Potential for transmission of *Elizabethkingia anophelis* by *Aedes albopictus* and the role of microbial interactions in Zika virus competence

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Abstract

Elizabethkingia anophelis has been the cause of four outbreaks with significant morbidity and mortality. Its transmission routes remain unknown and no point source of infection has been identified. Here we show that *E. anophelis* can be found in the saliva of *Aedes* mosquitoes, suggesting the novel possibility of vector-borne transmission of this bacterium. We additionally characterized diverse microbial communities in *Aedes* midguts, salivary glands and saliva. To the best of our knowledge, this represents the first description of the microbiome of *Aedes* saliva. Further, we demonstrate that increased abundance of *E. anophelis* is associated with decreased susceptibility and replication of Zika virus (ZIKV) in the midgut of *Aedes* mosquitoes, suggesting a novel transmission barrier for arboviruses transmitted by *Aedes* mosquitoes. Together, these results demonstrate the complex relationships between the mosquito, the midgut microbial community and arboviruses and offer insights into the epidemiology and control of emerging bacterial and viral pathogens.

Keywords: *Elizabethkingia anophelis*, Zika virus and *Aedes* mosquitoes

Author Summary

Elizabethkingia anophelis has in the recent past caused outbreaks different parts of the world resulting both in morbidity and mortality. Until now, to the best of our knowledge, no study has been able to demonstrate that this bacterium can be transmitted by mosquitoes. We have demonstrated for the first time that *Elizabethkingia anophelis* is present in the saliva of both infected and non-infected *Aedes* mosquitoes. Further, we have shown that it confers an inhibitory effect on Zika virus establishment in the midguts of *Aedes* mosquitoes. Together, these results potentially display the potential for vector borne transmission of *E. anophelis* as

46 well as a novel transmission barrier of ZIKV. Lastly, we have for the first time characterized
47 salivary microbes of *Aedes* mosquitoes necessitating the investigation of the impact of salivary
48 microbes in severity of disease in vertebrate hosts.

49
50
51 **Introduction**
52
53 *Elizabethkingia* is a gram-negative rod-like, aerobic, non-fermenting, non-motile and non-spore
54 forming bacteria widely distributed in natural environments, including soil and fresh water as
55 well as hospital environments [1]. It was originally called Flavobacterium or belonged to CDC
56 group IIa but later reclassified as Chryseobacterium in 1994 [2]. On the basis of shared
57 phenotypic and phylogenetic characteristics, *Chryseobacterium meningosepticum* and *C.*
58 *miricola* were re-classified to a new genus, *Elizabethkingia* [3]. The most clinically important
59 species of the genus *Elizabethkingia* include *E. anophelis, E. meningoseptica and E. miricola* [4].
60 *E. anophelis*, originally isolated from the midgut of *Anopheles gambiae* [5] has been the cause
61 of moderate to large-scale outbreaks resulting in morbidity and mortality [6–10] as well as
62 clinical cases across different geographical settings [1,4,11,12] . *E. anophelis* is associated with
63 clinically significant infections and high mortality [4] relative to *E. meningoseptica* [9].
64 The genus *Elizabethkingia* is prevalent in *Aedes* [13–15] and *Anopheles* [16–18] species of
65 mosquitoes and has been detected in both field-caught and laboratory-reared mosquitoes in
66 Africa, Europe and North America [15,18,19]. The microbe can be transmitted among
67 mosquitoes vertically, transstadially and horizontally [13,17]. In humans, evidence for perinatal
68 vertical transmission of *E. anophelis* has been identified and associated with neonatal
69 meningitis [11]. While some infections are thought to be nosocomial and mechanical

70 ventilation has been identified as a risk factor for transmission in hospital settings [1],
71 transmission routes have not been identified in a number of recent individual cases or clusters.
72 The transition of *Elizabethkingia* from a common environmental bacterial species to a human
73 pathogen of clinical significance, as well as the previous identification of *Elizabethkingia* in a
74 number of mosquito species, prompted us to assess the abundance and tropism of
75 *Elizabethkingia* bacteria in *Aedes* mosquitoes. We identified *Elizabethkingia* in midguts, salivary
76 glands and saliva of *Ae. albopictus*. Sequencing of *Elizabethkingia* identified in the salivary gland
77 demonstrated 100% homology to *E. anophelis*.
78 In order to assess the relative abundance of *Elizabethkingia* within microbial communities in
79 these tissues, we additionally performed 16S *rRNA* sequencing. Further, to better define
80 interactions with arboviruses vectored by *Aedes* mosquitoes, we assessed the correlation
81 between *Elizabethkingia* and other microbes with Zika virus (ZIKV) in the midgut of *Ae.*
82 *albopictus*. We measured a negative correlation between *E. anophelis* and ZIKV in the midgut
83 and in mosquito cell culture. These data together demonstrate the complex interactions
84 between microbial communities and arboviruses that could have important implications for the
85 emergence and transmission of both viral and bacterial pathogens.

86 **Results**
87
88 **Microbial profiles in *Aedes albopictus* are tissue-specific**

89 Illumina MiSeq 16S *rRNA* sequencing resulted in 2 369 009 reads. The number of reads for
90 individual samples ranged from 11 925 - 105 039 with a mean of 67 334. Both the positive
91 spike-in (PhiX) and negative controls (water and non-template control) had limited number of
92 sequences reads, ranging from 1620 – 2823, and hence were discarded in downstream analysis.

93 In order to control for alpha and beta diversity biases, each sample was rarefied to a total
94 frequency of 30 000 reads, hence two samples were discarded from downstream analysis. A
95 total of 154 OTUs were identified in this study, 59% were Bacteroidetes, 40% Proteobacteria
96 and 1% Cyanobacteria. In order to visualize the distribution of bacterial genera across individual
97 tissues, an additional cut-off read frequency of 7 000 was applied (Figure 1).
98 The microbial profile was diverse across tissues (PERMANOVA, F-value=0.9998, R^2= 0.3111; P
99 <0.001; Figure 2). Midguts were dominated by Bacteroidetes with a higher relative abundance
100 identified in ZIKV infected midguts relative to the unexposed midguts. Salivary glands, on the
101 other hand, were dominated by Proteobacteria. Surprisingly, bacterial taxa from mosquito
102 saliva was much more diverse with a range of Bacteroidetes, Proteobacteria and Cyanobacteria
103 (Chao1 [ANOVA], P < 0.001, F-value= 20.58). This analysis clearly demonstrates the dominance
104 of specific genera in individual tissues. For instance, ZIKV infected midguts and salivary glands
105 were overwhelmingly dominated by *Elizabethkingia*. Uninfected midguts were dominated by
106 either *Elizabethkingia* or *Wolbachia*, yet salivary glands from uninfected individuals were
107 dominated by Enterobacteriaceae (Figure 1).
108
109 ***Aedes* saliva contains diverse microbiota including *Elizabethkingia anophelis***
110
111 The core microbes in the saliva included: *Pseudomonas*, *Cytophagaceae*, *Chitinophagaceae*,
112 *Betaproteobacteria*, *Gammaproteobacteria*, *Comamonadaceae*, *Chryseobacterium*,
113 *Flavisolibacter*, *Bradyrhizobiaceae*, *MLE1_12* and *Sphingomonas*.
114 The most abundant taxa present in the saliva (*Elizabethkingia*, *Chitinophagaceae*,
115 *Enterobacteriaceae*, *Wolbachia*, *Pseudomonas* and *Betaproteobacteria*) were also present in
116 the salivary glands and midgut samples (Figure 1).

117 We observed presence of taxa of public health importance in the saliva of *Ae. albopictus*
118 exposed to infectious and non-infectious blood meals (Figure 1). These include *Elizabethkingia*,
119 *Enterobacteriaceae* and *Pseudomonas*.
120 We employed a quality control measure to assess potential sources of saliva contamination. A
121 cotton pad obtained from the container housing mosquitoes exposed to an infectious blood
122 meal, a non-infectious blood meal, a sterile cotton pad, filter sterilized 10% sucrose solution
123 used to feed mosquitoes, and filter sterilized mosquito diluent used to collect mosquito saliva,
124 were incubated overnight in LB media. The control was sterile LB media. Bacterial growth was
125 observed in the culture tube that had the cotton pad used to feed both mosquitoes exposed to
126 the infectious blood meal as well as the non-infectious blood meal. No growth occurred in the
127 culture tube with sterile cotton pad, sterile 10% sucrose solution, the mosquito diluent or the
128 negative control. Introduction of bacteria during feeding by mosquitoes cannot be ruled out for
129 taxa identified exclusively in saliva.
130 *Elizabethkingia* was present in both ZIKV -infected and uninfected midguts, salivary glands and
131 saliva. On the other hand, *Enterobacteriaceae* and *Pseudomonas* were present in the saliva and
132 salivary glands, but not midguts, of *Ae. albopictus* exposed to both ZIKV (infectious) and non-
133 infectious blood meals (Figure 1; Figure 3).
134
135 **Relationship between *Elizabethkingia* and ZIKV in *Ae. albopictus***
136 The 16S *rRNA* amplicons of saliva, salivary glands and midgut samples were sequenced on the
137 Illumina MiSeq platform. *Elizabethkingia* was identified in both saliva (10 out of 18) and
138 salivary gland (6 out of 6) samples of *Ae. albopictus* exposed to non-infectious and infectious

139 blood meals (Figure 1). *Elizabethkingia* sequences were also present in the midguts of *Ae.*
140 *albopictus* exposed to infectious or non-infectious blood meal (10 out of 10) (Figure 1).
141 Furthermore, a higher proportion of *Elizabethkingia* sequences were identified in saliva
142 collected following non-infectious blood meals relative to the saliva collected following ZIKV
143 infection (T- test; Mann Whitney test, P = 0.0256; Figure 3; Figure 4A). Conversely, while
144 *Elizabethkingia* was identified in both the ZIKV-infected and uninfected *Aedes* salivary glands,
145 ZIKV-infected salivary glands had a higher proportion of *Elizabethkingia* reads relative to their
146 uninfected counterparts (F test, P< 0.0001; Figure 4A). Overall, the ZIKV - infected *Ae.*
147 *albopictus* midguts had a higher relative amount of *Elizabethkingia* compared to other tissues
148 (ANOVA; Kruskal-Wallis test, P = 0.0027; Figure 4A).
149 Although exposure of *Ae. albopictus* midguts to non-infectious blood meal resulted in an
150 increase in the amount of *Elizabethkingia* in the midgut, Real-Time (RT) qPCR assay
151 demonstrated a higher load of *Elizabethkingia* in the midguts exposed to ZIKV infectious blood
152 meals as compared to the *Ae. albopictus* midguts exposed to non-infectious blood meals (T-
153 test, Mann Whitney test, P = 0.0051; Figure 4B).
154 To further investigate the relationship between *Elizabethkingia* and ZIKV measured in *Ae.*
155 *albopictus* midguts, *Elizabethkingia* bacterial loads were quantified together with ZIKV viral
156 loads both *in vivo* (ZIKV-infected midguts, (N = 22) and *in vitro* (U4.4 cells co-infected with
157 *Elizabethkingia* and ZIKV; three biological replicates, 1 biological replicate, n = 4 wells) using RT-
158 qPCR.
159 Despite the fact that feeding on a ZIKV infectious blood meal was associated with higher levels
160 of *Elizabethkingia* (Figure 4B), fold change in expression of the *Elizabethkingia rpoB* gene from

161 ZIKV-infected *Ae. albopictus* (N = 22) was negatively correlated to ZIKV absolute quantities

162 (Spearman r = -0.3985; P < 0.05; Figure 5A). ZIKV viral loads ranged from 3 - 11 $\log_{10}$ ZIKV RNA

163 copies/ml, while fold change of *Elizabethkingia* ranged from -1.8 - 2.2 $\log_{10}$ CFU/ml.

164 To clarify the potential for direct interaction between *Elizabethkingia* and ZIKV, we performed

165 an *in vitro* assay on the *Ae. albopictus* derived U4.4 cell line. Cells were co-infected with

166 *Elizabethkingia* and ZIKV or infected with ZIKV only, and viral loads were compared.

