DNA from
349 each barcoded library were pooled prior to sequencing. The samples were barcoded for
350 multiplexing using Nextera XT Index Kit v2. The pooled libraries were diluted to 4 pM and run on
351 the Illumina Miseq using a MiSeq Reagent Kit v2 (500-cycles).

352

353 To identify known bacteria, sequences were analyzed using the CLC Genomics Workbench
354 21.0.5 Microbial Genomics Module (CLC MGM). Reads containing nucleotides below the quality
355 threshold of 0.05 (using the modified Richard Mott algorithm) and those with two or more
356 unknown nucleotides or sequencing adapters were trimmed out. Reference-based Operational
357 Taxonomic Unit (OTU) picking was performed using the SILVA SSU v132 97% database [69].
358 Sequences present in more than one copy but not clustered to the database were placed into
359 de novo OTUs (97% similarity) and aligned against the reference database with an 80%
360 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. Differential abundance analysis was performed using CLC MGM at the genus level to compare the differences between the groups using trimmed mean of M-values. Each OTU was modeled as a separate generalized linear model, where it is assumed that abundances follow a negative binomial distribution. The Wald test was used to determine significance between groups. Multiple comparisons were corrected for with Bonferroni correction. Tables of differentially abundant taxa are on the complete unfiltered data set (Supplemental Tables 2).

Abundance profiling was performed using MicrobiomeAnalyst [70, 71]. 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 [72]. 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 6. After removing OTUs belonging to the negative control, 87 OTUs remained. These final 87 OTUs were used for the analysis in the MicrobiomeAnalyst.

387

388 **Pupal development**

389 To determine the rate of pupation, pupae were counted from the onset of pupation (Day 5) until
390 pupation finished (Day 10) in the same triplicate flasks used for the adult viral challenge assays.
391 The leftover larvae were counted and considered as total amount of individuals for the statistical
392 analysis. For the pupal development, data from two independent experiments was used, each
393 with three internal replicates (three replicate flasks). Graphpad was used to generate a simple
394 logistic regression that computed the day that 50% of larvae pupated (PD_{50}). An ANOVA was
395 run on the summary statistics of the PD_{50} generated from the logistic regression to determine if
396 the PD_{50} was dependent on the bacterial isolate, the mosquito genotype, or an interaction
397 between the two. Multiple comparison by-two-way-ANOVAs were performed to compare the
398 mean PD_{50} between populations (Sidak's test), and between each of three different bacterial
399 treatments within population (Tukey's test) and between each bacterium in the two populations
400 (Sidak's test). Data are a summary of two biological experiments done each time in triplicates.
401 The number of larvae used per experiment along with statistical information associated with
402 each comparison are listed in Supplementary Table 1.

403

404 **Mosquito infections**

405 Mosquito infection assays was conducted using the ZIKV DAKAR 41524 isolate received from
406 the World Reference Center for Emerging Viruses and Arboviruses at UTMB. After pupation,
407 pupae were transferred to a 1-pint carton box with netting and 10% sucrose solution until adult
408 emergence. After adult emergence, 4 to 5-day-old females were sorted and transferred into a
409 new cup with netting and deprived of sucrose solution for 24 hours and transferred to an
410 Arthropod Containment level 2 facility (ACL-2). Females were offered an artificial blood meal for
411 15 minutes using the Hemotek system with de-salted pig intestine as the membrane. The
412 infectious blood meal consisted of a 2:1 mixture of defibrinated sheep blood (Colorado Serum

