Zika virus infection and temperature significantly impact *Aedes albopictus* gut microbiota

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Abstract

Background: Vector-borne pathogens must survive and replicate in the hostile environment in the insect gut before successful transmission. The insect gut bacteria can have a modulatory effect on their vectorial capacity. The relationship between infection, temperature and the mosquito gut microbiome are not well characterized.

Methodology/ Principal findings: This study aimed to delineate the effect of Zika virus (ZIKV) infection and temperature variation on the microbial profile of *Aedes albopictus* mosquitoes. *Ae. albopictus* were reared at diurnal temperature of day 28°C/ night 24°C and day 30°C/ night 26°C. Mosquitoes were given infectious blood meals with 8.3 $\log_{10}$ PFU/ml ZIKV and 16S *rRNA* sequencing was performed on guts at 7 days post-infectious bloodmeal exposure.

Blood meal acquisition and ZIKV exposure significantly reduced the gut taxa diversity while increase in temperature significantly altered the bacterial composition. A number of individual bacterial genera, most notably *Elizabethkingia* and *Neoasaia*, were found to be associated with blood meal digestion, ZIKV exposure and susceptibility to infection. To the best of our knowledge, we identified *Neoasaia* for the first time in the midgut of an arthropod vector.

The findings of this study demonstrate that both temperature and ZIKV infection modulate the midgut microbial profile of *Ae. albopictus* mosquitoes. This may impact arbovirus transmission dynamics in future climate change scenarios and inform novel control strategies.

Key words: Zika virus, *Aedes albopictus*, microbiome, *Elizabethkingia*, *Neoasaia*

47 **Introduction**

48 The microbiota of mosquitoes has been shown to interfere with mosquito pathogen infection
49 and influence mosquito fitness, hence affecting their vectorial capacity (Romoli & Gendrin,
50 2018). The importance of the microbiota in the modulation of vector competence due to
51 complex interactions between the pathogen, the mosquito and its bacterial microbiota has
52 become an area of interest and target for development of novel biological control tools
53 (Guégan et al., 2018). Abundant communities of diverse microorganisms reside in insects with a
54 major concentration in the intestinal sections (Dillon & Dillon, 2004).

55 The major bottleneck of pathogen transmission by the mosquito is pathogen invasion of the
56 midgut epithelium (Romoli & Gendrin, 2018) and the gut microbiota may modulate pathogen
57 susceptibility and replication by activation of its basal immunity (Vallet-Gely et al., 2009).
58 Studies investigating the interaction of gut microbial taxa and arboviruses of medical
59 importance have mainly been limited to *Wolbachia* (Asad et al., 2018; Lindsey et al., 2019;
60 Mohanty et al., 2018; Moretti et al., 2018). *Wolbachia* has subsequently been utilised for large
61 scale control of dengue virus (DENV) and Zika virus (ZIKV) (Callaway, 2016; Nguyen et al., 2015).
62 Despite the effectiveness of *Wolbachia*, it is important to further explore other microbes that
63 could potentially be used as novel biological markers and tools. Our study provides insights into
64 the interactions of ZIKV with the resident gut microbiota of *Ae. albopictus*, considered as a
65 secondary vector of DENV and ZIKV.

66 In addition, the relationship between increases in temperature and gut microbiota of
67 mosquitoes is not well-characterized. This information is important to understand the potential
68 impact of future climate change on mosquito-pathogen interactions. This study sought to
69 delineate the impact of ZIKV infection and temperature shifts on the gut microbiota of *Ae.*

70 *albopictus*. The identification of symbionts that are associated with altered vector competence
71 or altered temperatures can potentially act as targets for informing novel vector control
72 measures.

73 **Materials and Methods**

74 **Mosquito sampling and DNA preparation**

75 *Ae. albopictus* (ALB LI) were collected from Suffolk County, Long island, NY in 2015 (kindly
76 provided by I. Rochlin) (Figure 1). Field-collected mosquitoes were colonized at the New York
77 State Department of Health (NYSDOH) Arbovirus Laboratory while being maintained at 27°C
78 under standard rearing conditions.

79 Mosquitoes were hatched and reared under two thermal conditions; 14 hours light at 30°C: 10
80 hours dark at 26°C (M temperature regimen) and 14 hours light at 28°C: 10 hours dark at 24°C
81 (L temperature regimen). The L temperature regime modeled current average temperatures
82 during peak transmission in New York State and the M temperature regime modeled a
83 predicted 2°C increase, as has been suggested will occur with climate change (Kjellstrom et al.,
84 2018).

85 To obtain mosquito midgut samples for microbiome sequencing of ZIKV - infected guts, the F15
86 ALB LI eggs were vacuum-hatched. Before hatching, the water was incubated for 3 h at the two
87 different day time temperatures. The immature stages of the mosquitoes were reared at the
88 two diurnal temperature regimes. At eight days post mating, the female adults were orally
89 exposed to blood meals containing 8.3 $\log_{10}$ PFU/ml ZIKV. Subsets of the same population of
90 mosquitoes were either not offered blood meals (UNFED) or offered non-infectious blood
91 meals (UNEXP).

92 A total of 15 pools (three mosquitoes/pool) of *Ae. albopictus* female mosquito midguts
93 collected 7 days post infectious (dpi) blood meal were stored at -80°C until total RNA and
94 genomic DNA extraction were carried out using the Trizol protocol (ThermoFisher scientific,
95 Massachusetts, USA). A ZIKV-specific quantitative PCR was utilised to screen the guts for ZIKV
96 infection as described by Ciota A.T et al., 2017. The ZIKV strain utilised in this study was HND
97 (2016-19563, GenBank accession no. KX906952). In addition to ZIKV exposed midguts (EXP),
98 midguts from UNF and UNEXP midguts were collected at equivalent timepoints for comparison.
99

100 **Sequencing of Microbiome of *Aedes* mosquito guts and Analysis**

101 To determine the gut microbial profile of ZIKV-exposed individuals reared at the two
102 temperature regimes, barcoded high-throughput amplicon sequencing of the bacterial 16S V3-
103 V4 hypervariable *rRNA* was done. PCR reactions were carried out in a total volume of 50 µl,
104 5µM of 16S *rRNA* V3-V4 hypervariable region primers (Table 1), 10µl genomic DNA and 36 µl
105 AccuStart II PCR supermix (Quanta biosciences, Beverly, USA). A fragment size of ~460 bp of
106 each sample was submitted to the Wadsworth Center Applied Genomics Core for sequencing.
107 Automated cluster generation and paired-end sequencing (250-bp reads) was performed on the
108 Illumina MiSeq system.
109 Analysis of the data was carried out on QIITA (https://qiita.ucsd.edu/) and MicrobiomeAnalyst
110 (Dhariwal et al., 2017). In summary, to convert the de-multiplexed FASTQ files to the format
111 used by QIITA, the command split libraries FASTQ was used, the samples were then trimmed to
112 100 bp size to allow for meta-analysis. The clustering of the sequences into operational
113 taxonomic units (OTUs), was done by the utilization of a closed-reference OTU picking based on
114 the GreenGenes 16S reference database which matches the sequences based on a 97%