167 At 2 dpi, we measured high amounts of ZIKV in the control, ZIKV/*Elizabethkingia* OD 1.0 (8.5

168 Logs CFU/ ml) and ZIKV/ *Elizabethkingia* OD0.5 (8.0 Logs CFU/ml) co-infected cells while the

169 ZIKV/ *Elizabethkingia* OD0.2 (7.7 Logs CFU/ml) co-infected cells had significantly lower ZIKV

170 titers (ANOVA; Kruskal-Wallis test, P = 0.0070). On the other hand, at 4 dpi, we measured a

171 significant reduction in ZIKV titers in all ZIKV/*Elizabethkingia* co-infected U4.4 cells relative to

172 the control (ANOVA, Kruskal-Wallis test, P = 0.0079; Figure 5B).

173

174 **Phylogenetic analysis of *Elizabethkingia***

175 To taxonomically classify the *Elizabethkingia* strain that we identified we sequenced the

176 *Elizabethkingia rpoB* gene amplified from the cDNA of the *Ae. albopictus* salivary gland. The

177 phylogenetic analysis demonstrated that the *Elizabethkingia anophelis* strain identified in our

178 study has a 100% sequence identity to *Elizabethkingia anophelis* which was originally isolated

179 from the midgut of *Anopheles gambiae* [20]. This strain has a 99% identity to *Elizabethkingia*

180 *endophytica*, originally isolated from sweet corn [21]. It was closely related to the

181 *Elizabethkingia genomosp*. 4 [22], *Elizabethkingia miricola,* isolated from condensation water in

182 space station [23] and *Elizabethkingia bruuniana*[24]. *E. anophelis* has a 92% identity to

183 *Elizabethkingia meningosepticum*, originally isolated from human cerebrospinal fluid [25]
184 (Figure 6).
185
186 **Discussion**
187 This study has demonstrated *Elizabethkingia* in the saliva of *Ae. albopictus* mosquitoes. Further,
188 phylogenetic analysis has identified the strain of *Elizabethkingia* isolated from an *Ae. albopictus*
189 salivary gland to have 100% sequence identity to *Elizabethkingia anophelis,* first isolated from
190 the midgut of the mosquito *Anopheles gambiae*[26]. The *Elizabethkingia* genus was previously
191 found to be the dominant bacterial genera in the salivary glands of both *An. gambiae* and *An. coluzii*
192 [27]. To the best of our knowledge, this is the first study to demonstrate *E. anophelis* in the
193 saliva of *Aedes* mosquitoes. Our findings bear clinical significance as *E. anophelis* has been
194 identified as a cause of sepsis in adults and children, as well as a cause of neonatal meningitis
195 [9]. By 2016, *E. anophelis* had caused an outbreak in Central African Republic [6], Singapore [7]
196 and two outbreaks in the Midwest, United States that resulted in morbidity and mortality of ~
197 30% [8–10]. The Midwest outbreak occurred primarily in community settings [8] and despite
198 extensive investigations, no point source of infection has been identified in either the Midwest
199 or Singapore outbreaks [9,10]. While there is currently no evidence of mosquito-borne
200 transmission with any of these outbreaks, our data demonstrate the potential for involvement
201 of mosquitoes in the maintenance of *E. anophelis* and other bacterial pathogens.
202 The identification of *Elizabethkingia* in the saliva of *Ae. albopictus* infected with ZIKV
203 necessitates the investigation of the impact of *Elizabethkingia* on transmission efficiency of
204 ZIKV and other arboviruses, and additionally on how co-transmission of bacteria could influence

205 the early events, progression and severity of viral infections in the host. Our results present a
206 new dimension into the vector-host interface dynamics. Several molecules present in mosquito
207 saliva have been shown to suppress the host inflammatory or immune responses [28–33] thus
208 enhancing dissemination [34]. Specific salivary proteins such as biogenic amine-binding D7
209 protein [35], saliva serine protease CLIPA3 [36], salivary factor LTRIN [37] and saliva protein
210 AgBR1[38] modulate vertebrate infection. However, a gap in knowledge exists on the
211 contributory role of mosquito salivary microbes in potentiating pathogenicity or transmission
212 efficiency in vertebrate hosts. Salivary microbes may provide crucial targets for the control of
213 disease-causing pathogen transmitted by mosquitoes.
214 In addition to *Elizabethkingia*, we observed a proliferation of other taxa associated with human
215 disease throughout *Aedes* mosquitoes following exposure to a blood meal. This included
216 bacteria belonging to the family Enterobacteriaceae, which have been shown to be
217 opportunistic pathogens [39–41]. Enterobacteriaceae are gram negative bacteria that includes
218 *Escherichia coli*, *Klebsiella*, *Salmonella*, *Shigella* and *Yersinia pestis*. *Enterobacter* spp., which we
219 identified as among the primary bacteria associated with *Ae. albopictus*. These bacteria are
220 known to produce molecules with hemolytic activity [42,43] and the increase in abundance of
221 Enterobacteriaceae in the saliva of *Ae. albopictus* may therefore be an evolutionary mechanism
222 that exploits blood digestion to ensure their horizontal transfer as well as infection of new
223 hosts.
224 *Wolbachia* was identified in the saliva, salivary gland and midguts of *Ae. albopictus* in this study.
225 *Wolbachia* is an intracellular endosymbiont primarily transmitted vertically through the female
226 germline[44,45]. It can also undergo transfer between somatic and germ line cells. Horizontal

227 transfer independent of cell to cell contact utilizing host cell phagocytic and clathrin/dynamin-
228 dependent endocytic machinery is possible [46]. Our results provide further proof of the
229 possibility of horizontal transfer of *Wolbachia* through saliva. *Wolbachia* has been identified in
230 the phloem vessels and in some novel "reservoir" spherules along the phloem after *Wolbachia*-
231 infected whiteflies fed on cotton leaves [47]. These findings could have important implications
232 for the exploitation of *Wolbachia* as a biological control of tropical diseases.

233 A significant reduction in *Ae. albopictus* midgut microbial diversity resulting from ZIKV
234 infection was evident, yet the effect on individual genera was highly variable. Available
235 evidence demonstrates that commensal microbiota regulate and are in turn regulated by
236 invading viruses [48–60]. Viral infection often causes disturbance of the commensal host
237 microbiota, resulting in dysbiosis and subsequently affecting viral infectivity of the host
238 [50,51,56,61–65]. The dysbiosis of the commensal microbiota due to virus infection is mainly
239 due to modulation of the immune responses by the virus resulting in immune suppression
240 [66,67]. Our results are consistent with the findings of a study on the response of the total gut
241 microbiota diversity of *A. japonicus* and *A. triseriatus* to infection with La Crosse virus, which
242 revealed higher midgut bacterial richness among the midguts obtained from newly emerged
243 individuals and lower richness among midguts exposed to viral infection or a non-infectious
244 blood meals [50]. In addition, our study corroborates previous findings with West Nile virus
245 infection on the bacterial diversity of *Culex pipiens*, which demonstrated that lower diversity
246 was associated with virus exposure[51].

247 It is also notable that *Elizabethkingia* and *Wolbachia* appeared to have an exclusive presence in
248 the midgut of the *Ae. albopictus* mosquitoes. It is possible that there is competitive exclusion of

249 *Wolbachia* in the midgut by *Elizabethkingia*. Studies have shown that *Ae. albopictus* and *Cx.*
250 *quinquefasciatus* naturally infected by both *Asaia* and *Wolbachia* have a co-exclusion
251 relationship[51]. In species uninfected by *Wolbachia* such as *An. gambiae*, *An. stephensi* and
252 *Ae. aegypti*, *Asaia* is capable of colonizing reproductive organs and salivary glands [68]. The
253 predominance of *E. meningosepticum* in the *An. stephensi* midgut was shown to be due to its
254 antimicrobial properties suppressing the growth of other midgut bacteria [69].
255 We demonstrated a significant negative correlation between ZIKV and *Elizabethkingia* as well
256 as a reduction in the ZIKV titer in U4.4 cells co-infected with *Elizabethkingia* and ZIKV. Similarly,
257 it has been demonstrated that *E. meningoseptica* metabolites obtained from the midgut of
258 *Anopheles stephensi* exhibited antiparasitic effect on *P. falciparum* parasites *in vitro* and
259 demonstrated *in vitro* toxicity on gametocytes[69]. We also measured a significant increase in
260 *Elizabethkingia* bacterial load in the midgut infected with ZIKV relative to those exposed to a
261 non-infectious blood meal. These findings corroborate the results of a previous study aimed at
262 understanding the effect of ZIKV on the dynamic bacterial community harbored by *Ae. aegypti*
263 [70], which demonstrated that the Flavobacteriacae family was the most abundant bacterial
264 taxa. This was particularly the case among the ZIKV-exposed blood fed individuals, yet
265 Flavobacteriacae decreased in abundance among ZIKV-exposed gravid individuals [70]. The
266 interactions between bacteria and viruses are clearly complex. The mechanisms of interaction
267 mostly benefit virus infiltration into cells directly by virus binding to a bacterial cell or viral
268 utilization of a bacterial product [71]. Indirect mechanisms include: virus-induced increase of
269 bacterial receptor concentrations; virus damage to underlying epithelial cells; virus
270 displacement of commensal bacteria and virus suppression of the host immune system[71]. In

271 the direct interactions, the commensal bacteria are considered capable of outcompeting viral
272 pathogens and limiting tissue accessibility by competing for receptor binding sites reducing the
273 binding of pathogenic bacteria or viral attachment. This may potentially explain the negative
274 correlation that we observed in *Ae. albopictus* midguts. Our *In vitro* results which demonstrate
275 decreased viral loads with *Elizabethkingia* co-infection, particularly at day 4 post-infection,
276 corroborate previous studies [70] and clearly demonstrate the capacity for this bacterium to
277 independently suppress viral replication and/or persistence. Further studies delineating the
278 mechanism that *Elizabethkingia* utilizes to inhibit viral fitness in *Ae. albopictus* are needed, yet
279 these data suggest the identification of a novel transmission barrier that could ultimately be
280 exploited in the design of novel control measures.
281 Overall, to the best of our knowledge, this is the first study to demonstrate and characterize the
282 microbiome of the saliva of mosquitoes. These data may have significant implications on the
283 pathogen - host interface and our understanding of the impact of these microbes on the
284 severity of disease in the vertebrate host. We have also demonstrated that the salivary gland
285 and saliva of *Aedes* mosquitoes exposed to infectious or non-infectious bloodmeals contain the
286 bacterial pathogen *Elizabethkingia anophelis*. Finally, our study has demonstrated both by *in*
287 *vivo* and *in vitro* studies that *Elizabethkingia* and ZIKV are negatively correlated in the mosquito
288 midgut. These findings together have implications for our understanding of the interaction of
289 bacterial and viral agents in mosquitoes that could ultimately improve our understanding of
290 disease epidemiology and inform novel control measures.
291
292
293
294

295 **Materials and methods**
296
297 **Virus**
298
299 This study utilized frozen virus sample. The ZIKV strain was HND (2016-19563, GenBank
300 accession no. KX906952).
301
302 **Insect sampling and DNA preparation.**
303 *Ae. albopictus* were collected from Suffolk County, NYS in 2015 (kindly provided by I. Rochlin)
304 and colonized at the New York State Department of Health (NYSDOH) Arbovirus Laboratory.
305 F15 *Ae. albopictus* were vacuum-hatched and maintained at 27°C under standard rearing
306 conditions. At eight days post mating, female adults were orally exposed to blood meals. The
307 blood meal consisted of either 1:1 dilution of defibrinated sheep blood plus 2.5% sucrose;
308 sodium bicarbonate (to adjust pH to 8.0) and virus, or a non-infectious blood meal containing a
309 final concentration of 2.5% sucrose solution. Infectious blood meals contained 8.3 $\log_{10}$ PFU/ml
310 ZIKV HND [72]. The female mosquitoes were exposed to blood meals in a 37°C pre-heated
311 Hemotek membrane feeding system (Discovery Workshops, Acrington, UK) with a porcine
312 sausage casing membrane. After an hour, the mosquitoes were anaesthetized with CO_2 and
313 immobilized on a pre-chilled tray connected to 100% CO_2. Engorged females were separated
314 and placed in three separate 0.6 L cardboard cartons (30 individuals per carton). In addition, 1
315 ml of each blood meal was transferred to a 1.5 ml Safe Seal microtube (Eppendorf, Hamburg
316 Germany) and stored at -80°C. Blood fed females were maintained on 10% sucrose solution
317 provided *ad libitum*.
318 At 14 dpi, the female mosquitoes were immobilized using triethylamine (Sigma Aldrich, St.
319 Louis, MO, USA). To examine for ZIKV dissemination in the mosquito, the legs were removed