413 Company) and virus at a final concentration of 1.49 x 10^7 focus-forming units (FFU)/ml. The
414 blood meal was supplemented with 10 mM adenosine triphosphate (ATP). Prior to addition to
415 the blood, sodium bicarbonate was mixed with the virus stock at 1% final concentration.
416 Following exposure to an infectious bloodmeal, fully engorged females were sorted into 1-pint
417 carton boxes with *ad libidum* access to 10% sucrose solution and kept in an incubator under
418 controlled conditions (28°C, 12h:12h light: dark cycle). After 7 days of incubation, the head and
419 body of ZIKV-exposed mosquitoes were separated to determine infection rate (the proportion of
420 blood-fed mosquitoes with ZIKV-positive body) and dissemination titer (the amount of virus in
421 the head tissues of ZIKV-infected mosquitoes). To determine the infection rate, female bodies
422 were homogenized in 200 µl of a crude RNA extraction buffer (10 mM Tris HCl, 50 mM NaCl,
423 1.25 mM EDTA, fresh 0.35 g/L proteinase K) during two rounds of 3 minutes at a 30Hz/s
424 frequency in a TissueLyser II grinder (Qiagen). Total RNA was converted into complementary
425 DNA (cDNA) using M-MLV reverse transcriptase (Invitrogen) and random hexamers, the
426 reaction was carried out as follows: 10 min at 25°C, 50 min at 37°C, and 15 min 70°C. The
427 cDNA was amplified by PCR carried out in a 25µl reaction containing 12.5µl of 1x DreamTaq
428 DNA polymerase (Thermo Fisher Scientific) and 10 µM of each ZIKV primer (forward: 5'-
429 GTATGGAATGGAGATAAGGCCCA-3', and reverse: 5'-ACCAGCACTGCCATTGATGTGC-3').
430 Cycling conditions were as follow, 2 min at 95°C, followed by 35 cycles of 30s at 95°C, 30s at
431 60°C, and 30s at 72°C with a final extension step of 7 min at 72°C. Amplicons were visualized
432 on a 2% agarose gel. The proportion of ZIKV-infected females was analyzed by binomial logistic
433 regression as a function of treatment, colony, and their interaction in R.
434

435 To determine the dissemination titer, the heads of females with positive ZIKV-infected bodies
436 were titrated by focus-forming assay in Vero cells. Heads were homogenized individually in 200
437 µl of Vero cell media (DMEM 1X) supplemented with 2% heat inactivated fetal bovine serum

438 (FBS) and 1X Antibiotic-Antimycotic (Life Technologies) for 3 minutes at a 30Hz/s frequency in
439 a TissueLyser II grinder (Qiagen). Vero cells were seeded in 24-well plates and incubated for 24
440 hours to reach confluency. Each well was inoculated with 200µl of head homogenate in 10-fold
441 dilutions (from 10^1 to 10^6) and incubated at 37°C (5% CO^2) for 1 hour, rocking every 15 minutes.
442 Infected cells were overlaid with α-MEM media supplemented with 1.25% carboxymethyl
443 cellulose, 5% FBS, and 1% Pen-Strep. After three days of incubation at 37°C, infected cells
444 were fixed with 10 % formaldehyde for at least 1 hour and cells were washed three times in 1X
445 PBS. Approximately 500 µl of blocking solution (5% w/v non-fat powdered milk in 1X PBS) was
446 added to each well and the plates were placed on the plate rocker for 30 minutes. The blocking
447 solution was discarded and 200 µl of primary antibody solution (ZIKV antibody diluted 1:1000 in
448 blocking solution) was added to each well and plates were placed on plate rocker overnight. The
449 primary antibody solution was discarded, and plates were washed three times with 1X PBS
450 again, and 200 µl of secondary antibody (peroxidase-labeled goat anti-mouse IgG human
451 serum KPL-474-1806) solution (secondary antibody diluted 1:2000 in blocking solution) was
452 added to each well. Plates were placed on plate rocker for 1 hour. The secondary antibody
453 solution was discarded, and plates were washed three times with 1X PBS. To develop visible
454 foci, 100µl of TrueBlue peroxidase substrate (KPL 5510-0050) was added to each well, and
455 plates were placed on the plate rocker until foci could be seen, around 10 min. Plates were
456 washed with deionized water and FFU was counted with the help of a light. Focus-forming units
457 were Log_{10} transformed to represent the concentration of infectious ZIKV particles detected in
458 *Ae. aegypti* heads. Head titer data were analyzed by two-way ANOVA as a function of bacterial
459 treatment, mosquito genotype, and their interaction in R. Infection rate data was analyzed by a
460 two-way ANOVA on a binomial logistic regress in R.