115 sequence identity threshold before assigning a taxonomy. A BIOM-formatted OTU table was
116 subsequently generated, to reduce the potential of alpha and beta diversity biases, the OTU
117 data was rarefied to a sample depth of 44000 OTUs per sample. Finally, to visualize the
118 taxonomic profiles of each sample, taxa bar plots were generated using the rarefied data
119 artifact.
120 The alpha diversity profiling (the number of species of taxa in a single sample) was calculated
121 using the Chao1 diversity measure and significance testing was done using the T-test/ANOVA
122 method in Microbiome Analyst. Chao1 index estimates the richness of taxa in a sample by
123 extrapolating out the number of rare organisms that may have been omitted due to under
124 sampling. The index assumes that the number of observations for a taxon has a Poisson
125 distribution and hence corrects for variance (Chao, 1984). Shannon diversity index was also
126 utilised to calculate the alpha diversity (Shannon, 1964). This diversity index accounts for both
127 abundance and evenness of the species present. The proportion of species *i* relative to the total
128 number of species (p_i) is calculated, this is then multiplied by the natural logarithm of the
129 proportion ($\ln p_i$).
130 Beta diversity, a comparison of microbial community composition between two samples, was
131 analyzed by utilizing the Bray-Curtis dissimilarity statistic (Bray & Curtis, 1957). The distance
132 matrix generated after comparing each sample to the other was visualized for dissimilarity
133 between samples using the Principle Coordinate Analysis ordination method.
134
135 **Clustering and Biomarker analysis**
136 Patterns of relative abundance of taxa species in response to different experimental factors was
137 analyzed in Microbiome Analyst (Dhariwal et al., 2017). The distance measure was Euclidean

138 (Motzkin, 1949) and the clustering algorithm utilised was Ward, an agglomerative hierarchical
139 clustering procedure (Ward & Hook, 1963). The Euclidean distance, a measure of the true
140 straight-line distance between two points in Euclidean space, examines the root of square
141 differences between coordinates of a pair of objects.
142 To provide an estimate of the most important taxa for classification of data resulting from
143 different experimental factors, biomarker analysis was carried out using the random forest
144 algorithm within Microbiome Analyst (Dhariwal et al., 2017). The default setting of the number
145 of trees to grow and number of predictors to try was applied (500 and 7 respectively) with the
146 randomness setting left on. Random forest algorithm (Svetnik et al., 2003) is a supervised
147 classification algorithm of trees created by using bootstrap samples while training data and
148 random feature selection in tree induction. It is an ensemble of unpruned classification or
149 regression trees trained with the bagging method (Liaw & Wiener, 2002).
150
151 **Phylogenetic analysis of the *Neoasaia* and *Elizabethkingia rpoB* genes**
152 The *Neoasaia rpoB* (502 bp) and *Elizabethkingia rpoB* (588 bp) genes were sequenced from PCR
153 products excised from agarose gel and cleaned using QIAquick gel extraction kit (Qiagen, UK) at
154 the Wadsworth Center Applied Genomics Core. The sequences were manually edited using
155 Bioedit v7.1.9 and aligned using MUSCLE (Edgar, 2004; Hall, 2011). Upon trimming the primer
156 region and the low-quality regions of the sequence, the FASTA sequence of the respective
157 genes were blasted against the NCBI database. A total of 11 *rpoB* gene sequences published
158 with the following GenBank accession numbers: KY587657.1; FJ999737.1; KY587659.1;
159 KY587658.1; FJ999739.1; FN391773.1; FN391805.1; KF537505.1; FN391750.1; FN391807.1,
160 HG329623.1 and NC_00913.3 were added to the phylogenetic analysis.

161 Bayesian analysis was performed on the sequences using MR. BAYES v3.1.2 (Ronquist et al.,
162 2012). The analysis was run for 1000000 generations using the general time reversible (GTR)
163 substitution model, sampling for convergence every 5 000 generations.
164
165 **Results**
166 **Gut taxa microbial profile of *Aedes* mosquitoes**
167 Next generation sequencing resulted in a total of 7419965 reads with a maximum read length
168 of 301 bp and minimum read length of 280 bp. After trimming the reads to 100 bp to allow
169 efficient comparison, a total of 6062 OTUs were derived from a closed reference OTU picking
170 with a frequency of 6036630 across the 34 individuals ranging between 44255 and 583632. The
171 OTU were rarefied to 44000 to allow downstream analysis.
172 The OTU generated in this study belonged to a total of 35 phyla; Bacteroidetes (0.58),
173 Proteobacteria (0.36), Actinobacteria (0.03), Firmicutes (0.02), Cyanobacteria (0.01) and OD1
174 (0.004) were the dominant taxa while the rest of the phyla had a frequency of < 0.004 (Figure
175 1). To generate a taxa bar plot to visualize the distribution of bacterial genera across individual
176 guts in each group, a cut-off taxa frequency of 150 was applied and a taxa bar plot generated
177 (Figure 2).
178
179 **Blood feeding alters *Ae. albopictus* gut microbiota diversity and composition**
180 Blood feeding significantly altered the gut taxa abundance of *Ae. albopictus* regardless of
181 grouping ([PERMANOVA] F-value: 15.686, R-squared, 0.18095, P<0.001; Figure 2) and the
182 overall alpha diversity decreased independent of ZIKV exposure or infection (chao1 T-
183 test/ANOVA P(0.006) [T-test] statistic -3.1216; Figure 3A). Although less apparent at the higher

184 temperature regime (M), in all groups the decrease in diversity was driven primarily by an
185 increase the relative abundance of the genus *Elizabethkingia* (Figure 2). The Random forest
186 classifier, used to determine the association of differentially abundant taxa in the microbial
187 community with certain states, confirmed that low relative abundance of *Elizabethkingia* was
188 strongly associated with UNFED individuals (Figure 4). Numerous taxa decreased or were
189 eliminated as a result of blood feeding. Most notable were *Acidovorax, Bdellovirio* and
190 *Pseudomonas* (Figures 2 and 4).
191
192 **ZIKV exposure alters *Ae. albopictus* gut microbiota diversity and composition.**
193 Comparisons of pools of midguts from ZIKV exposed individuals (FED EXP) to those fed non-
194 infectious blood meals (FED UNEXP) demonstrates that ZIKV exposure independently decreases
195 alpha diversity at 7 days post-feeding (chao1 T-test/ANOVA P (0.005) [T-test] statistic -3.1084;
196 Figure 3B). These differences in diversity at the group level were also visible at the individual
197 sample level (Figure 2). Taxa for which relative abundance was decreased as a result of ZIKV
198 exposure included *Delftia*, *Chryseobacterium*, *Pseudomonas*, *Moraxellaceae*, and *Acidovorax*
199 (Figures 2 and 4).
200 There was no significant difference in taxa richness within and between pools that
201 disseminated the virus and those that were uninfected following exposure (EXP UNINF v. EXP
202 DIS; Alpha diversity Chao1 Index P[7.574e-05(ANOVA) F-value: 10.379]; Beta diversity
203 [PERMANOVA] F-value: 2.7121; R-squared: 0.14892; P [<0.041]; Figure 3C). Despite this, some
204 individual taxa were associated with infection status. Although blood feeding alone increased
205 the relative amount of *Elizabethkingia*, the highest abundance of this genus was associated
206 with exposed uninfected midguts (EXP UNINF; Figure 2, Figure 4A). This was particularly the

207 case with individuals reared at the lower temperature regime (EXP UNINF[L]; Figure 2).

208 *Elizabethkingia* is only taxa associated with 'resistance' to infection. Pools of guts with

209 individuals that disseminated the virus demonstrated higher relative amounts of *Neoasaia* and

210 additonal taxa belonging to the order Rhodospirillales, family Acetobacteraceae (*Roseomonas*,

211 and *Swaminathania*). Other taxa associated with infection and dissemination included *E. coli*,

212 *Salmonella* and *Tanticharoenia* (Figures 2 and 4; Supplementary Figure 1).