320 from the mosquitoes before dissecting the gut and salivary gland from every individual. Saliva
321 was collected under sterile conditions by inserting the proboscis of the female mosquitoes into
322 a capillary tube containing ~20 µl fetal bovine serum plus 50% sucrose 1:1 for 30 minutes and
323 subsequently ejecting the mixture into 125 µl Mosquito Diluent (MD; 20% heat-inactivated fetal
324 bovine serum in Dulbecco phosphate-buffered saline plus 50 µg/ml penicillin/streptomycin, 50
325 µg/ml gentamicin and 2 µg/ml Fungizone (Sigma Aldrich, St. Louis, MO, USA). The midgut was
326 dissected from individual mosquitoes and rinsed twice in sterile PBS before transfer to sterile
327 microcentrifuge tubes and storage at -80°C until tested. Mosquito carcass and legs were then
328 placed in individual tubes containing 500 µl MD and a bead. All samples were held at -80°C until
329 assayed. A ZIKV-specific quantitative PCR assay that targets the NS1 region was utilized to
330 obtain viral titer [73] from the legs, carcass, saliva, salivary glands and midguts as described by
331 [72].
332 As a precautionary measure to prevent contamination of saliva, all the reagents used during the
333 rearing and blood feeding of mosquitoes were filter sterilized and cotton pads were autoclaved.
334 To test for potential contamination during rearing, we incubated overnight (in LB media) a
335 piece of cotton pad obtained from the gallon housing mosquitoes exposed to an infectious
336 blood meal, mosquitoes exposed to a non-infectious blood meal, a sterile cotton pad not
337 exposed to mosquitoes, filter sterilized 10% sucrose solution that was used to feed the
338 mosquitoes, filter sterilized mosquito diluent, and sterile LB Media used as a control.
339
340 **Sequencing and analysis of microbiome of *Aedes* mosquito saliva, salivary glands and**
341 **midguts.**

342 To determine the microbial profile in the *Ae. albopictus* saliva, salivary glands and midguts of
343 each individual mosquito, midgut, legs, carcass and saliva samples were screened for virus. The
344 individuals that were successfully infected and or transmitting the virus were identified. The
345 cDNA was prepared from the RNA samples using iScript cDNA kit (Biorad, Hercules, California).
346 To amplify starting material for downstream analysis, the saliva, salivary gland and midgut
347 samples were individually whole transcriptome amplified according to the REPLI-g WTA single
348 cell kit protocol (Hilden, Germany). The resulting amplified cDNA was diluted 1:100. Thereafter,
349 16S *rRNA* amplicon were amplified using the 16S primer set and conditions in Table 1.
350 The barcoded high-throughput amplicon sequencing of the bacterial 16S V3-V4 hypervariable
351 *rRNA* was done at Wadsworth Center Applied Genomics Core (WCAGC). PCR reactions were
352 carried out in a total volume of 50 µl, 5µM of 16S *rRNA* V3-V4 hypervariable region primers
353 consisting of an Illumina barcode (Table 1) [51], 2 µl WTA cDNA, 8 µl deionized filter sterilized
354 water and 36 µl AccuStart II PCR supermix (Quanta biosciences, Beverly, USA). To ensure that
355 any potential contamination either at the PCR stage or at the sequencing step would be
356 identified, two negative controls: water and non-template control were included at the PCR
357 step and sequenced as well as a positive spike included during the sequencing run. A fragment
358 size of ~460 bp of each sample was submitted to the WCAGC for sequencing together with the
359 negative controls. Automated cluster generation and paired-end sequencing (250-bp reads)
360 was performed on the Illumina MiSeq 500 cycle.
361 A total of 37 samples [6 infected midguts (INF MG); 4 non-infected midguts (NINF MG); 12
362 infected saliva (INF SAL); 6 non-infected saliva (NINF SAL); 3 infected salivary glands (INF SALG);

363 3 non- infected salivary gland (NINF SALG); 1 negative control (NC); 1 non-template control
364 (NTC) and 1 positive spike (PC) were included in this study.
365 Analysis of the data was carried out on QIITA (https://qiita.ucsd.edu/) and MicrobiomeAnalyst
366 [74]. In summary, to convert the de-multiplexed FASTQ files to the format used by QIITA, the
367 command split libraries FASTQ was used. The clustering of the sequences into operational
368 taxonomic units (OTUs), was done by the utilization of a closed-reference OTU picking based on
369 the GreenGenes 16S reference database which matches the sequences based on a 97%
370 sequence identity threshold before assigning a taxonomy. A BIOM-formatted OTU table was
371 subsequently generated, to reduce the potential of alpha and beta diversity biases, the data
372 was rarefied to a depth of 30 000 reads per sample. Finally, to visualize the taxonomic profiles
373 of each sample, taxa bar plots were generated using the rarefied data artifact. Following the
374 generation of the relative quantity of each taxon, we generated a taxa bar plot based on taxa
375 with sequences ≥ 4 000.
376 The reads have been deposited in NCBI GenBank Short Read Archives with accession numbers:
377 PRJNA608507, PRJNA608505, PRJNA608503, PRJNA608501, PRJNA608500, PRJNA608499,
378 PRJNA608472, PRJNA608496, PRJNA608495, PRJNA608493, PRJNA608492, PRJNA608491,
379 PRJNA608489, PRJNA608483, PRJNA608471, PRJNA608482, PRJNA608470, PRJNA608456,
380 PRJNA608450, PRJNA608481, PRJNA608449, PRJNA608448, PRJNA608480, PRJNA608479,
381 PRJNA608477, PRJNA608437, PRJNA608434, PRJNA608427, PRJNA608428, PRJNA608429,
382 PRJNA608375, PRJNA608426, PRJNA608424, PRJNA608416, PRJNA608418, PRJNA608422,
383 PRJNA608436 and PRJNA608419.

384 The alpha diversity profiling (the number of species of taxa in a single sample) was calculated
385 using the Chao1 diversity measure and significance testing was done using the ANOVA method
386 in Microbiome Analyst. Chao1 index estimates the richness of taxa in a sample by extrapolating
387 out the number of rare organisms that may have been omitted due to under sampling. The
388 index assumes that the number of observations for a taxon has a Poisson distribution and
389 hence corrects for variance [75].
390 Beta diversity, a comparison of microbial community composition between two samples or two
391 conditions, was analyzed by utilizing the Bray-Curtis dissimilarity statistic [76]. The distance
392 matrix generated after comparing each sample to the other was visualized for dissimilarity
393 between samples using the Principle Coordinate Analysis ordination method.
394
395 **Clustering and Biomarker analysis**
396 Patterns of relative abundance of taxa species in response to different experimental factors was
397 analyzed in Microbiome Analyst [74]. The distance measure was Euclidean [77] and the
398 clustering algorithm utilized was Ward, an agglomerative hierarchical clustering procedure [78].
399 The Euclidean distance, a measure of the true straight-line distance between two points in
400 Euclidean space, examines the root of square differences between coordinates of a pair of
401 objects.
402 To provide an estimate of the most important taxa for classification of data resulting from
403 different experimental factors, biomarker analysis was carried out using the random forest
404 algorithm within Microbiome Analyst [74]. The default setting of the number of trees to grow
405 and number of predictors to try was applied (500 and 7, respectively) with the randomness

406 setting left on. Random forest algorithm [79] is a supervised classification algorithm of trees
407 created by using bootstrap samples while training data and random feature selection in tree
408 induction. It is an ensemble of unpruned classification or regression trees trained with the
409 bagging method [80].
410
411 **Quantification of *Elizabethkingia* and Zika virus**
412 Both *in vitro* and *in vivo* analyses to quantify the *Elizabethkingia* loads were performed using
413 the primer pairs *rpoB* gene specific to *Elizabethkingia* using the PCR conditions described in
414 Table 1. The ribosomal protein *S7* gene was utilized to normalize transcript levels (Table 1).
415 Absolute quantity of ZIKV in individual midguts was obtained by isolating RNA using Trizol
416 (ThermoFisher scientific, Massachusetts, USA). A ZIKV-specific quantitative PCR assay that
417 targets the NS1 region was used for detection [73] and serially diluted ZIKV of known quantities
418 was used to generate the standard curve and extrapolate viral load.
419
420 **Phylogenetic analysis of the *Elizabethkingia* isolated from *Ae. albopictus* salivary gland.**
421 In order to validate the identity of *Elizabethkingia* taxa identified in the *Ae. albopictus* salivary
422 gland and saliva samples, *rpoB* gene (GenBank Accession number: MT096047) was amplified
423 from the salivary gland cDNA using the *rpoB* primer pairs and PCR conditions as described in
424 Table 1. The 558 bp PCR amplicon was excised from Agarose gel and cleaned using QIAquick gel
425 extraction kit (Qiagen, UK). The amplicons were Sanger sequenced at the WCAGC.
426 The sequences were manually trimmed using Bioedit v7.1.9 and aligned using MUSCLE [81,82].
427 Upon trimming the primer region and the low-quality regions of the sequence, the FASTA

428 sequence of the respective genes were blasted against the NCBI database. A total of 9 gene
429 sequences published with the following GenBank accession numbers: CP023010.2, KY587659.1,
430 CP016377.1, KY587657.1, CP035811.1, CP035809.1, CP011059.1, CP039929.1 and MN327643.1
431 were added to the phylogenetic analysis.
432 To construct the *Elizabethkingia rpoB* phylogenetic tree, Molecular Evolutionary Genetic
433 Analysis (MEGA) software [83] was utilized. The phylogenetic tree was constructed using the
434 Maximum Likelihood statistical method [84] with nucleotide substitution type.
435 The test of phylogeny was by bootstrap method [85] with 1000 bootstrap replications. The
436 nucleotide substitution model was the Tamura 3-parameter model [86]. The rates among sites
437 were uniform rates while the Heuristic method was the Nearest-Neighbor-Interchange.
438
439 ***In vitro* assay of *Elizabethkingia* and ZIKV co-infection**
440 In order to study the growth kinetics of ZIKV co-infected with *Elizabethkingia*, U4.4 cells (*Ae.*
441 *albopictus*), were co-infected with *Elizabethkingia* (kindly provided by Steven Blaire, The
442 Connecticut Agricultural Experiment Station) and ZIKV.
443 *Elizabethkingia* bacterial strain was streaked on Luria-Bertani (LB) plates and incubated in a 10%
444 CO_2 incubator at 28°C overnight. A single colony was picked using a sterile plastic loop and used
445 to inoculate 500 ml of sterile LB broth. The inoculated broth media was incubated overnight at
446 28°C in an orbital shaker shaking at 225 rpm. The *Elizabethkingia* overnight culture was
447 subsequently diluted to OD 1.0 (8.5 $\log_{10}$CFU/ml), OD 0.5 (8.0 $\log_{10}$CFU/ml) and OD 0.2 (7.7
448 $\log_{10}$CFU/ml).