461

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469

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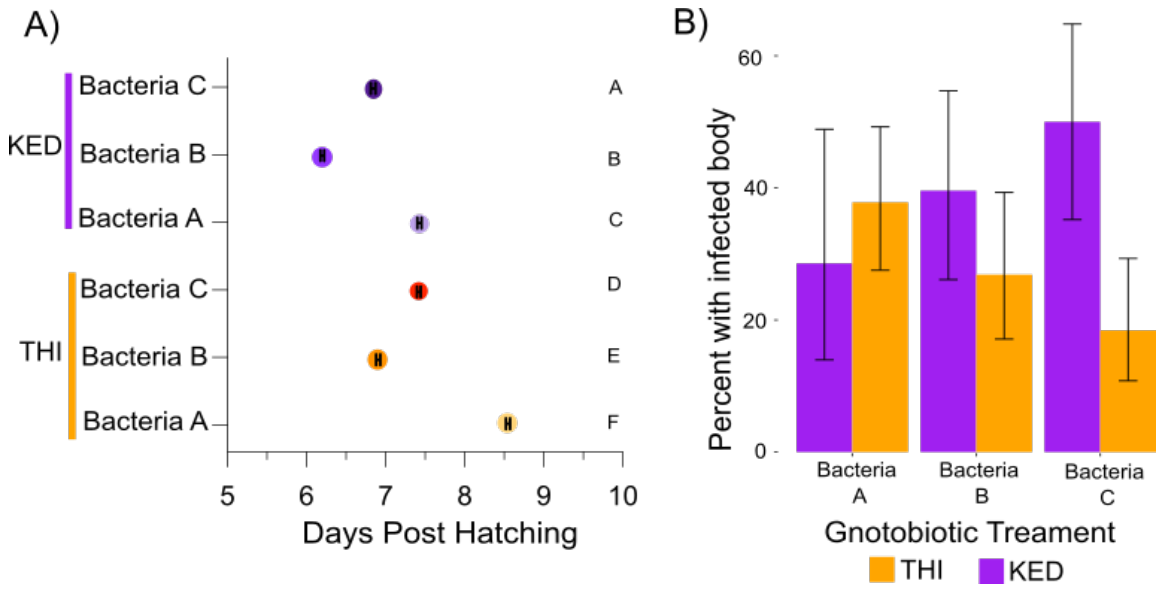
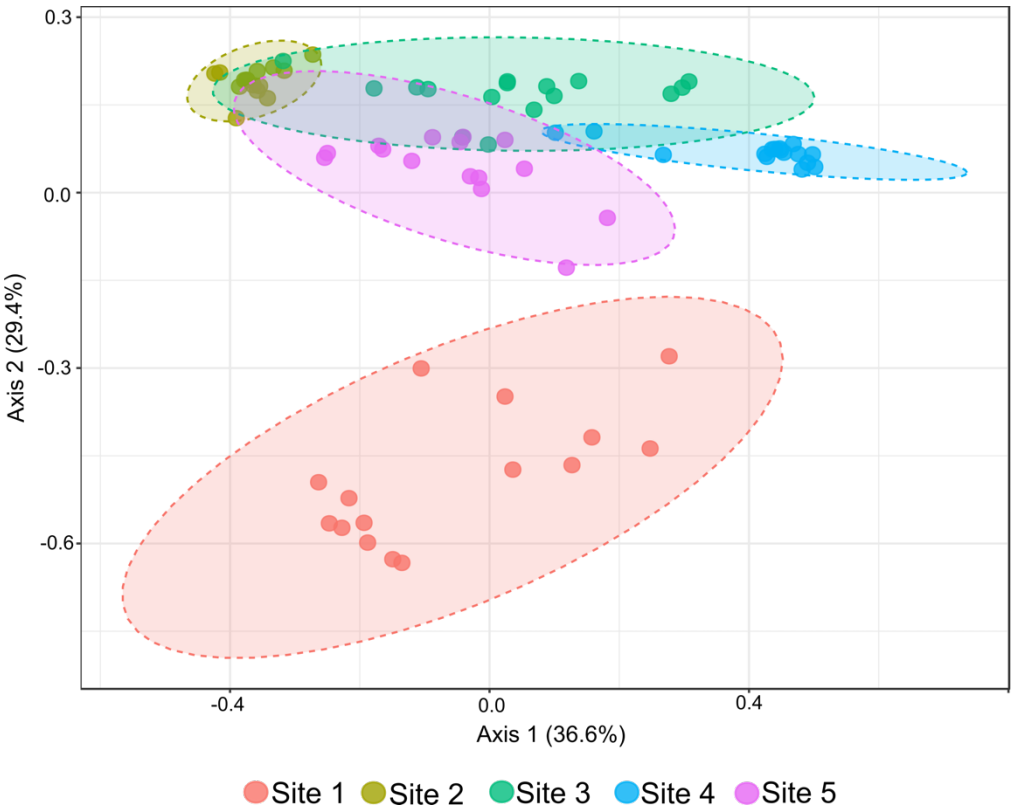