213

214 **Higher temperature enriches the gut microbial community of *Ae. albopictus***

215 Increased temperatures altered the microbial composition and increased overall microbial

216 diversity in the absence of ZIKV exposure ([Alpha diversity, Shannon index P (0.022377); T-test

217 statistic: -2.4172]; [Beta diversity [PERMANOVA] F-value: 3.1477; R-squared: 0.089557; P

218 (<0.045)]; Figure 3E). The higher diversity is primarily due to a lower relative abundance of

219 *Elizabethkingia* at the higher (M) temperature regime (Figure 2), which is also the primary

220 driver of the measured difference in beta diversity (Figure 3F). The fact that this difference in

221 alpha diversity among temperature regimes in the UNEXP mosquitoes is significant only when

222 using the Shannon, rather than Chao1 index, is due to the increased likelihood of rare taxa in

223 the M temperature regime (Figure 3D/E). Regardless of index, this difference is lost following

224 ZIKV exposure (Figure 3D/E), where diversity is significantly lower at both L and M temperature

225 regimes relative to UNEXP mosquitoes. Despite this lack of temperature-dependent variability

226 in alpha diversity among ZIKV EXP mosquitoes, the relative abundance of *Elizabethkingia*

227 remains lower at higher temperatures (Figure 2).

228

229 ***rpoB* gene sequencing of bacterial strains isolated from *Aedes* mosquitoes**

230 Given the relative abundance of *Elizabethkingia* and *Neoasaia*, together with their association
231 with phenotypes of interest (resistance to infection and dissemination, respectively) these taxa
232 were chosen for further sequencing.
233 To the best of our knowledge, this is the initial reporting of detection of *Neoasaia* bacterial
234 strain from an insect. The only other strain present in the literature was derived from a plant
235 (*Neoasaia chiangmaiensis*)(Yukphan et al., 2005). The *Neoasaia* strain detected in mosquitoes
236 in this study clustered with this previously identified strain (Figure 5A). The *Elizabethkingia*
237 strain isolated from the gut of the *Ae. albopictus* in this study clustered with pathogenic strain
238 of medical importance. Unexpectedly, it did not share a clade with the *Elizabethkingia*
239 *anophelis*, which was previously isolated from Anopheline mosquitoes (Kämpfer et al., 2011)
240 (Figure 5B).
241
242 **Discussion**
243 This study has demonstrated a significant reduction in *Ae. albopictus* gut microbial diversity
244 resulting from blood feeding which is further exacerbated by ZIKV exposure and modified at
245 different temperature regimes. These data further identified individual gut microbes in *Aedes*
246 mosquitoes that potentially play a modulating role in ZIKV susceptibility and competence.
247 Recent studies have confirmed the importance of microbiota in the modulation of vector
248 competence (Apte-Deshpande et al., 2012; Bourtzis et al., 2014; Dickson et al., 2017; Ramirez et
249 al., 2014; Weiss & Aksoy, 2011). Microbiota can influence competence through inhibition of
250 the pathogen by secreted enzymes or toxins, or by the induction of the host immune system
251 targeting the invading pathogenic microbe (Weiss & Aksoy, 2011).

252 With the aim of delineating possible taxa modulating ZIKV infection of the mosquito midguts,
253 we characterized the midgut microbial profile before and after feeding on non-infectious and
254 infectious blood meals and further compared profiles based on infection and dissemination
255 status. In addition, we measured the effect of temperature on the midgut microbial community
256 in an effort to understand how climate change might ultimately alter microbial taxa that
257 influence vectorial capacity for ZIKV and other arboviruses.
258 Our data support previous studies demonstrating that bacterial composition of the mosquito
259 midgut was significantly altered by blood meal acquisition and digestion. This is likely brought
260 about by an oxidative shift in the midgut after blood digestion (Champion & Xu, 2017; Zouache
261 et al., 2012). While blood feeding significantly decreased overall microbial diversity, substantial
262 increases in the relative abundance of *Elizabethkingia* were measured, clearly indicating that
263 the gut environment is more amenable to the propagation of this species following blood meal
264 digestion. This alteration in microbial composition could be beneficial to *Ae. albopictus.*
265 Specifically, *Elizabethkingia anophelis* has been shown to produce OxyR regulon and
266 antioxidants that in turn potentially protect the mosquitoes from the oxidative stress brought
267 about by blood digestion. The bacteria also displays hemolytic activity and hence may assist in
268 erythrocyte digestion in the mosquito midgut (Kukutla et al., 2014).
269 Our data additionally demonstrated that ZIKV exposure significantly altered microbial
270 composition independent of the effect of blood feeding. Specifically, ZIKV exposure was
271 associated with elimination of a number of taxa that were present in the mosquito midgut
272 before and after non-infectious blood meals. This resulted in a few select taxa dominating the
273 midgut microbial community of the ZIKV exposed midguts. While *Elizabethkingia* was the

274 dominant genus in all ZIKV exposed guts, the relative abundance of this microbe was higher in
275 guts that did not acquire infections.
276 *Elizabethkingia* species have been previously identified with *Anopheles* mosquitoes, *Aedes*
277 mosquitoes and most recently, *Culicoides* (Mee et al., 2017, Boissière et al., 2012; Kukutla et al.,
278 2014; Terenius et al., 2012; Wang et al., 2011). The association of *Elizabethkingia* abundance
279 with ZIKV resistance suggests a potential antiviral effect. While further studies are needed to
280 define this relationship, one potential mechanism is the interaction of *Elizabethkingia* with the
281 Reactive Oxygen Species (ROS) pathway, which is documented to influence *Aedes* competence
282 for arboviruses (Li et al., 2015). The genus *Elizabethkingia* (formerly Chryseobacterium or
283 Flavobacterium), a gram-negative bacilli, is additionally a source of public health concern (Janda
284 & Lopez, 2017). Three species of this genus have been shown to cause human infections of
285 medical importance: *E. meningoseptica*, *E. anopheles* and *E. miricola* (Janda & Lopez, 2017; Lau
286 et al., 2016). Its existence in a similar clade as these medically important strains (*E. miricola* and
287 *E. meningoseptica*), as shown by our *rpoB* phylogenetic analysis, demonstrates that further
288 investigation into its pathogenicity in humans is warranted.
289 To the best of our knowledge, our study is the initial report of an isolation of *Neoasaia spp* from
290 an arthropod vector, with a single previous isolation of *Neoasaia* reported from a red ginger
291 flower (Yukphan et al., 2005). While the mechanistic basis for a potential interaction is unclear,
292 we found *Neoasaia* positively associated with the development of disseminated ZIKV infections,
293 underscoring the need for further transcriptomic or metabolomic studies. *Neoasaia spp*, an
294 acetic acid bacteria, belongs to the family Acetobacteraceae which were initially recovered
295 from bees (Bruce White, 1921). The acetic acid bacteria have been found to colonize insects
296 that mainly rely on sugar-based diets such as nectars, fruit sugars or phloem (Russell et al.,