449 Individual wells were seeded with 3 ml of 2 X 10^5 U4.4 cells/ml suspended in M&M medium
450 without FBS and incubated at 28°C for 1 h before adding FBS to each well at a final
451 concentration of 20%. The cells were then incubated for 5 days in a 10% CO_2 incubator at 28°C.
452 After 5 days, supernatant was removed carefully, and the plates were infected with 100 µl of
453 *Elizabethkingia* at OD 1.0 (8.5 $\log_{10}$ CFU/ml), OD 0.5(8.0 $\log_{10}$ CFU/ml) and OD 0.2 (7.7 $\log_{10}$
454 CFU/ml) for three biological replicates. The negative control plates were infected with 100 µl of
455 8.3 $\log_{10}$ PFU/ml. After 1 h of incubation at 28°C, 3 ml of M&M media containing 20% fetal
456 bovine serum (FBS) were added to each well and incubated at 28°C.
457 To determine whether the U4.4 cells were successfully infected with *Elizabethkingia,* a single
458 well from each replicate of the different dilutions of *Elizabethkingia* infection was harvested at
459 2 dpi.
460 The cells were spun at 1000 rpm and washed three times with sterile phosphate buffered saline
461 (PBS). After the third wash, 100 µl of the washed cells were used to inoculate 3 ml of LB media
462 and incubated overnight at 28°C in an orbital shaker at 225 rpm.
463 The experimental wells were infected with 100 µl of 8.3 $\log_{10}$ PFU/ml ZIKV after a 2 day
464 incubation with *Elizabethkingia*. Cells which were co-infected with *Elizabethkingia* and ZIKV, as
465 well as the ZIKV only control, were harvested at both 2 dpi and 4 dpi. RNA was extracted from
466 the cells using Trizol (ThermoFisher scientific, Massachusetts, USA). The ZIKV quantities were
467 measured as previously described.
468 **Statistical analysis**
469 Statistical analysis was performed with GraphPad Prism version 5.0. Differences in
470 *Elizabethkingia* quantities were assessed using two-tailed T test. Viral loads were compared

using an ANOVA test. Finally, the *Elizabethkingia* /ZIKV correlation analysis was carried out using Pearson tests.

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References

1. Choi MH, Kim M, Jeong SJ, Choi JY, Lee I, Yong T, et al. Risk Factors for Elizabethkingia Acquisition and Clinical Characteristics of Patients , South Korea. Emerg Infect Dis. 2019;25: 42–51.
2. Vandamme P, Bernardet J-F, Segers P, Kersters K, Holmes B. New Perspectives in the Classification of the Flavobacteria: Description of Chryseobacterium gen. nov ., Bergeyella gen. nov ., and Empedobacter nom. rev. Int J Syst Bacteriol. 1994;44: 827–831.
3. Kim KK, Kim MK, Lim JH, Park HY, Lee S. Transfer of Chryseobacterium meningosepticum and Chryseobacterium miricola to Elizabethkingia gen . nov . as Elizabethkingia

495 meningoseptica. Int J Syst Evol Microbiol. 2005;55: 1287–1293. doi:10.1099/ijs.0.63541-0
496 4. Lau SKP, Chow WN, Foo CH, Curreem SOT, Lo GCS, Teng JLL, et al. Elizabethkingia
497 anophelis bacteremia is associated with clinically significant infections and high
498 mortality. Sci Rep. 2016;6: 8–17. doi:10.1038/srep26045
499 5. Janda JM, Lopez DL. Mini review: New pathogen profiles: Elizabethkingia anophelis.
500 Diagn Microbiol Infect Dis. 2017;88: 201–205. doi:10.1016/j.diagmicrobio.2017.03.007
501 6. Bobossi-Serengbe G, Gody J, Beyam N, Bercion R. First documented case of
502 Chryseobacterium meningosepticum meningitis in Central African Republic. Med Trop.
503 2006;66: 182–184.
504 7. Teo J, Tan SY-Y, Tay M, Ding Y, Kjelleberg S, Givskov M, et al. First case of E anophelis
505 outbreak in an intensive-care unit. Lancet. 2013;382: 855–856. doi:10.1016/S0140-
506 6736(13)61858-9
507 8. Perrin A, Larsonneur E, Nicholson AC, Edwards DJ, Gundlach KM, Whitney AM, et al.
508 Evolutionary dynamics and genomic features of the Elizabethkingia anophelis 2015-2016
509 Wisconsin outbreak strain. Nat Commun. 2017;8: 1–12. doi:10.1038/ncomms15483
510 9. Janda JM, Lopez DL. Mini review: New pathogen profiles: Elizabethkingia anophelis.
511 Diagn Microbiol Infect Dis. 2017;88: 201–205. doi:10.1016/j.diagmicrobio.2017.03.007
512 10. Navon L, Clegg W, Morgan J, Austin C, McQuiston J, Blaney D, et al. Notes from the field:
513 Investigation of Elizabethkingia anophelis cluster - Illinois, 2014-2016. 2016.
514 11. Lau SKP, Wu AKL, Teng JLL, Tse H, Curreem SOT, Tsui SKW, et al. Evidence for
515 Elizabethkingia Anophelis transmission from mother to infant, Hong Kong. Emerg Infect
516 Dis. 2015;21: 232–241. doi:10.3201/eid2102.140623
517 12. Reed TAN, Watson G, Kheng C, Tan P, Roberts T, Ling CL, et al. Elizabethkingia anophelis
518 Infection in infants, Cambodia, 2012-2018. Emerg Infect Dis. 2020;26: 320–322.
519 13. Chen S, Bagdasarian M, Walker ED. Elizabethkingia anophelis: Molecular manipulation
520 and interactions with mosquito hosts. Appl Environ Microbiol. 2015;81: 2233–2243.
521 doi:10.1128/AEM.03733-14
522 14. Perrin A, Larsonneur E, Nicholson AC, Edwards DJ, Gundlach KM, Whitney AM, et al.
523 Evolutionary dynamics and genomic features of the Elizabethkingia anophelis 2015 to

524 2016 Winconsin outbreak strain. Nat Commun. 2017;8: 1–12.
525 doi:10.1038/ncomms15483
526 15. Terenius O, Lindh JM, Eriksson-Gonzales K, Bussière L, Laugen AT, Bergquist H, et al.
527 Midgut bacterial dynamics in Aedes aegypti. FEMS Microbiol Ecol. 2012;80: 556–565.
528 doi:10.1111/j.1574-6941.2012.01317.x
529 16. Bahia AC, Dong Y, Blumberg BJ, Mlambo G, Tripathi A, Chandra R, et al. Exploring
530 Anopheles gut bacteria for Plasmodium blocking activity. Environ Microbiol. 2015;16:
531 2980–2994. doi:10.1111/1462-2920.12381.Exploring
532 17. Akhouayri IG, Habtewold T, Christophides GK. Melanotic Pathology and Vertical
533 Transmission of the Gut Commensal Elizabethkingia meningoseptica in the Major Malaria
534 Vector Anopheles gambiae. PLoS One. 2013;8: 1–12. doi:10.1371/journal.pone.0077619
535 18. Boissière A, Tchioffo MT, Bachar D, Abate L, Marie A, Nsango SE, et al. Midgut microbiota
536 of the malaria mosquito vector Anopheles gambiae and interactions with Plasmodium
537 falciparum infection. PLoS Pathog. 2012;8: 1–12. doi:10.1371/journal.ppat.1002742
538 19. Wang Y, Gilbreath TM, Kukutla P, Yan G, Xu J. Dynamic gut microbiome across life history
539 of the malaria mosquito anopheles gambiae in Kenya. PLoS One. 2011;6: 1–9.
540 doi:10.1371/journal.pone.0024767
541 20. Kämpfer P, Matthews H, Glaeser SP, Martin K, Lodders N, Faye I. Elizabethkingia
542 anophelis sp. nov., isolated from the midgut of the mosquito Anopheles gambiae. Int J
543 Syst Evol Microbiol. 2011;61: 2670–2675. doi:10.1099/ijs.0.026393-0
544 21. Kämpfer P, Busse HJ, McInroy JA, Glaeser SP. Elizabethkingia endophytica sp. nov.,
545 isolated from Zea mays and emended description of Elizabethkingia anophelis Kämpfer
546 et al. 2011. Int J Syst Evol Microbiol. 2015;65: 2187–2193. doi:10.1099/ijs.0.000236
547 22. Holmes B, Steigerwalt AG, Nicholson AC. DNA-DNA hybridization study of strains of
548 Chryseobacterium, Elizabethkingia and Empedobacter and of other usually indole-
549 producing non-fermenters of CDC groups IIc, IIe, IIh and IIi, mostly from human clinical
550 sources, and proposals of Chryseobacterium be. Int J Syst Evol Microbiol. 2013;63: 4639–
551 4662. doi:10.1099/ijs.0.054353-0
552 23. Li Y, Kawamura Y, Fujiwara N, Naka T, Hongsheng L, Huang X, et al. Chryseobacterium

553 miricola sp. nov., A novel species isolated from condensation water of space station Mir.
554 Syst Appl Microbiol. 2003;26: 523–528.
555 24. Nicholson AC, Gulvik CA, Whitney AM, Humrighouse BW, Graziano J, Emery B, et al.
556 Revisiting the taxonomy of the genus Elizabethkingia using whole-genome sequencing,
557 optical mapping, and MALDI-TOF, along with proposal of three novel Elizabethkingia
558 species: Elizabethkingia bruuniana sp. nov., Elizabethkingia ursingii sp. nov., and Eliz.
559 Antonie van Leeuwenhoek, Int J Gen Mol Microbiol. 2018;111: 55–72.
560 doi:10.1007/s10482-017-0926-3
561 25. King E. Studies on a group of previously unclassified bacteria associated with meningitis
562 in infants. Am J Clin Pathol. 1959;31: 241–247.
563 26. Ka P, Matthews H, Glaeser SP, Martin K, Lodders N, Faye I. Elizabethkingia anophelis sp .
564 nov ., isolated from the midgut of the mosquito Anopheles gambiae. 2019; 2670–2675.
565 doi:10.1099/ijs.0.026393-0
566 27. Mancini M V, Damiani C, Accoti A, Tallarita M, Nunzi E, Cappelli A, et al. Estimating
567 bacteria diversity in different organs of nine species of mosquito by next generation
568 sequencing. BMC Microbiol. 2018;18: 1–10.
569 28. Lombardo F, Capurro ML. Salivary gland-specific gene expression in the malaria vector
570 Anopheles gambiae. 1999.
571 29. Arca B, Lombardo F, Lanfrancotti A, Spanos L, Veneri M, Louis C, et al. A cluster of four
572 D7-related genes is expressed in the salivary glands of the African malaria vector
573 Anopheles gambiae. Insect Mol Biol. 2002;11: 47–55.
574 30. Kerlin R., Hughes S. Enzymes in saliva from four parasitic arthropods. Med Vet Entomol.
575 1992;6: 121–126.
576 31. Mellink JJ, Vos BJ. Primary Lymph Nodes Response to Mosquito Bites. Zeitschrift fur
577 Parasitenkd Parasitol Res. 1977;51: 187–188.
578 32. Ribeiro J. Characterization of a vasodilator from the salivary glands of the yellow fever
579 mosquito Aedes aegypti. J exp Biol. 1992;165: 61–71.
580 33. Schneider B., Soong L, Zeidner N., Higgs S. Aedes aegypti Salivary Gland Extracts
581 Modulate Anti-Viral and TH1/TH2 Cytokine Responses to Sindbis Virus Infection. Viral

582 Immunol. 2004;17: 565–573.
583 34. Titus RG, Bishop J V, Mejia JS. The immunomodulatory factors of arthropod saliva and
584 the potential for these factors to serve as vaccine targets to prevent pathogen
585 transmission. 2006; 131–141. doi:10.1111/j.1365-3024.2006.00807.x
586 35. Conway MJ, Londono-renteria B, Troupin A, Watson AM, Klimstra WB, Fikrig E, et al.
587 Aedes aegypti D7 Saliva Protein Inhibits Dengue Virus Infection. PLoS Negl Trop Dis.
588 2016; 1–19. doi:10.1371/journal.pntd.0004941
589 36. Conway MJ, Watson AM, Colpitts TM, Dragovic SM, Li Z, Wang P, et al. Mosquito Saliva
590 Serine Protease Enhances Dissemination of Dengue. J Virol. 2014;88: 164–175.
591 doi:10.1128/JVI.02235-13
592 37. Jin L, Guo X, Shen C, Hao X, Sun P, Li P, et al. Salivary factor LTRIN from Aedes aegypti
593 facilitates the transmission of Zika virus by interfering with the lymphotoxin-β receptor.
594 Nat Immunol. 2018;19: 342–353. doi:10.1038/s41590-018-0063-9
595 38. Uraki R, Hastings AK, Marin-lopez A, Sumida T, Takahashi T, Grover JR, et al. Aedes
596 aegypti AgBR1 antibodies modulate early Zika virus infection of mice. Nat Microbiol.
597 doi:10.1038/s41564-019-0385-x
598 39. Rubinstien E, Klevjer-Anderson P, Smith C, Drouin M, Patterson J. Enterobacter taylorae,
599 a New Opportunistic Pathogen: Report of Four Cases. J Clin Microbiol. 1993;31: 249–254.
600 40. Hurrell E, Kucerova E, Loughlin M, Caubilla-barron J, Hilton A, Armstrong R, et al.
601 Neonatal enteral feeding tubes as loci for colonisation by members of the
602 Enterobacteriaceae. BMC Infect Dis. 2009;9: 1–9. doi:10.1186/1471-2334-9-146
603 41. Guentzel M. Escherichia, Klebsiella, Enterobacter, Serratia, Citrobacter and Proteus. 4th
604 ed. In: Samuel Barron, editor. Medical Microbiology. 4th ed. Galveston, Texas: University
605 of Texas Medical Branch at Galveston; 1996.
606 42. Gaio ADO, Gusmão DS, Santos A V, Berbert-molina MA, Pimenta PFP, Lemos FJA.
607 Contribution of midgut bacteria to blood digestion and egg production in Aedes aegypti (
608 diptera: culicidae) (L .). 2011; 1–10.
609 43. Simi S, Carbonell G, Falcon R, Gatti M, Joazeiro P, Darini A, et al. A low molecular weight
610 enterotoxic hemolysin from clinical Enterobacter cloacae. Can J Microbiol. 2003;49: 479–