471

472 **Figure 1: Larval exposure to different bacteria isolates alters pupation rate and**
473 **ZIKV infection rates.** Variation in pupation rate (A) and ZIKV infection rates (B) are
474 shown for *Ae. aegypti* reared in the presence of single bacteria isolates in a gnotobiotic
475 system. (A) The PD_{50} (day where approximately 50% of the larvae have pupated) is
476 shown for two distinct genotypes of *Ae. aegypti* [7] KED and THI reared in the presence
477 of each bacterial isolate (Bacteria A, Bacteria B, and Bacteria C). Statistical significance
478 of differences between PD_{50} was determined by multiple comparisons with two-way-
479 ANOVA and Tukey's test (between bacterial treatments in each mosquito genotype) and

480 Sidak's test (between bacterial treatments within the same mosquito genotype) and is
481 designated by the letters. Error bars are shown within each point. (B) The proportion of
482 KED and THI lines of *Ae. aegypti* with a ZIKV positive body 7 days post-infection
483 following larval rearing in single bacteria isolates, Bacteria A, Bacteria B, and Bacteria C.
484 Error bars represent the 95% confidence intervals of the proportions. Data were
485 analyzed by a two-way ANOVA on a binomial logistic regression (bacteria isolate: p-
486 value = 0.614, mosquito genotype: p-value = 0.372, bacterial isolate x mosquito
487 genotype: p-value = 0.010).
488