297 2017). The closely related *Asaia spp* has also been found in association with the pathogen-
298 transmitting mosquitoes *An. Stephensis*, *An. Maculipennnis*, *An. gambiae* and *Ae. aegypti*
299 (Crotti et al., 2009; Damiani et al., 2010; Favia et al., 2007).
300 In addition to these taxa, data suggested other bacterial species are associated with ZIKV
301 competence. For instance, *Escherichia coli* was found to have a higher relative abundance in
302 midguts of individuals with disseminated infections. *E. coli* has been shown to induce hypoxia in
303 the midgut of mosquitoes through cytochrome bd oxidase (Coon et al., 2017) and it is feasible
304 that this could indirectly influence ZIKV competence. It is also notable that the *Ae. albopictus*
305 midgut samples had very little detectable *Wolbachia*, however whole-body samples were found
306 to have very high amounts of *Wolbachia* (data not shown). This is consistent with the majority
307 of *Wolbachia* being in the germline cells. It is possible that there is competitive exclusion of
308 *Wolbachia* in the midgut by another microbe. Studies have shown that *Ae. albopictus* and *Cx.*
309 *quinquefasciatus* naturally infected by both *Asaia* and *Wolbachia* have a co-exclusion type of a
310 relationship. In species not colonized by *Wolbachia* such as *An. gambiae*, *An. stephensi* and *Ae.*
311 *aegypti*, *Asaia* is capable of colonizing reproductive organs and salivary gland (Rossi et al.,
312 2015).
313 It is well-documented that temperature can significantly alter vectorial capacity of *Aedes*
314 mosquitoes for arboviruses including ZIKV (Ali et al., 2017;Paz & Semenza, 2016; Onyango et al.,
315 under review), yet the relationship between altered vectorial capacity, temperature and
316 microbial communities is not well documented. Temperature is an important component in
317 shaping the microbial communities of organisms (Fuhrman et al., 2008; Lokmer & Wegner,
318 2015) and our data clearly demonstrated a complex relationship between *Ae. albopictus* midgut
319 microbial diversity and temperature. This complexity is partially due to the confounding effects

320 of blood meal digestion and virus exposure, but also clearly a result of temperature
321 differentially affecting unique bacterial taxa. For example, higher temperatures are associated
322 with a decrease in the relative abundance of *Elizabethkingia* and an increase in the relative
323 abundance of *Neoasaia*. Given the association of these taxa with ZIKV resistance and
324 dissemination, respectively, these data suggest that modifications to microbial communities at
325 higher temperatures could be a contributing factor to increased transmissibility of ZIKV and
326 other arboviruses with climate change. This also demonstrates that individual bacterial species
327 that may potentially act as biomarkers for infection and/or be utilized as novel vector control
328 tools could be differentially affected by climate change. These relationships should therefore be
329 considered when assessing the potential effectiveness of such strategies.
330

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339

340 **References**

341 Ali, S., Gugliemini, O., Harber, S., Harrison, A., Houle, L., Ivory, J., … Mordecai, E. A. (2017).
342 Environmental and Social Change Drive the Explosive Emergence of Zika Virus in the
343 Americas. *PLoS Neglected Tropical Diseases*, 11(2).

https://doi.org/10.1371/journal.pntd.0005135
Apte-Deshpande, A., Paingankar, M., Gokhale, M. D., & Deobagkar, D. N. (2012). Serratia odorifera a midgut inhabitant of aedes aegypti mosquito enhances its susceptibility to dengue-2 virus. *PLoS ONE*, *7*(7). https://doi.org/10.1371/journal.pone.0040401
Asad, S., Hussain, M., Hugo, L., Osei-Amo, S., Zhang, G., Watterson, D., & Asgari, S. (2018). Suppression of the pelo protein by Wolbachia and its effect on dengue virus in Aedes aegypti. *PLoS Neglected Tropical Diseases*, *12*(4), 1–20. https://doi.org/10.1371/journal.pntd.0006405
Bourtzis, K., Dobson, S. L., Xi, Z., Rasgon, J. L., Calvitti, M., Moreira, L. A., … Gilles, J. R. L. (2014). Harnessing mosquito-Wolbachia symbiosis for vector and disease control. *Acta Tropica*, *132*(1), S150–S163. https://doi.org/10.1016/j.actatropica.2013.11.004
Bray, R. J., & Curtis, J. T. (1957). An Ordination of the Upland Forest Communities of Southern Wisconsin. *Ecological Monographs*, *27*(4), 325–349.
Bruce White, P. (1921). The Normal Bacterial Flora of the Bee. *The Journal of Pathology*, *24*(1).
Callaway, E. (2016). Infected mosquitoes fight Zika. *Nature*, *539*(7627), 17–18. https://doi.org/10.1038/nature.2016.20878
Champion, C. J., & Xu, J. (2017). The impact of metagenomic interplay on the mosquito redox homeostasis. *Free Radical Biology and Medicine*, *105*(August 2016), 79–85. https://doi.org/10.1016/j.freeradbiomed.2016.11.031
Chao, A. (1984). Nonparametric Estimation the Number of Classes in a Population. *The Annals of Mathematical Statistics*, *20*(4), 265–270. https://doi.org/10.1214/aoms/1177729949
Ciota, A., Bialosuknia, S., Zink, S., Brecher, M., Ehrbar, D., Morrissette, M., & Kramer, L. D. (2017). Effects of Zika Virus Strain and Aedes Mosquito Species on Vector Competence. *Emerging Infectious Diseases*, *23*(7), 1110–1117.
Coon, K. L., Valzania, L., McKinney, D. A., Vogel, K. J., Brown, M. R., & Strand, M. R. (2017). Bacteria-mediated hypoxia functions as a signal for mosquito development. *Proceedings of the National Academy of Sciences*, *114*(27), E5362–E5369. https://doi.org/10.1073/pnas.1702983114
Crotti, E., Damiani, C., Pajoro, M., Gonella, E., Rizzi, A., Ricci, I., … Daffonchio, D. (2009). Asaia, a versatile acetic acid bacterial symbiont, capable of cross-colonizing insects of phylogenetically distant genera and orders. *Environmental Microbiology*, *11*(12), 3252–3264. https://doi.org/10.1111/j.1462-2920.2009.02048.x
Damiani, C., Ricci, I., Crotti, E., Rossi, P., Rizzi, A., Scuppa, P., … Favia, G. (2010). Mosquito-Bacteria Symbiosis: The Case of Anopheles gambiae and Asaia. *Microbial Ecology*, *60*(3), 644–654. https://doi.org/10.1007/s00248-010-9704-8
Dhariwal, A., Chong, J., Habib, S., King, I. L., Agellon, L. B., & Xia, J. (2017). MicrobiomeAnalyst: A web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. *Nucleic Acids Research*, *45*(W1), W180–W188. https://doi.org/10.1093/nar/gkx295
Dickson, L. B., Jiolle, D., Minard, G., Moltini-Conclois, I., Volant, S., Ghozlane, A., … Lambrechts, L. (2017). Carryover effects of larval exposure to different environmental bacteria drive adult trait variation in a mosquito vector. *Science Advances*, *3*(8), 1–15. https://doi.org/10.1126/sciadv.1700585
Dillon, R. J., & Dillon, V. M. (2004). The Gut Bacteria of Insects: Nonpathogenic Interactions. *Annual Review of Entomology*, *49*, 71–92.
Edgar, R. C. (2004). MUSCLE: Multiple sequence alignment with high accuracy and high

throughput. *Nucleic Acids Research*, *32*(5), 1792–1797. https://doi.org/10.1093/nar/gkh340

Engla, N. E. W., & Journal, N. D. (2010). New engla nd journal, 1061–1063.

Favia, G., Ricci, I., Damiani, C., Raddadi, N., Crotti, E., Marzorati, M., … Daffonchio, D. (2007). Bacteria of the genus Asaia stably associate with Anopheles stephensi, an Asian malarial mosquito vector. *Proceedings of the National Academy of Sciences*, *104*(21), 9047–9051. https://doi.org/10.1073/pnas.0610451104

Fuhrman, J. A., Steele, J. A., Hewson, I., Schwalbach, M. S., Brown, M. V., Green, J. L., & Brown, J. H. (2008). A latitudinal diversity gradient in planktonic marine bacteria. *Proceedings of the National Academy of Sciences*, *105*(22), 7774–7778. https://doi.org/10.1073/pnas.0803070105

Guégan, M., Zouache, K., Démichel, C., Minard, G., Tran Van, V., Potier, P., … Valiente Moro, C. (2018). The mosquito holobiont: fresh insight into mosquito-microbiota interactions. *Microbiome*, *6*(1), 49. https://doi.org/10.1186/s40168-018-0435-2

Hall, T. (2011). BioEdit: An important software for molecular biology. *GERF Bulletin of Biosciences*, *2*(1), 60–61.