611 482.
612 44. Serbus LR, Sullivan W. A Cellular Basis for Wolbachia Recruitment to the Host Germline.
613 PLoS Pathog. 2007;3: 1930–1937. doi:10.1371/journal.ppat.0030190
614 45. Werren JH, Baldo L, Clark ME. Wolbachia: master manipulators of invertebrate biology.
615 Nat Rev Microbiol. 2008;6: 741–751. doi:10.1038/nrmicro1969
616 46. White PM, Pietri JE, Debec A, Russell S, Patel B, Sullivan W. Mechanisms of Horizontal
617 Cell-to-cell Transfer of Wolbachia spp. in Drosophila melanongaster. Appl Environ
618 Microbiol. 2017;83: 1–12.
619 47. Li S, Ahmed MZ, Lv N, Shi P, Wang X, Ji-Lei H, et al. Plant – mediated horizontal
620 transmission of Wolbachia between whiteflies. ISME J. 2017;11: 1019–1028.
621 doi:10.1038/ismej.2016.164
622 48. Bourtzis K, Dobson SL, Xi Z, Rasgon JL, Calvitti M, Moreira LA, et al. Harnessing mosquito-
623 Wolbachia symbiosis for vector and disease control. Acta Trop. 2014;132: S150–S163.
624 doi:10.1016/j.actatropica.2013.11.004
625 49. Dickson LB, Jiolle D, Minard G, Moltini-Conclois I, Volant S, Ghozlane A, et al. Carryover
626 effects of larval exposure to different environmental bacteria drive adult trait variation in
627 a mosquito vector. Sci Adv. 2017;3: 1–15. doi:10.1126/sciadv.1700585
628 50. Muturi EJ, J BJ, Rooney AP, K. HA. Midgut fungal and bacterial microbiota of Aedes
629 triseriatus and Aedes japonicus shift in response to La Crosse virus infection. Mol Ecol.
630 2016;25: 4075–4090. doi:10.1111/mec.13741
631 51. Zink SD, van Slyke GA, Palumbo MJ, Kramer LD, Ciota AT. Exposure to west nile virus
632 increases bacterial diversity and immune gene expression in culex pipiens. Viruses.
633 2015;7: 5619–5631. doi:10.3390/v7102886
634 52. Zouache K, Michelland RJ, Failloux AB, Grundmann GL, Mavingui P. Chikungunya virus
635 impacts the diversity of symbiotic bacteria in mosquito vector. Mol Ecol. 2012;21: 2297–
636 2309. doi:10.1111/j.1365-294X.2012.05526.x
637 53. Ramirez JL, Short SM, Bahia AC, Saraiva RG, Dong Y, Kang S, et al. Chromobacterium
638 Csp_P Reduces Malaria and Dengue Infection in Vector Mosquitoes and Has
639 Entomopathogenic and In Vitro Anti-pathogen Activities. PLoS Pathog. 2014;10.

640 doi:10.1371/journal.ppat.1004398
641 54. Apte-Deshpande A, Paingankar M, Gokhale MD, Deobagkar DN. Serratia odorifera a
642 midgut inhabitant of aedes aegypti mosquito enhances its susceptibility to dengue-2
643 virus. PLoS One. 2012;7. doi:10.1371/journal.pone.0040401
644 55. Weiss B, Aksoy S. Microbiome influences on insect host vector competence. Trends
645 Parasitol. 2011;27: 514–522. doi:10.1016/j.pt.2011.05.001
646 56. Li N, Ma W, Pang M, Fan Q, Hua J. The Commensal Microbiota and Viral Infection: A
647 Comprehensive Review. Front Immunol. 2019;10: 1–16. doi:10.3389/fimmu.2019.01551
648 57. Karst SM. The influence of commensal bacteria on infection with enteric viruses. Nat Rev
649 Microbiol. 2016;14: 197–204. doi:10.1038/nrmicro.2015.25.The
650 58. Berger AK, Mainou BA. Interactions between Enteric Bacteria and Eukaryotic Viruses
651 Impact the Outcome of Infection. Viruses. 2018;10: 4–6. doi:10.3390/v10010019
652 59. Pfeiffer JK, Virgin HW. Transkingdom control of viral infection and immunity in the
653 mammalian intestine. Science (80-). 2017;351: 1–12.
654 doi:10.1126/science.aad5872.Transkingdom
655 60. Abt MC, Osborne LC, Monticelli LA, Doering TA, Alenghat T, Sonnenberg GF, et al.
656 Commensal Bacteria Calibrate the Activation Threshold of Innate Antiviral Immunity. Cell.
657 2012;37: 158–170. doi:10.1016/j.immuni.2012.04.011
658 61. Kumar A, Vlasova AN, Deblais L, Huang H, Wijeratne A, Kandasamy S, et al. Impact of
659 nutrition and rotavirus infection on the infant gut microbiota in a humanized pig model.
660 BMC Gastroenterol. 2018;18: 1–17.
661 62. Jang J, Kim S, Kwon M, Lee J, Yu D, Song R, et al. Rotavirus-mediated alteration of gut
662 microbiota and its correlation with physiological characteristics in neonatal calves. J
663 Microbiol. 2019;57: 113–121. doi:10.1007/s12275-019-8549-1
664 63. Ma X, Wang Q, Li H, Xu C, Cui N, Zhao X. 16S rRNA genes Illumina sequencing revealed
665 differential cecal microbiome in specific pathogen free chickens infected with diff erent
666 subgroup of avian leukosis viruses. Vet Microbiol. 2017;207: 195–204.
667 doi:10.1016/j.vetmic.2017.05.016
668 64. Zhao N, Li M, Luo J, Wang S, Liu S, Wang S, et al. Impacts of canine distemper virus

669 infection on the giant panda population from the perspective of gut microbiota. Sci Rep.
670 2017; 1–10. doi:10.1038/srep39954
671 65. Ding ZF, Cao MJ, Zhu XS, Xu GH, Wang RL. Changes in the gut microbiome of the Chinese
672 mitten crab (Eriocheir sinensis) in response to White spot syndrome virus (WSSV)
673 infection. J Fish Dis. 2017;40: 1561–1571. doi:10.1111/jfd.12624
674 66. Mayuzumi H, Okamoto Y. Interleukin- 17A is required to suppress invasion of Salmonella
675 enterica serovar Typhimurium to enteric mucosa. 2010; 377–385. doi:10.1111/j.1365-
676 2567.2010.03310.x
677 67. Deriu E, Boxx GM, He X, Pan C, Benavidez SD, Cen L, et al. Influenza Virus Affects
678 Intestinal Microbiota and Secondary Salmonella Infection in the Gut through Type I
679 Interferons. PLoS Pathog. 2016; 1–26. doi:10.1371/journal.ppat.1005572
680 68. Rossi P, Ricci I, Cappelli A, Damiani C, Ulissi U, Mancini MV, et al. Mutual exclusion of
681 Asaia and Wolbachia in the reproductive organs of mosquito vectors. Parasites and
682 Vectors. 2015;8: 1–10. doi:10.1186/s13071-015-0888-0
683 69. Ngwa C, Glockner V, Abdelmohsen U, Scheuermayer M, Fischer R, Hentschel U, et al. 16S
684 rRNA gene-based identification of Elizabethkingia meningoseptica
685 (Flavobacteriales:Flavobacteriaceae) as a dominant midgut bacterium of the Asian
686 malaria vector Anopheles stephensi (Diptera: Culicidae) with antimicrobial activities. J
687 Med Entomol. 2013;50: 404–14.
688 70. Villegas LEM, Campolina TB, Barnabe NR, Orfano AS, Chaves BA, Norris DE, et al. Zika
689 virus infection modulates the bacterial diversity associated with Aedes aegypti as
690 revealed by metagenomic analysis. PLoS One. 2018;13: e0190352.
691 doi:10.1371/journal.pone.0190352
692 71. Almand E, Moore M, Jaykus L. Virus-Bacteria Interactions: An Emerging Topic in Human
693 Infection. Viruses. 2017;9: 1–10. doi:10.3390/v9030058
694 72. Ciota A, Bialosuknia S, Zink S, Brecher M, Ehrbar D, Morrissette M, et al. Effects of Zika
695 Virus Strain and Aedes Mosquito Species on Vector Competence. Emerg Infect Dis.
696 2017;23: 1110–1117.
697 73. Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ, Vorndam a V, Vorndamt a V. Rapid

698 detection and typing of dengue viruses from clinical samples by using reverse
699 transcriptase-polymerase chain reaction. J Clin Microbiol. 1992;30: 545–51.
700 74. Dhariwal A, Chong J, Habib S, King IL, Agellon LB, Xia J. MicrobiomeAnalyst: A web-based
701 tool for comprehensive statistical, visual and meta-analysis of microbiome data. Nucleic
702 Acids Res. 2017;45: W180–W188. doi:10.1093/nar/gkx295
703 75. Chao A. Nonparametric Estimation the Number of Classes in a Population. Ann Math
704 Stat. 1984;20: 265–270. doi:10.1214/aoms/1177729949
705 76. Bray RJ, Curtis JT. An Ordination of the Upland Forest Communities of Southern
706 Wisconsin. Ecol Monogr. 1957;27: 325–349.
707 77. Motzkin T. The euclidean algorithm. Bulletin of the American Society. 1949. pp. 1142–
708 1146.
709 78. Ward J, Hook M. Application of an hierarchical grouping procedure to a problem of
710 grouping profiles. Educ Psychol Meas. 1963;XXIII.
711 79. Svetnik V, Liaw A, Tong C, Christopher Culberson J, Sheridan RP, Feuston BP. Random
712 Forest: A Classification and Regression Tool for Compound Classification and QSAR
713 Modeling. J Chem Inf Comput Sci. 2003;43: 1947–1958. doi:10.1021/ci034160g
714 80. Liaw A, Wiener M. Classification and Regression by randomForest. R news. 2002;2: 18–
715 22. doi:10.1023/A:1010933404324
716 81. Hall T. BioEdit: An important software for molecular biology. GERF Bull Biosci. 2011;2:
717 60–61.
718 82. Edgar RC. MUSCLE: Multiple sequence alignment with high accuracy and high
719 throughput. Nucleic Acids Res. 2004;32: 1792–7. doi:10.1093/nar/gkh340
720 83. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular
721 evolutionary genetics analysis using maximum likelihood, evolutionary distance, and
722 maximum parsimony methods. Mol Biol Evol. 2011;28: 2731–9.
723 doi:10.1093/molbev/msr121
724 84. Felsenstein J. Evolutionary Trees from DNA Sequences: A Maximum Likelihood
725 Approach. J Mol Evol. 1981;17: 368–376.
726 85. Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap.