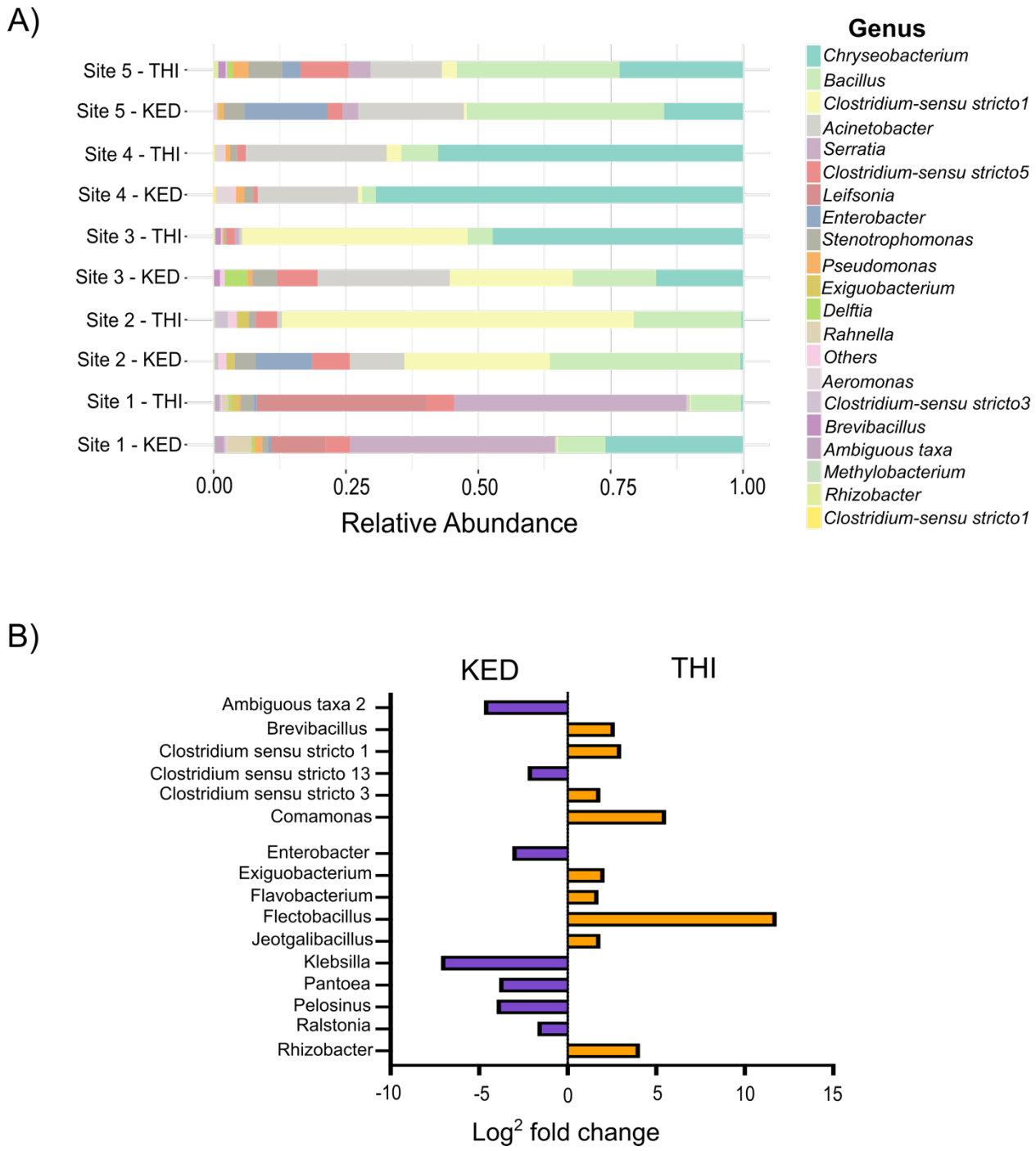


489
490 **Figure 2: The bacterial community structure differs between the gnotobiotic**
491 **larvae.** Structure of bacterial communities was determined by deep sequencing the V3-
492 V4 region of the 16S gene in individual larvae reared in a gnotobiotic system inoculated
493 with complex microbiomes generated from larvae homogenates collected in Senegal

494 (Site1-Site5). Bacterial structure is represented by PCoA of a Bray-Curtis dissimilarity

495 matrix based on mean genera abundance (PERMANOVA p = 0.001).

496

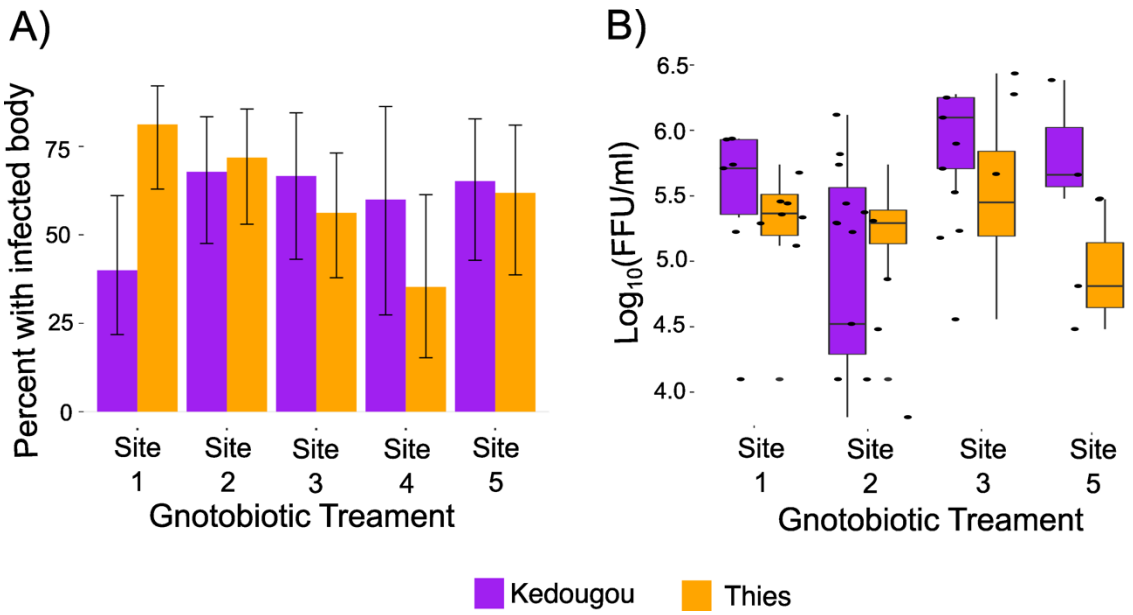


497

498 **Figure 3: Abundance of specific taxa differs between lines of gnotobiotic larvae**.