Janda, J. M., & Lopez, D. L. (2017a). Mini review: New pathogen profiles: Elizabethkingia anophelis. *Diagnostic Microbiology and Infectious Disease*, *88*(2), 201–205. https://doi.org/10.1016/j.diagmicrobio.2017.03.007

Janda, J. M., & Lopez, D. L. (2017b). Mini review: New pathogen profiles: Elizabethkingia anophelis. *Diagnostic Microbiology and Infectious Disease*, *88*(2), 201–205. https://doi.org/10.1016/j.diagmicrobio.2017.03.007

Kämpfer, P., Matthews, H., Glaeser, S. P., Martin, K., Lodders, N., & Faye, I. (2011). Elizabethkingia anophelis sp. nov., isolated from the midgut of the mosquito Anopheles gambiae. *International Journal of Systematic and Evolutionary Microbiology*, *61*(11), 2670–2675. https://doi.org/10.1099/ijs.0.026393-0

Kjellstrom, E., Nikulin, G., Strandberg, G., Christensen, O., Jacob, D., Keuler, K., … Vautard, R. (2018). European climate change at global mean temperature increases of 1.5 and 2C above pre-industrial conditions as simulated by the EURO-CORDEX regional climate models. *Earth System Dynamics*, *9*, 459–478. https://doi.org/ethz-b-000265761

Kukutla, P., Lindberg, B. G., Pei, D., Rayl, M., Wanqin, Y., Steritz, M., … Xu, J. (2014). Insights from the genome annotation of Elizabethkingia anophelis from the malaria vector anopheles gambiae. *PLoS ONE*, *9*(5). https://doi.org/10.1371/journal.pone.0097715

Lau, S. K. P., Chow, W. N., Foo, C. H., Curreem, S. O. T., Lo, G. C. S., Teng, J. L. L., … Woo, P. C. Y. (2016). Elizabethkingia anophelis bacteremia is associated with clinically significant infections and high mortality. *Scientific Reports*, *6*(March), 8–17. https://doi.org/10.1038/srep26045

Li, Y., Liu, Y., Chew, S. C., Tay, M., Margarette, M., Salido, S., … Yang, L. (2015). Complete Genome Sequence and Transcriptomic Analysis of the Novel Pathogen Elizabethkingia anophelis in Response to Oxidative Stress, *7*(6), 1676–1685. https://doi.org/10.1093/gbe/evv101

Liaw, A., & Wiener, M. (2002). Classification and Regression by randomForest. *R News*, *2*(December), 18–22. https://doi.org/10.1023/A:1010933404324

Lindsey, A. R. I., Bhattacharya, T., Newton, I. L. G., & Hardy, R. W. (2018). Conflict in the intracellular lives of endosymbionts and viruses: A mechanistic look at Wolbachia-

mediated pathogen-blocking. *Viruses*, *10*(4). https://doi.org/10.3390/v10040141

Lokmer, A., & Mathias Wegner, K. (2015). Hemolymph microbiome of Pacific oysters in response to temperature, temperature stress and infection. *ISME Journal*, *9*(3), 670–682. https://doi.org/10.1038/ismej.2014.160

McLean, B. J., Dainty, K. R., Flores, H. A., & O'Neill, S. L. (2019). Differential suppression of persistent insect specific viruses in trans-infected wMel and wMelPop-CLA Aedes-derived mosquito lines. *Virology*, *527*(August 2018), 141–145. https://doi.org/10.1016/j.virol.2018.11.012

Mee, P. T., Lynch, S. ., Walker, P. J., Melville, L., & Duchemin, J.-B. (2017). Detection of Elizabethkingia spp. in Culicoides Biting Midges, Australia. *Emerging Infectious Diseases*, *23*(8), 1409–1410. https://doi.org/10.1093/tropej/49.5.308

Mohanty, I., Rath, A., Swain, S. P., Pradhan, N., & Hazra, R. K. (2018). Wolbachia Population in Vectors and Non-vectors: A Sustainable Approach Towards Dengue Control. *Current Microbiology*, *0*(0), 0. https://doi.org/10.1007/s00284-018-1596-8

Moretti, R., Yen, P. S., Houé, V., Lampazzi, E., Desiderio, A., Failloux, A. B., & Calvitti, M. (2018). Combining Wolbachia-induced sterility and virus protection to fight Aedes albopictus-borne viruses. *PLoS Neglected Tropical Diseases*, *12*(7), 1–20. https://doi.org/10.1371/journal.pntd.0006626

Motzkin, T. (1949). The euclidean algorithm. In *Bulletin of the American Society* (pp. 1142–1146).

Nguyen, T. H., Nguyen, H. Le, Nguyen, T. Y., Vu, S. N., Tran, N. D., Le, T. N., … Hoffmann, A. A. (2015). Field evaluation of the establishment potential of wmelpop Wolbachia in Australia and Vietnam for dengue control. *Parasites and Vectors*, *8*(1), 1–14. https://doi.org/10.1186/s13071-015-1174-x

Paz, S., & Semenza, J. C. (2016). Correspondence El Niño and climate. *The Lancet*, *387*(10020), 745. https://doi.org/10.1016/S0140-6736(16)00256-7

Ramirez, J. L., Short, S. M., Bahia, A. C., Saraiva, R. G., Dong, Y., Kang, S., … Dimopoulos, G. (2014). Chromobacterium Csp_P Reduces Malaria and Dengue Infection in Vector Mosquitoes and Has Entomopathogenic and In Vitro Anti-pathogen Activities. *PLoS Pathogens*, *10*(10). https://doi.org/10.1371/journal.ppat.1004398

Romoli, O., & Gendrin, M. (2018). The tripartite interactions between the mosquito, its microbiota and Plasmodium. *Parasites and Vectors*, *11*(1), 1–8. https://doi.org/10.1186/s13071-018-2784-x

Ronquist, F., Teslenko, M., Van Der Mark, P., Ayres, D. L., Darling, A., Höhna, S., … Huelsenbeck, J. P. (2012). Mrbayes 3.2: Efficient bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology*, *61*(3), 539–542. https://doi.org/10.1093/sysbio/sys029

Rossi, P., Ricci, I., Cappelli, A., Damiani, C., Ulissi, U., Mancini, M. V., … Favia, G. (2015). Mutual exclusion of Asaia and Wolbachia in the reproductive organs of mosquito vectors. *Parasites and Vectors*, *8*(1), 1–10. https://doi.org/10.1186/s13071-015-0888-0

Russell, J. A., Sanders, J. G., & Moreau, C. S. (2017). Hotspots for symbiosis: Function, evolution, and specificity of ant-microbe associations from trunk to tips of the ant phylogeny (Hymenoptera: Formicidae). *Myrmecological News*, *24*(21), 43–69. https://doi.org/10.1128/AEM.01336-10

Shannon, C. E. (1964). A mathematical theory of communication. *ACM SIGMOBILE Mobile*