727 Evolution (N Y). 1985;39: 783–791.
728 86. Tamura K, Nei M. Estimation of the Number of Nucleotide Substitutions in the Control
729 Region of Mitochondrial DNA in Humans and Chimpanzees. Mol Biol Evol. 1993;10: 512–
730 526.
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732
733 **Author Contributions**
734 MGO: Involved in the study design, conducted the experiments, analyzed the results and
735 drafted the paper
736 PA: Trained me in the cell culture, was involved in the antibiotic treatment of the Aedes
737 mosquitoes and assisted in mosquito manipulation exercises
738 SJ: Involved in the mosquito rearing, assisted in mosquito manipulation exercises
739 DC: Assisted in mosquito manipulation exercises
740 KL: Generated and propagated the ZIKV infectious clone utilized in this study
741 KLD: Involved in the study design, participated in the writing of the paper
742 CAT: Involved in the study design, participated in the writing of the paper
743 **Competing Interests**
744 No competing interests
745 **Materials and correspondence**
746 Correspondence and material requests should be addressed to CAT
747
748
749
750

Tables and figures

List of tables

Table 1. List of Primers used in this study

Primer name	Primer sequence	Annealing temperature
Ribosomal protein S7 _F	GAG ATC GAG TTC AAC AGC AAG A	
Ribosomal protein S7 _R	GAG AAC TTC TTC TCC AGC TCA C	55°C
*Elizabethkingia*_rpoB_F	CTC CGG AAG GAC CAA ACA TTG	
*Elizabethkingia*_rpoB_R	CAA CCG TCC AGT CAG ATC C	55°C
*Elizabethkingia*_qrpoB_F	GCT AGG ACA CAT GGT TGA TGA	
*Elizabethkingia*_qrpoB_R	ACC ACC GAA CTG AGC TTT AC	55°C
16S Microbiome_F	TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG	
16S Microbiome_R	GTC TCG TGG GCT CGG AGA TCT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C	55°C

Figures

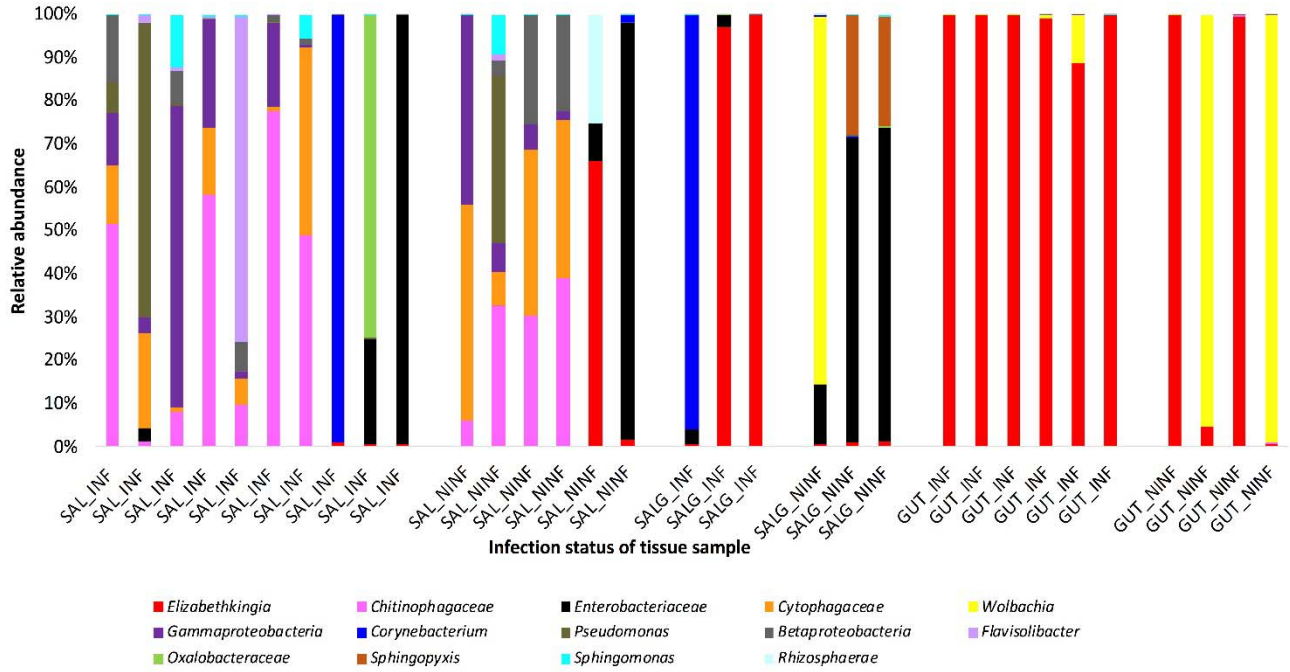


Figure 1. Relative abundance of bacterial taxa in individual *Aedes* saliva, salivary gland and midgut samples. Illumina MiSeq 16S rRNA sequencing was used to identify bacterial taxa and distributions were visualized using a cut-off of 7000 reads. Each stacked bar plot illustrates the taxonomic distribution of bacteria identified in individual *Aedes* saliva, salivary gland or midgut samples. Samples were taken at 7 days post-feeding. Blood fed individuals were further separated into NINF (fed non-infectious blood meal [BM]) and INF (fed Zika virus-positive BM and acquired an infection). SAL_INF represents saliva samples obtained from individuals fed on INF BM and successfully transmitting the virus; SAL_NINF represent saliva samples obtained from individuals exposed to NINF BM; SALG_INF represent individual salivary gland samples exposed to INF BM and infected; SALG_NINF represent salivary glands from individuals fed on NINF BM; GUT_INF represent midgut samples exposed to INF BM and infected, and GUT_NINF represent midgut samples from individuals fed on NINF BM.

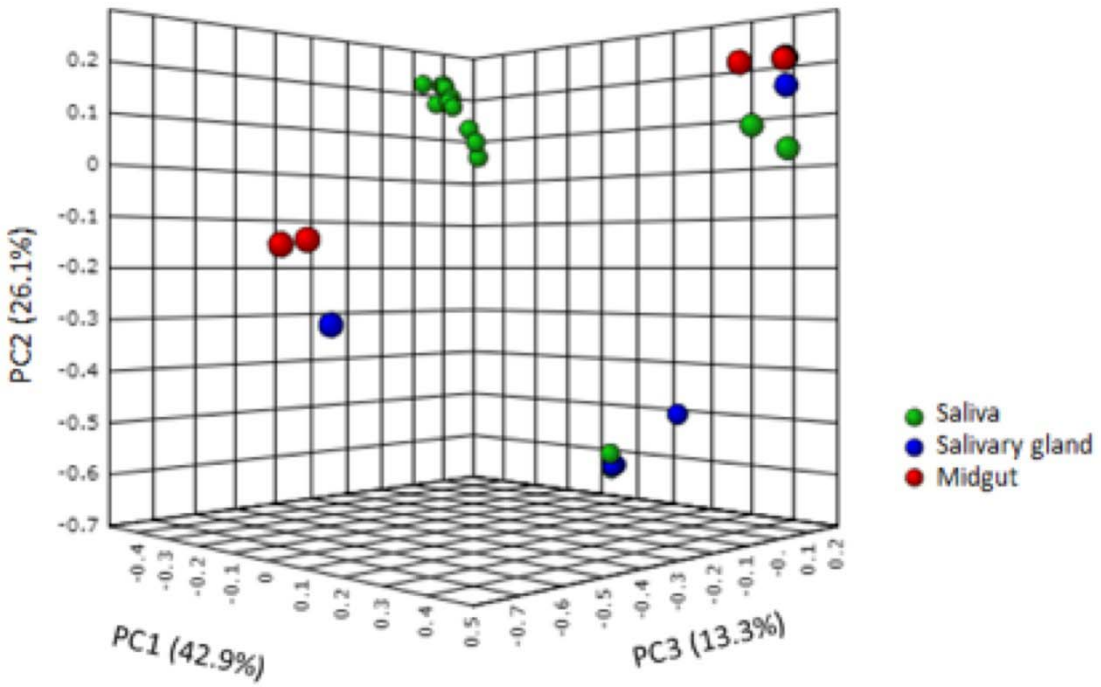


Figure 2. Tissue-specific diversity of microbes in *Ae. albopictus*. Principal component analyses were completed to assess the genetic relationships among and between bacterial taxa identified in different tissue types. Despite a number of shared individual bacterial genera, significant separation by tissue type was identified [PERMANOVA; F-value:6.9998; R-squared: 0.3111; P <0.001].

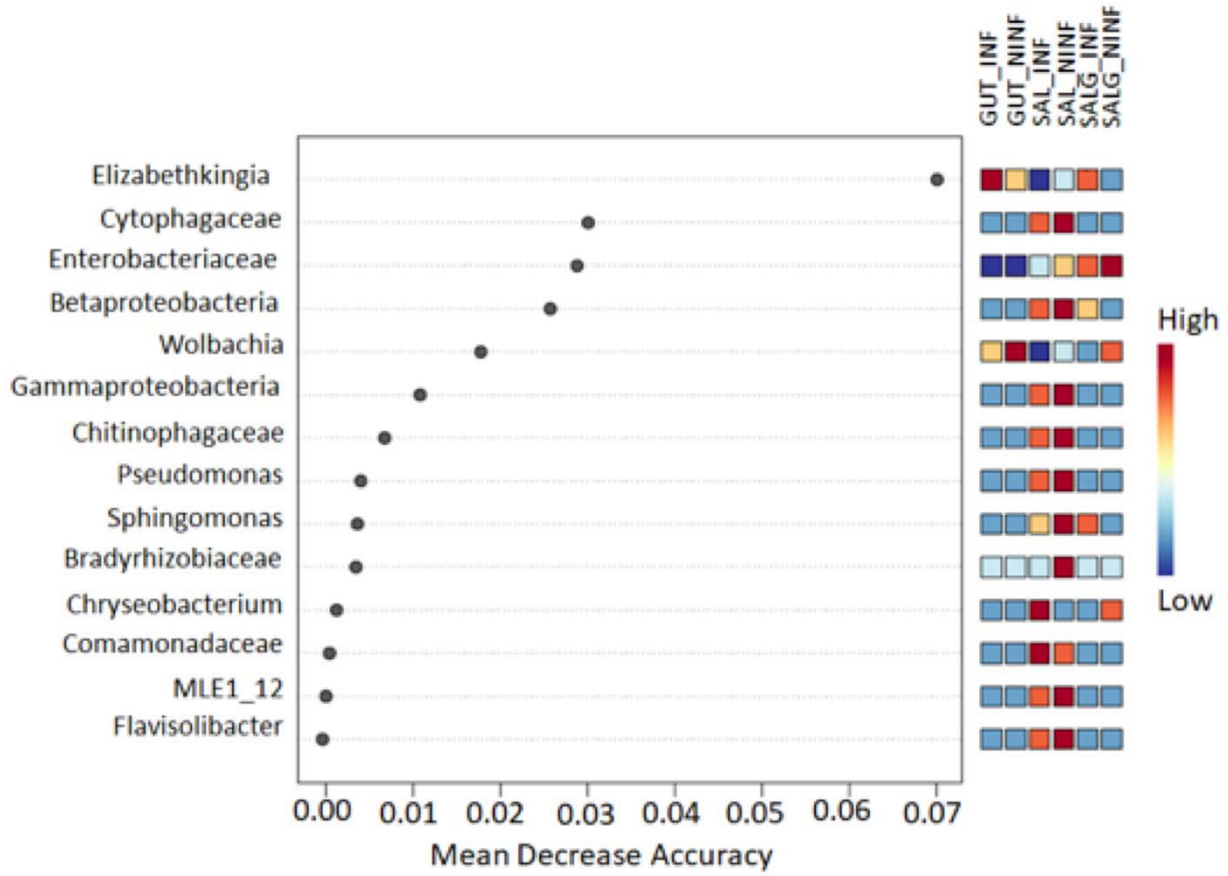


Figure 3. Association of bacterial taxa with both tissue and Zika virus infection status in *Ae. albopictus* mosquitoes. The Random forest classifier was used to determine the association of differentially abundant microbial genera with both tissue-type and Zika virus infection status of *Aedes* mosquitoes. Mean Decrease Accuracy is reported for each of the taxa. This measure 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. The highest mean decrease in accuracy was identified for *Elizabethkingia*. In addition, *Elizabethkingia* and *Enterobacteriaceae*, taxa associated with human disease, were found to be present in the midguts, saliva and salivary glands of *Aedes* mosquitoes exposed to both infectious (INF) and noninfectious (NINF) blood meals (BM).