499 (A) The percent abundance of the top 20 most abundant genera is plotted by larval

500 treatment (Site1-5) and mosquito line (KED or THI). (B) The log_2fold change of bacteria
501 genera with significant pairwise differences between
502 KED and THI is plotted. Pairwise differential analysis was performed between larvae
503 from the KED or THI after receiving identical complex bacterial communities. The
504 bacteria associated with negative value are the one more likely to be associated to KED
505 and the one with positive value are the one associated with THI.
506

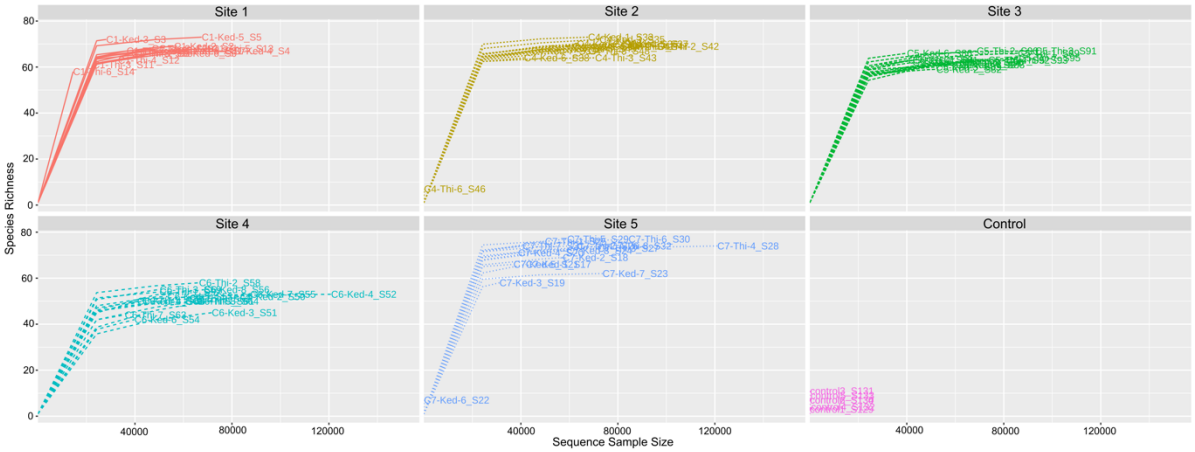


507

508 **Figure 4: Larval development in different complex microbiomes alters ZIKV**
509 **infection rates in a mosquito genotype dependent manner**. (A) The proportion of
510 blood-fed *Aedes aegypti* females from the KED and THI lines with a ZIKV-positive body
511 7 days post-infectious blood meal by RT-PCR following larval rearing in complex
512 microbiomes, Site1-5. The y-axis indicates the proportion of ZIKV-infected female bodies
513 and error bars represent the 95% confidence intervals of the proportions. Data were
514 analyzed by binomial logistic regression as a function of bacterial treatment, mosquito
515 genotype, and their interaction (bacterial treatment: p-value = 0.265, mosquito genotype:
516 p-value = 0.392, but was dependent on the specific pairing of bacterial treatment x