Computing and Communications Review, *5*(1), 3. https://doi.org/10.1145/584091.584093
Svetnik, V., Liaw, A., Tong, C., Christopher Culberson, J., Sheridan, R. P., & Feuston, B. P. (2003). Random Forest: A Classification and Regression Tool for Compound Classification and QSAR Modeling. *Journal of Chemical Information and Computer Sciences*, *43*(6), 1947–1958. https://doi.org/10.1021/ci034160g
Vallet-Gely, I., Novikov, A., Augusto, L., Liehl, P., Bolbach, G., Pechy-Tarr, M., … Lemaitre, B. (2009). Implication of the Mosquito Midgut Microbiota in the Defense against Malaria Parasites. *Applied and Environmental Microbiology*, *76*(3), 910–921. https://doi.org/10.1371/journal.ppat.1000423
Ward, J., & Hook, M. (1963). Application of an hierarchical grouping procedure to a problem of grouping profiles. *Educational and Psychological Measurement*, *XXIII*(1).
Weiss, B., & Aksoy, S. (2011). Microbiome influences on insect host vector competence. *Trends in Parasitology*, *27*(11), 514–522. https://doi.org/10.1016/j.pt.2011.05.001
Yukphan, P., Malimas, T., Potacharoen, W., Tanasupawat, S., Tanticharoen, M., & Yamada, Y. (2005). Neoasaia chiangmaiensis gen. nov., sp. nov., a novel osmotolerant acetic acid bacterium in the alpha-Proteobacteria. *J Gen Appl Microbiol*, *51*(5), 301–311. https://doi.org/10.2323/jgam.51.301
Zouache, K., Michelland, R. J., Failloux, A. B., Grundmann, G. L., & Mavingui, P. (2012). Chikungunya virus impacts the diversity of symbiotic bacteria in mosquito vector. *Molecular Ecology*, *21*(9), 2297–2309. https://doi.org/10.1111/j.1365-294X.2012.05526.x

Author Contributions

MGO: Involved in the study design, conducted the experiments, analyzed the results and drafted the paper
BS: Involved in the mosquito rearing, assisted in mosquito manipulation exercises
PA: Assisted in mosquito manipulation exercises
MN: Involved in the mosquito rearing, assisted in mosquito manipulation exercises
KL: Generated and propagated the ZIKV infectious clone utilised in this study
KLD: Involved in the study design, participated in the writing of the paper
CAT: Involved in the study design, participated in the writing of the paper

Figures

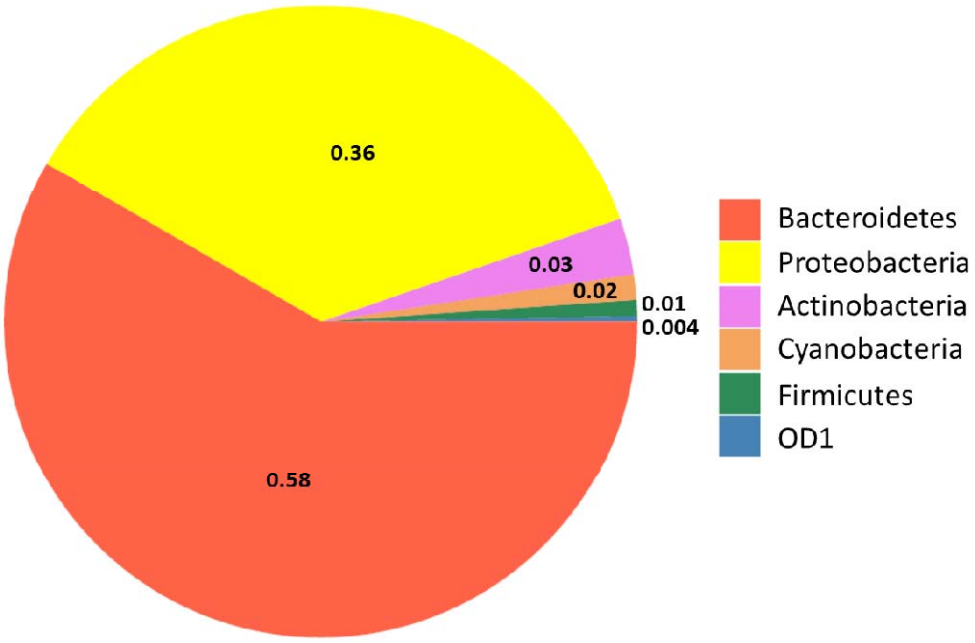

Figure 1. Proportions of bacterial phyla identified in *Ae. albopictus* midguts.

Data indicates proportions identified by 16s sequencing for all pools of midguts combined (n=34). Pools consisted of 3 individual midguts. All samples were acquired at 7 days post-feeding or at equivalent age for unfed individuals.