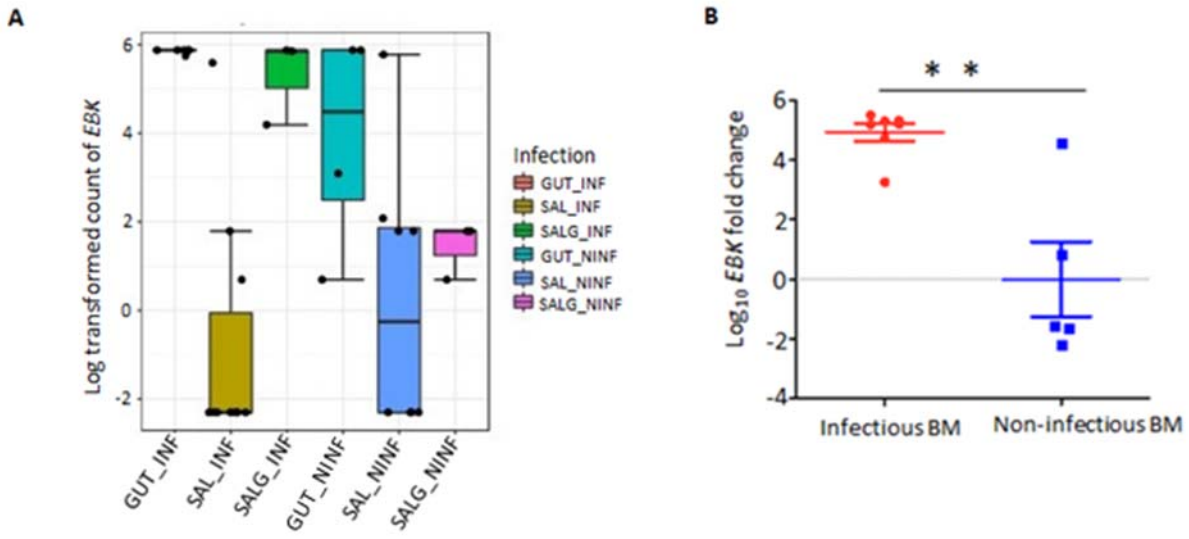


Figure 4. Relative *Elizabethkingia* levels in *Ae. albopictus.* A. Tissue tropism. Relative quantification of *Elizabethkingia* in the midgut, salivary gland and saliva of *Ae. albopictus*. Levels were tissue-specific and dependent on Zika virus (ZIKV) exposure. GUT_INF- infected gut; SAL_INF - infected saliva; SALG_INF - infected salivary gland; GUT_NINF – gut samples of *Ae. albopictus* exposed to NINF BM; SAL_NINF – saliva samples from *Ae. albopictus* exposed to NINF BM; SALG_NINF – salivary gland samples of *Ae. albopictus* exposed to NINF BM. **B. Relative levels of *Elizabethkingia* in Zika virus infected and uninfected *Ae. albopictus* midguts.** Infection of *Ae. albopictus* midguts by ZIKV (INF) was associated with higher *Elizabethkingia rpoB* transcript levels (T-test; Mann Whitney test P = 0.0051) relative to midguts following non-infectious blood meals (NINF BM) at 7 days post-feeding.

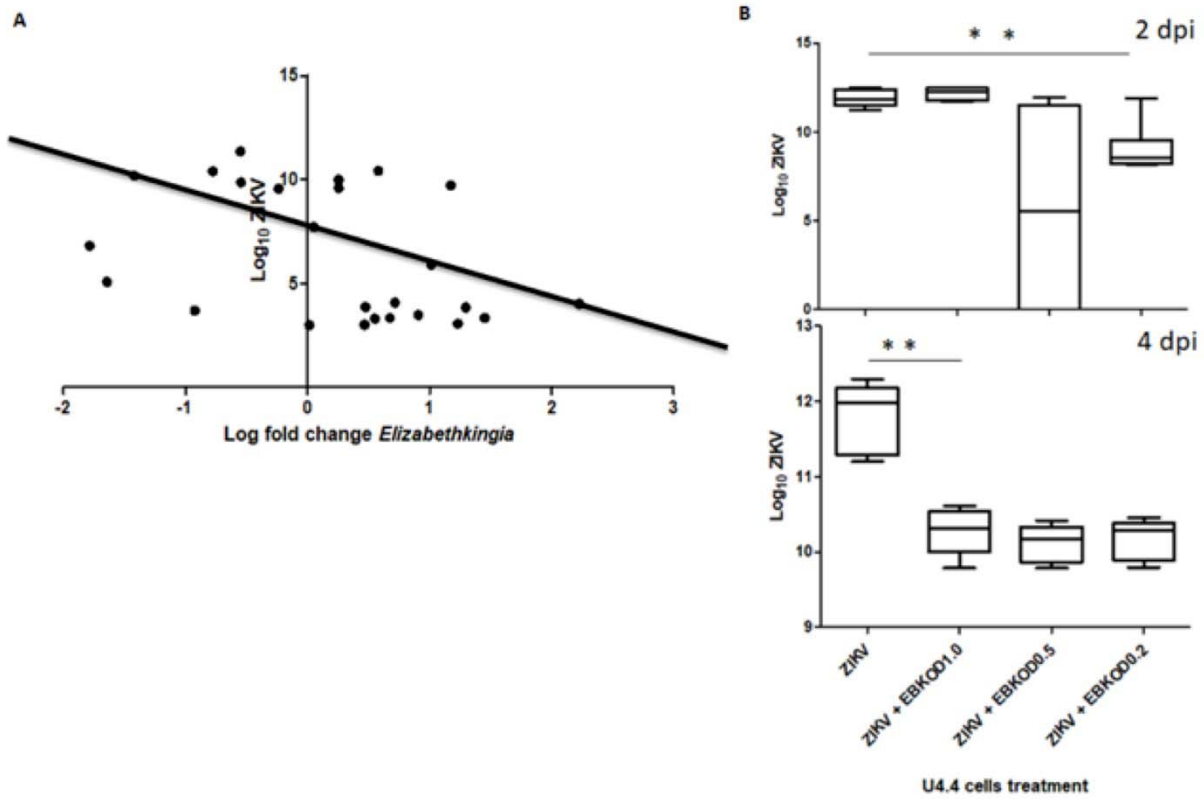


Figure 5. Relationship between *Elizabethkingia* and Zika virus proliferation. A. *In vivo* comparison in *Ae. albopictus* midguts. *Aedes albopictus* were infected with a bloodmeal containing 8.3 $\log_{10}$ PFU/ml ZIKV and of both expression of *Elizabethkingia rpoB* gene and ZIKV levels were assessed at 7 days post infection. The RT qPCR results were normalized using S7 gene. The X axis represents the log fold change of *Elizabethkingia*, while Y axis represents $\log_{10}$ ZIKV PFU/midgut. A negative correlation between ZIKV and *Elizabethkingia* was demonstrated (Spearman r = -0.3985; $P < 0.05$). **B. *In-vitro* assay of *Elizabethkingia* and Zika virus in U4.4 cells** U4.4 cells were infected with 8.3 $\log_{10}$ PFU/ml ZIKV following 2 days exposure to *Elizabethkingia* (EBK, experimental) or media alone (control). Experimental wells were previously exposed to either OD 1.0 (8.5 $\log_{10}$ CFU/ml EBK), OD 0.5 (8.0 $\log_{10}$ CFU/ml EBK) or OD 0.2 (7.7 $\log_{10}$ CFU/ml EBK). At both 2 and 4 days post infection (dpi) ZIKV levels were significantly higher in the ZIKV

only controls relative to the EBK co-infected cells (ZIKV + *Elizabethkingia*; ANOVA; Kruskal-Wallis test, **P < 0.01).

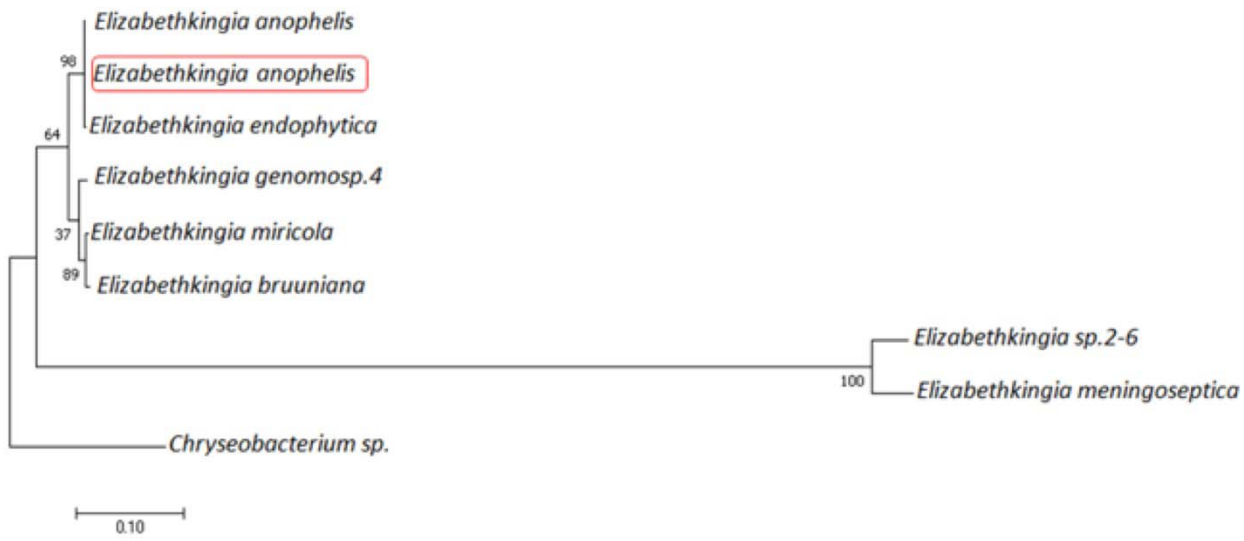


Figure 6. **Phylogenetic analysis of *Elizabethkingia rpoB* gene.** Phylogenetic affiliation of *Elizabethkingia* taxa from the salivary gland of *Ae. albopictus* based on a partial sequence of the *rpoB* gene (GenBank Accession number: MT096047). Phylogenetic dendrograms were constructed in MEGA. Bootstrap proportions are shown on branches. *Chryseobacterium* was used as an outgroup.

Figure 1.