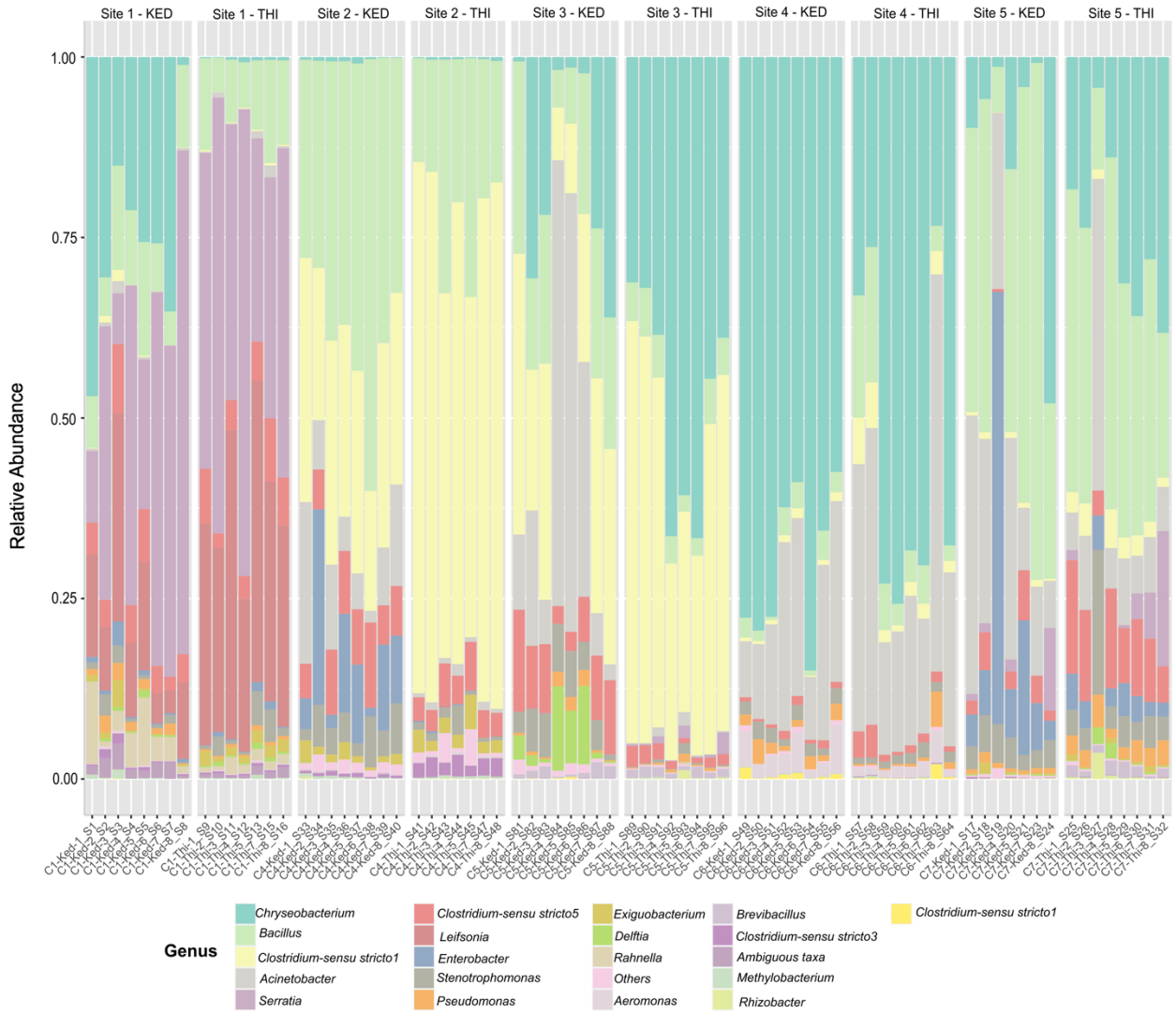
517 mosquito genotype: p-value = 0.017. (B) Boxplot showing the dissemination titers of
518 infectious ZIKV particles expressed as the Log10-transformed number of focus-forming
519 units (FFU) per ml detected in the *Ae. aegypti* head seven days post-infectious blood
520 meal. The points represent individuals and the mean is represented by a horizontal line.
521 The error bars represent the 95% confidence interval. Data were analyzed by Two-way
522 ANOVA as a function of bacterial treatment, mosquito genotype, and their interaction
523 (bacterial treatment: p-value = 0.0415, mosquito genotype: p-value = 0.057, bacterial
524 treatment x mosquito genotype: p-value = 0.538).
525