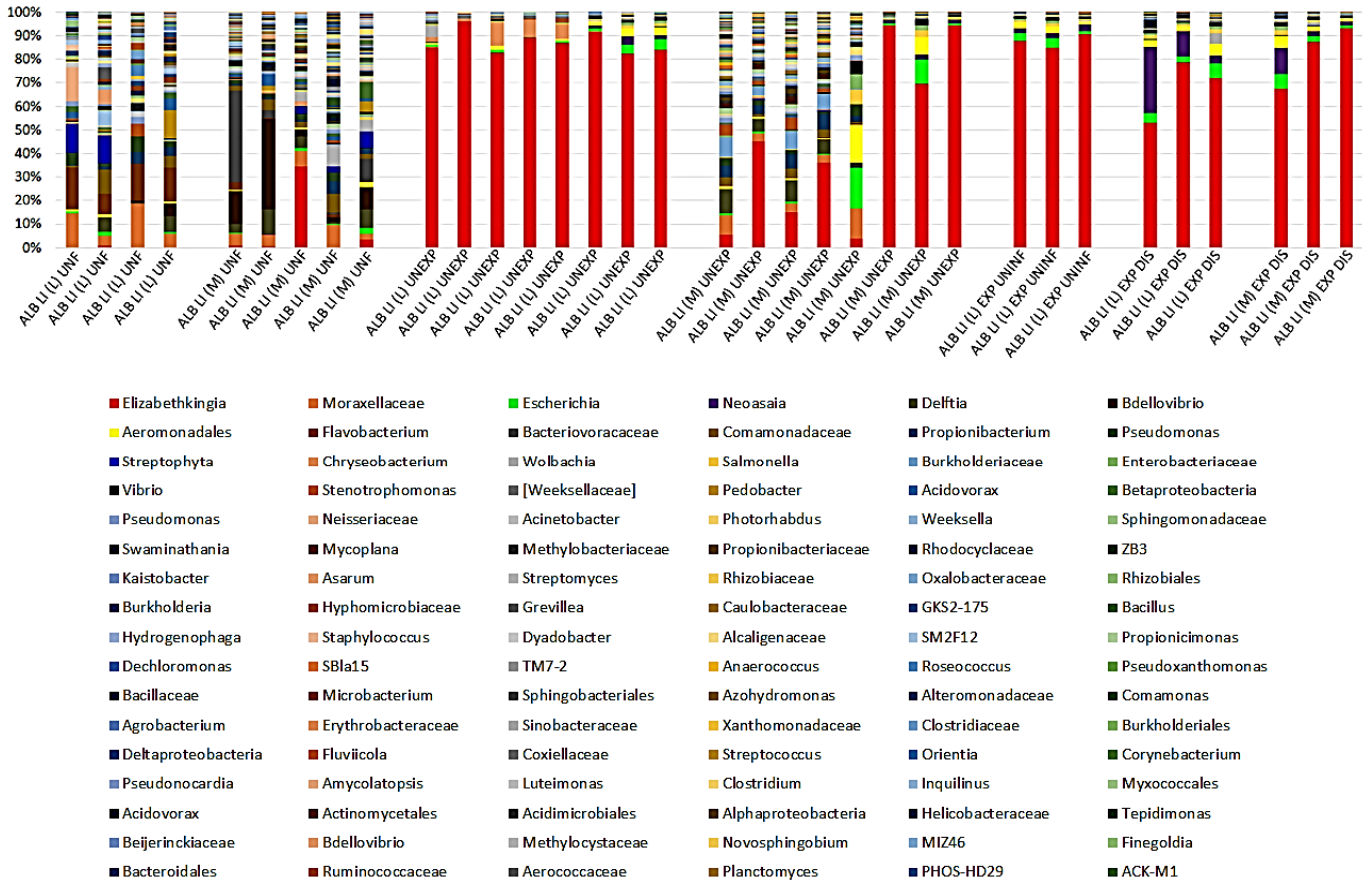

Figure 2. Relative abundance of bacterial genera in individual pools of *Ae. albopictus* midguts. Each stacked bar plot illustrates the taxa distribution at the genus level for pools of 3 *Ae. albopictus* midguts. Samples were taken at 7 days post-feeding or equivalent age in unfed (UNF) individuals. Blood fed individuals were further separated into UNEXP (fed non-infectious blood meal), EXP UNINF (fed ZIKV-positive blood meal but did not acquire infection) or EXP DIS (fed ZIKV positive blood meal and developed a disseminated infection). Mosquitoes were hatched and reared under two thermal conditions; 14 hours light at 30°C: 10 hours dark at 26°C (M temperature regimen) or 14 hours light at 28°C: 10 hours dark at 24°C (L temperature regimen).

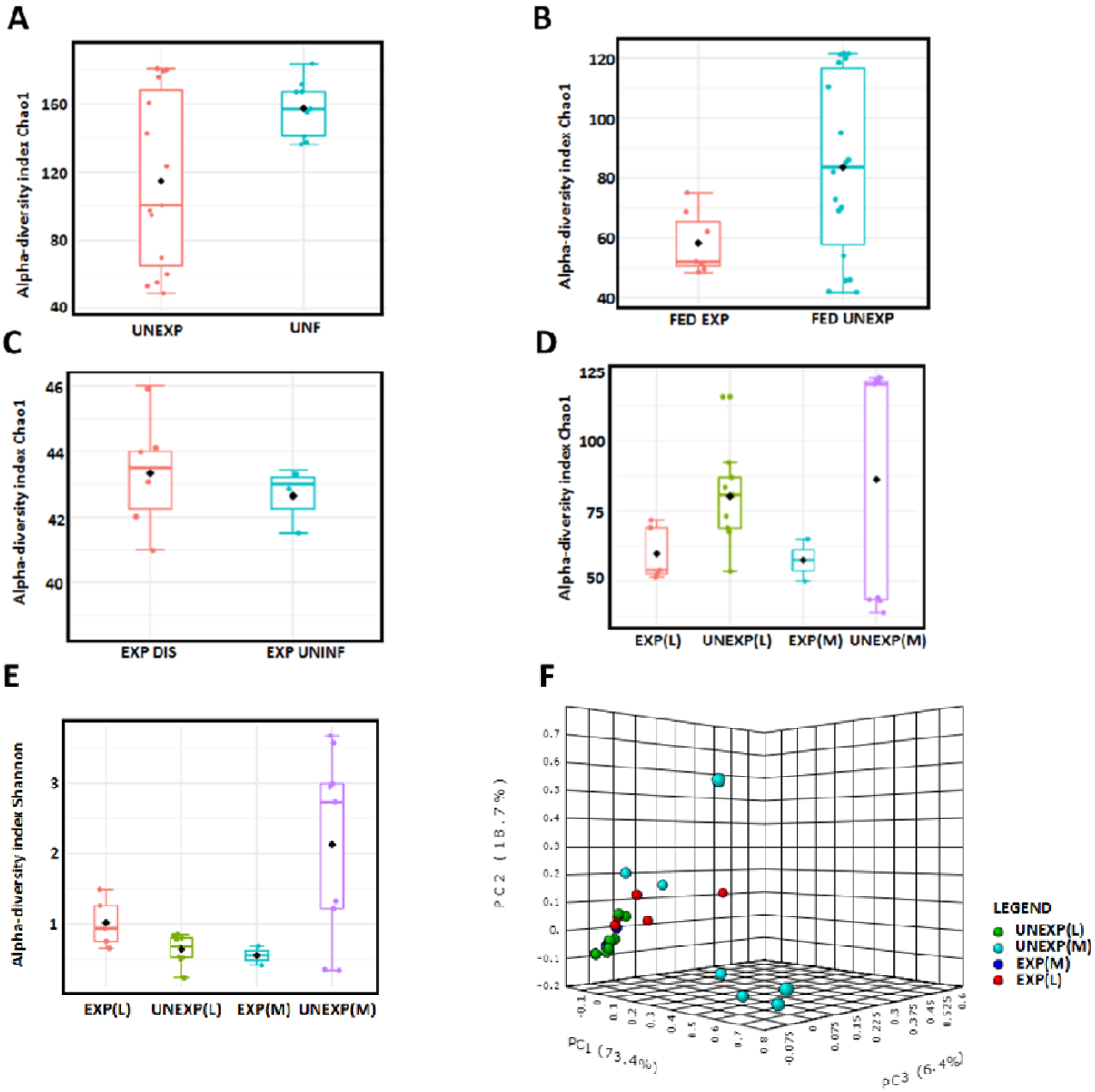

Figure 3. Blood feeding, ZIKV exposure and temperature have a significant effect on microbial diversity in *Ae. albopictus* midguts.

Distribution of alpha diversity within groups as measured by Chao1 or Shannon Index measures. The differences in diversity between groups was measured by Bray Curtis diversity

A. The effect of blood feeding on *Ae. albopictus* midgut microbial diversity. Comparisons of pools of midguts from unfed individuals (UNF) to those fed non-infectious blood meals demonstrates that blood meal acquisition independently decreases alpha diversity at 7 days post-feeding (chao1 T-test/ANOVA P(0.006) [T-test] statistic -3.1216). **B.** The effect of Zika virus exposure on *Ae. albopictus* midgut microbial diversity. Comparisons of pools of midguts from ZIKV exposed individuals (FED EXP) to those fed non-infectious blood meals demonstrates that ZIKV exposure independently decreases alpha diversity at 7 days post-feeding (chao1 T-test/ANOVA P(0.005) [T-test] statistic -3.1084). **C.** The effect of ZIKV infection on *Ae. albopictus* midgut microbial diversity. Comparisons of pools of midguts from ZIKV exposed individuals that were not infected (EXP UNINF) to those that were infected and developed disseminated infections (EXP DIS) demonstrates that ZIKV infection and dissemination do not independently decreases alpha diversity at 7 days post-feeding (chao1 T-test/ANOVA P(0.005) [T-test] statistic -3.1084). **D-F.** The effect of temperature on *Ae. albopictus* midgut microbial diversity. Although comparison of microbial diversity for individuals held at L and M temperature regimes demonstrates no statistical difference when using the Chao1 index (**D**), a significant temperature effect on alpha diversity is measured with the Shannon index (**E**; Shannon P(0.0065); [ANOVA] F-value: 5.4042) such that higher temperature are associated with decreased microbial diversity for ZIKV exposed individuals (EXP) but increased microbial diversity for individuals fed non-infectious blood meals (UNEXP). A significant effect of temperature on beta diversity was additionally measured (**F**; [PERMANOVA] F-value: 3.7463; R-squared: 0.34861; P[=0.018]).