526 **Supplemental Figures**

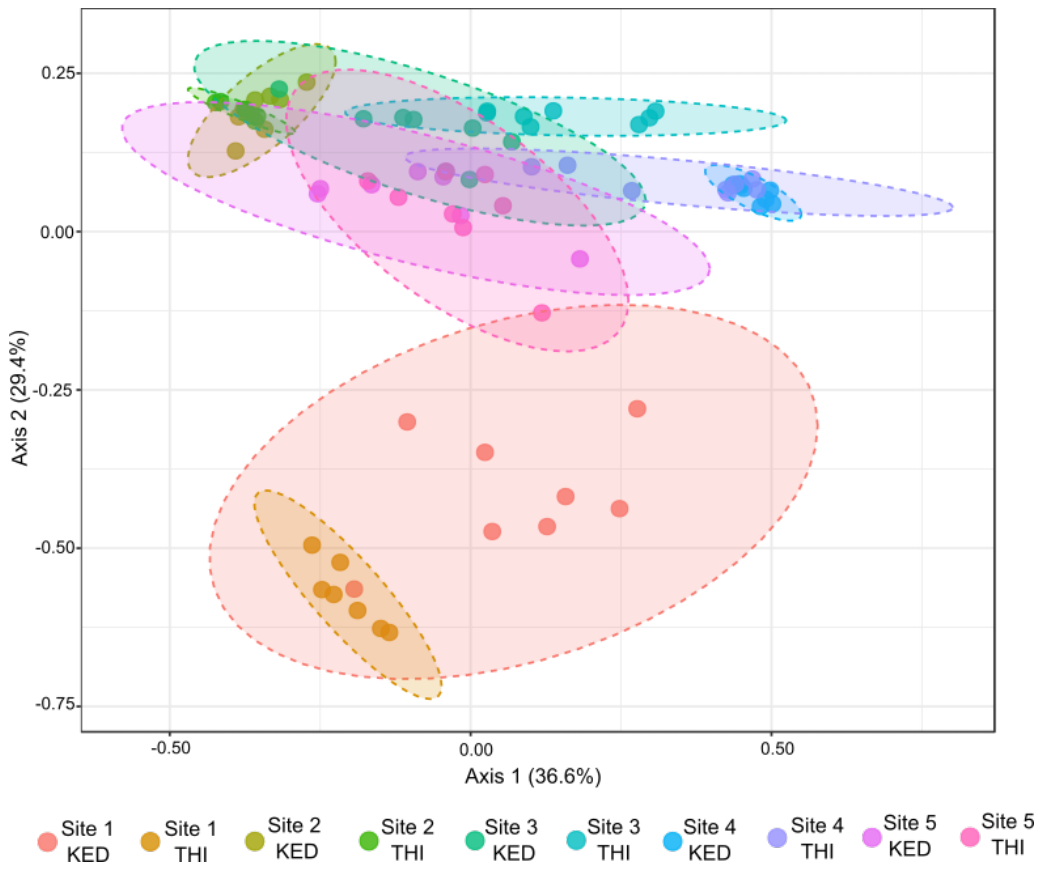


527

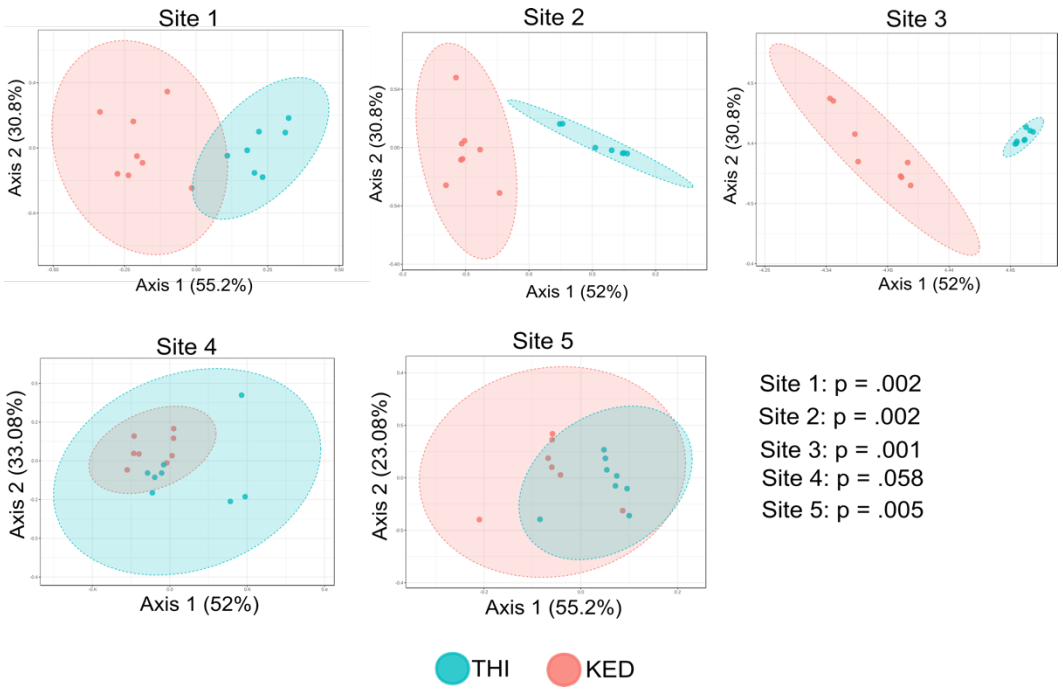
528 **Supplemental Figure 1:** Rarefaction curves showing the sequencing depth of each library. The
529 number of species is shown on the Y axis, and the number of sequencing reads is shown on the
530 X axis.
531



Supplemental Figure 2: 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.



Supplemental Figure 3: 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).



Supplemental Figure 4: 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 shown on the figure.

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