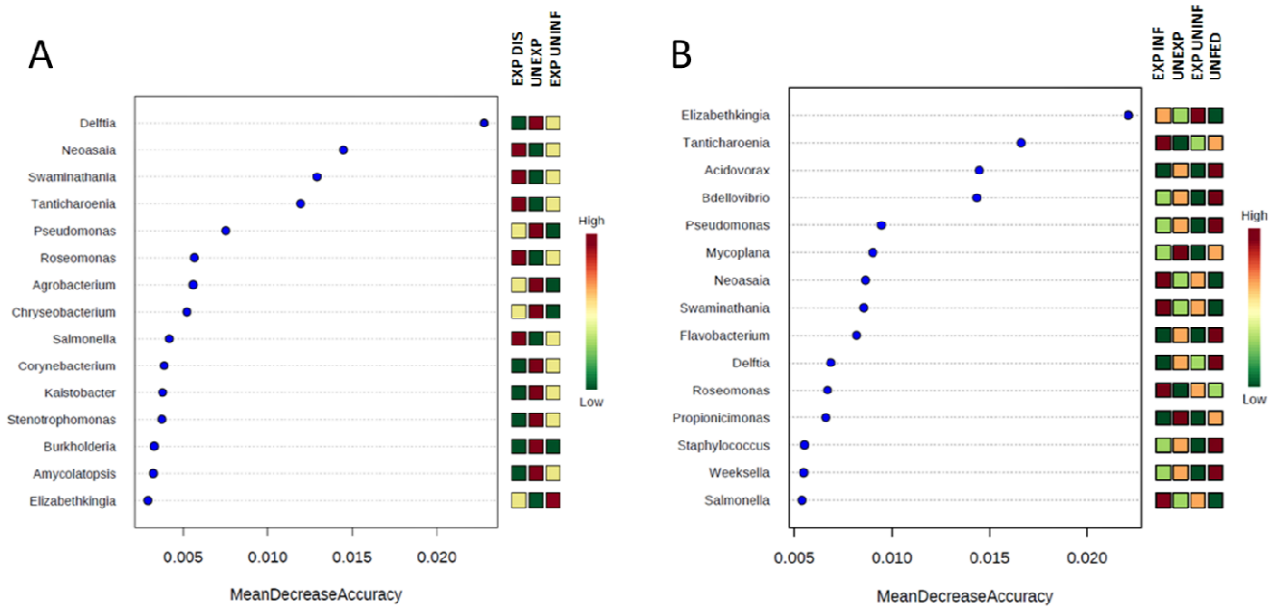

Figure 4. *Ae. albopictus* midgut microbial signatures associated with blood feeding, ZIKV exposure and ZIKV infection.

The Random forest classifier was used to determine the association of differentially abundant taxa in the microbial community with certain states. Mean Decrease Accuracy is reported for each of the taxa, which is obtained by removing the relationship of a taxa and measuring increase in error. The taxa with highest mean decrease in accuracy is considered to have the highest association with the state. **A.** Taxa were listed in order of the strength of their level of association with states including UNFED (no blood meal), UNEXP (blood fed non-infectious blood), EXP UNINF (infectious blood meal but no infection established) and EXP INF (ZIKV positive following infectious blood meal). **B.** Taxa were listed in order of the strength of their level of association with states including EXP DIS (fed ZIKV positive blood meal and developed a disseminated infection), EXP UNINF (fed ZIKV-positive blood meal but did not acquire infection) and UNEXP (fed non-infectious blood meal).

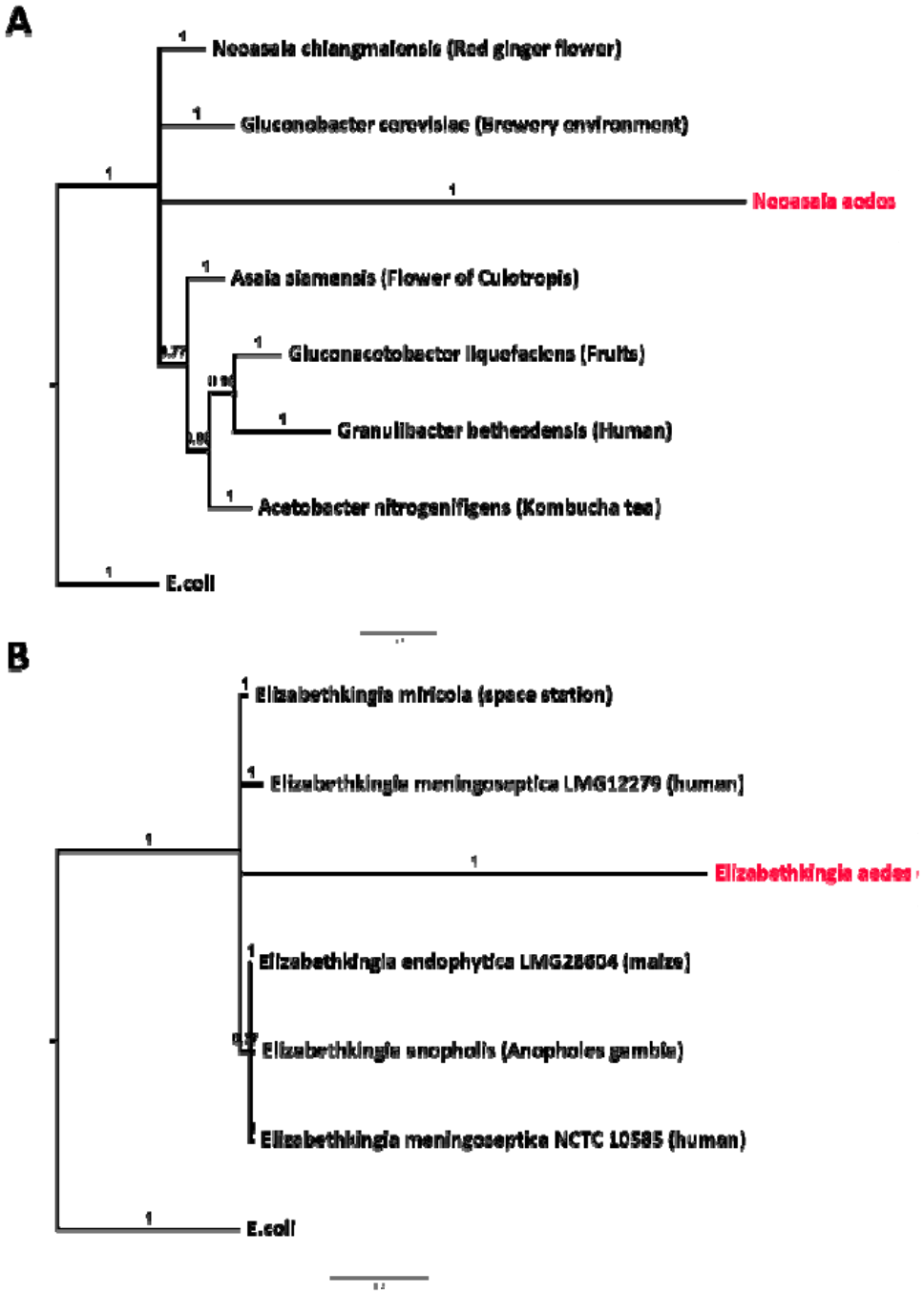

Figure 5. Phylogenetic analysis of *Neoasaia* and *Elizabethkingia rpoB* gene

Phylogenetic affiliation of the *Neoasaia* (**A**) and *Elizabethkingia* (**B**) taxa from the midgut of *Aedes* mosquitoes (shown in red) based on a partial sequence of the *rpoB* gene. Phylogenetic dendrograms were constructed in BAYES. Bootstrap proportions are shown on branches. *Escherichia coli* was used as an outgroup.

Supplementary Figure 1. A heat map showing the relative abundance of taxa at OTU level and their distribution across groups.