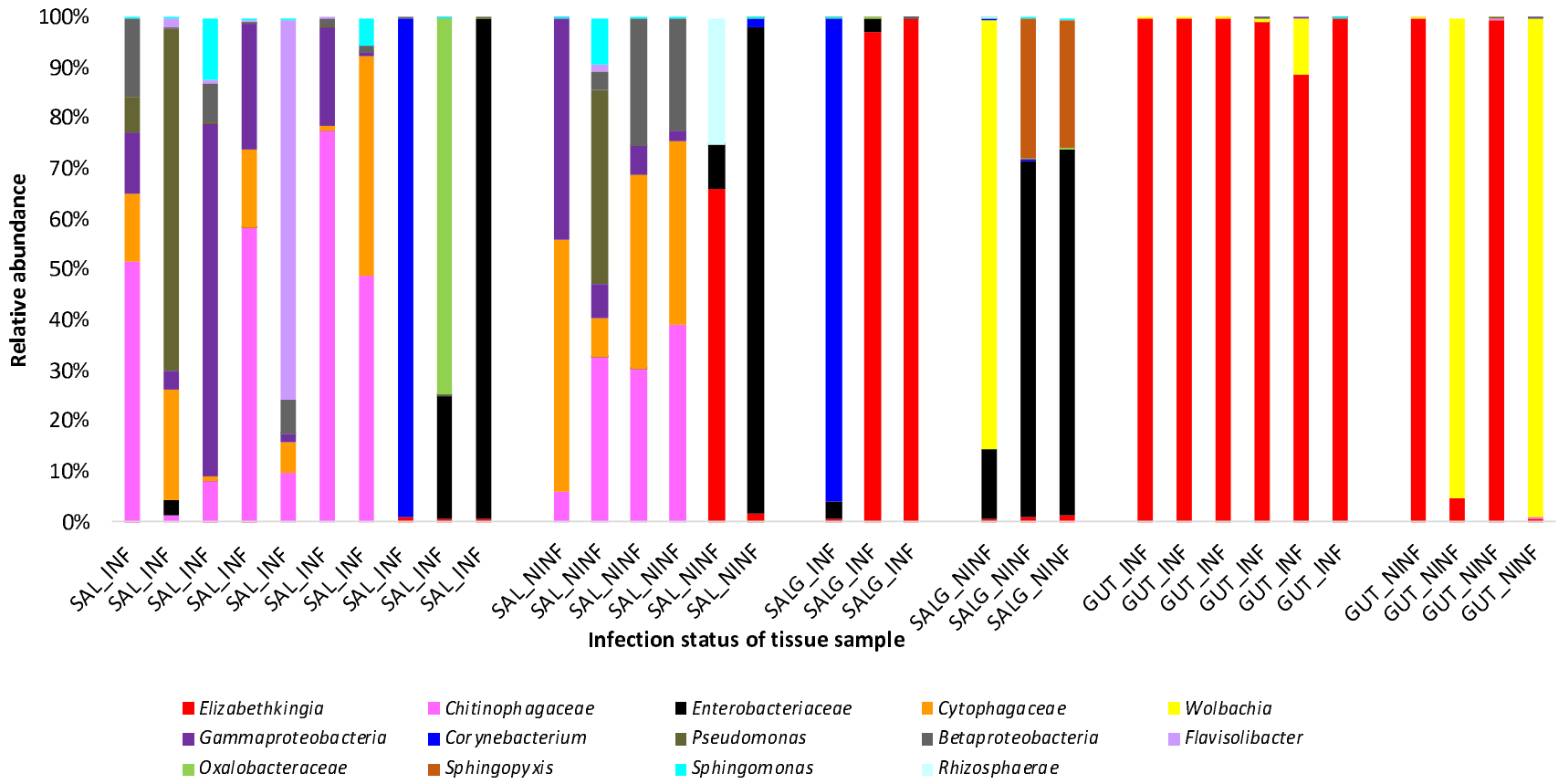

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Relative abundance
SAL_INF
SAL_NINF
SALG_INF
SALG_NINF
GUT_INF
GUT_NINF
Infection status of tissue sample
Elizabethkingia
Chitinophagaceae
Enterobacteriaceae
Cytophagaceae
Wolbachia
Gammaproteobacteria
Corynebacterium
Pseudomonas
Betaproteobacteria
Flavisolibacter
Oxalobacteraceae
Sphingopyxis
Sphingomonas
Rhizosphaerae

Figure 2

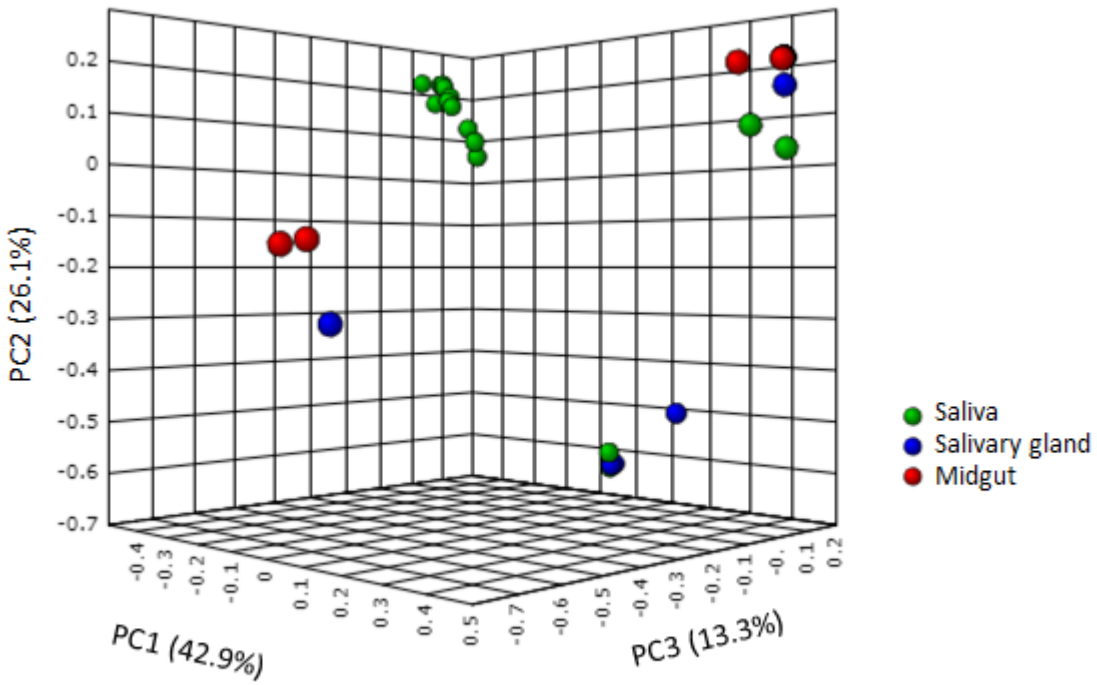


Figure 3

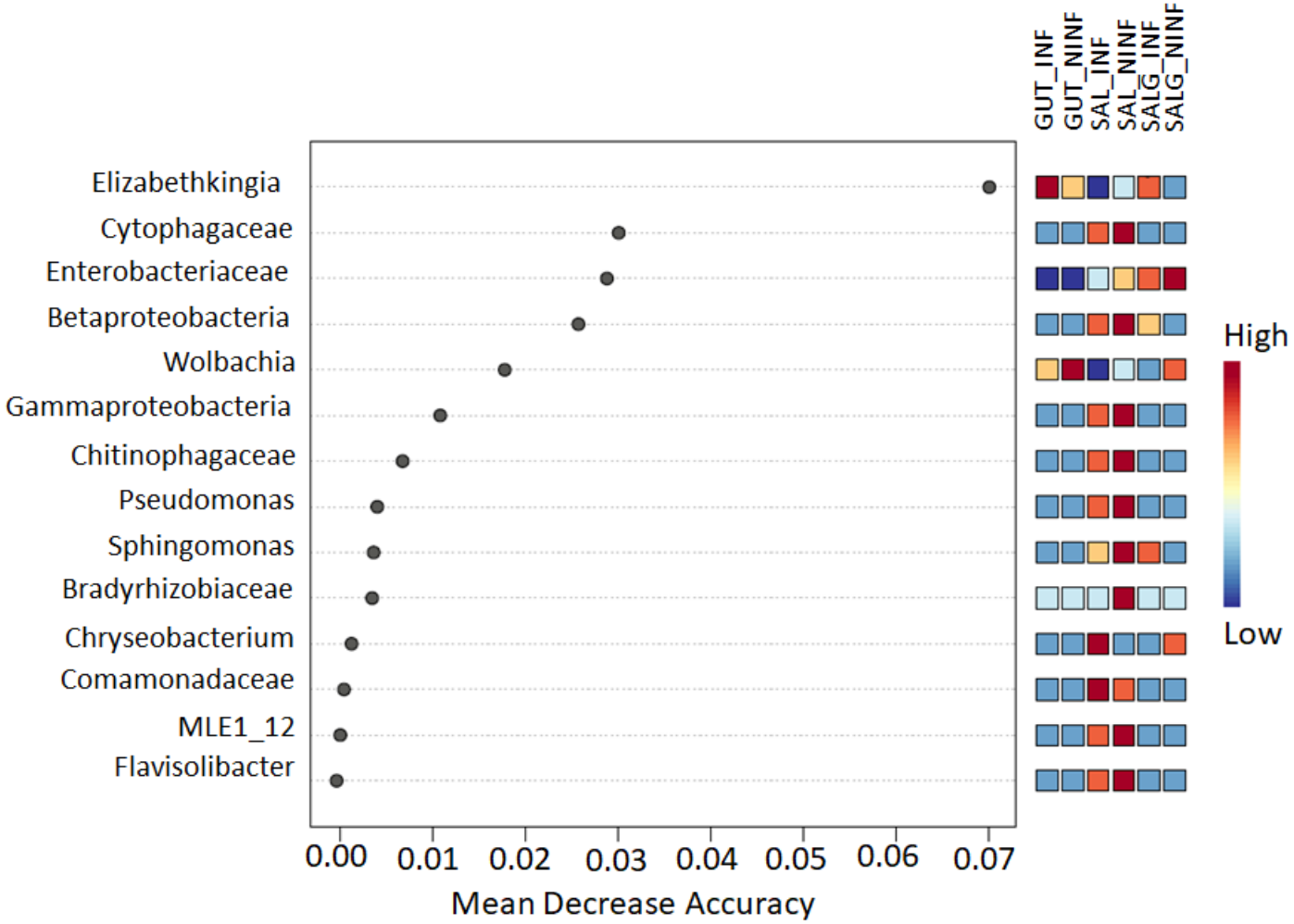

GUT_INF
GUT_NINF
SAL_INF
SAL_NINF
SALG_INF
SALG_NINF
Elizabethkingia
Cytophagaceae
Enterobacteriaceae
Betaproteobacteria
Wolbachia
Gammaproteobacteria
Chitinophagaceae
Pseudomonas
Sphingomonas
Bradyrhizobiaceae
Chryseobacterium
Comamonadaceae
MLE1_12
Flavisolibacter
0.00
0.01
0.02
0.03
0.04
0.05
0.06
0.07
Mean Decrease Accuracy
High
Low

Figure 4

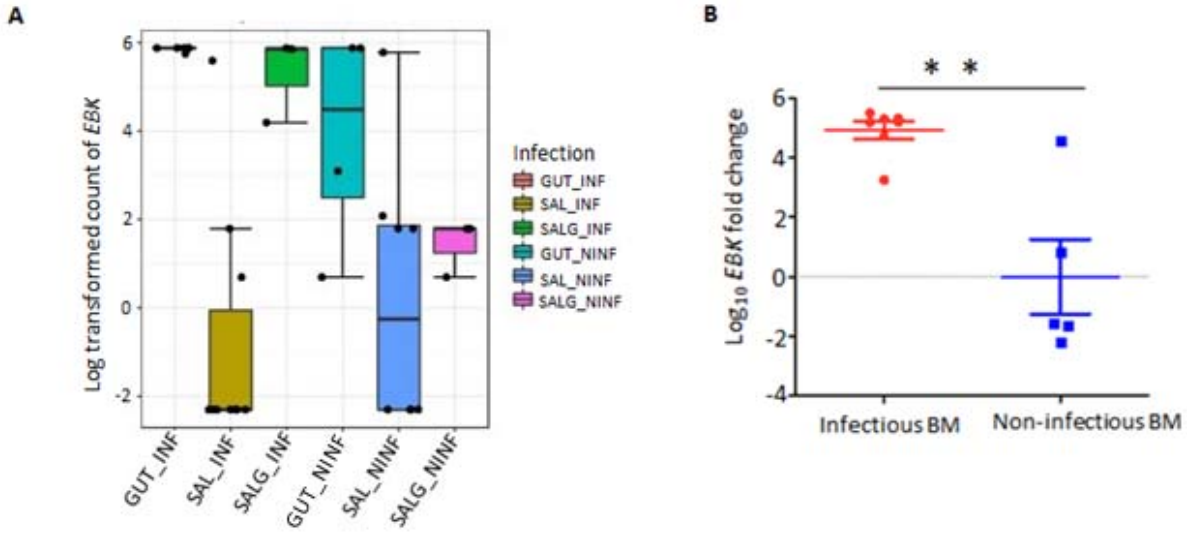

A
Log transformed count of EBK
6
4
2
0
-2
GUT_INF
SAL_INF
SALG_INF
GUT_NINF
SAL_NINF
SALG_NINF
Infection
GUT_INF
SAL_INF
SALG_INF
GUT_NINF
SAL_NINF
SALG_NINF
B
* *
Log10 EBK fold change
6
4
2
0
-2
-4
Infectious BM
Non-infectious BM

Figure 5

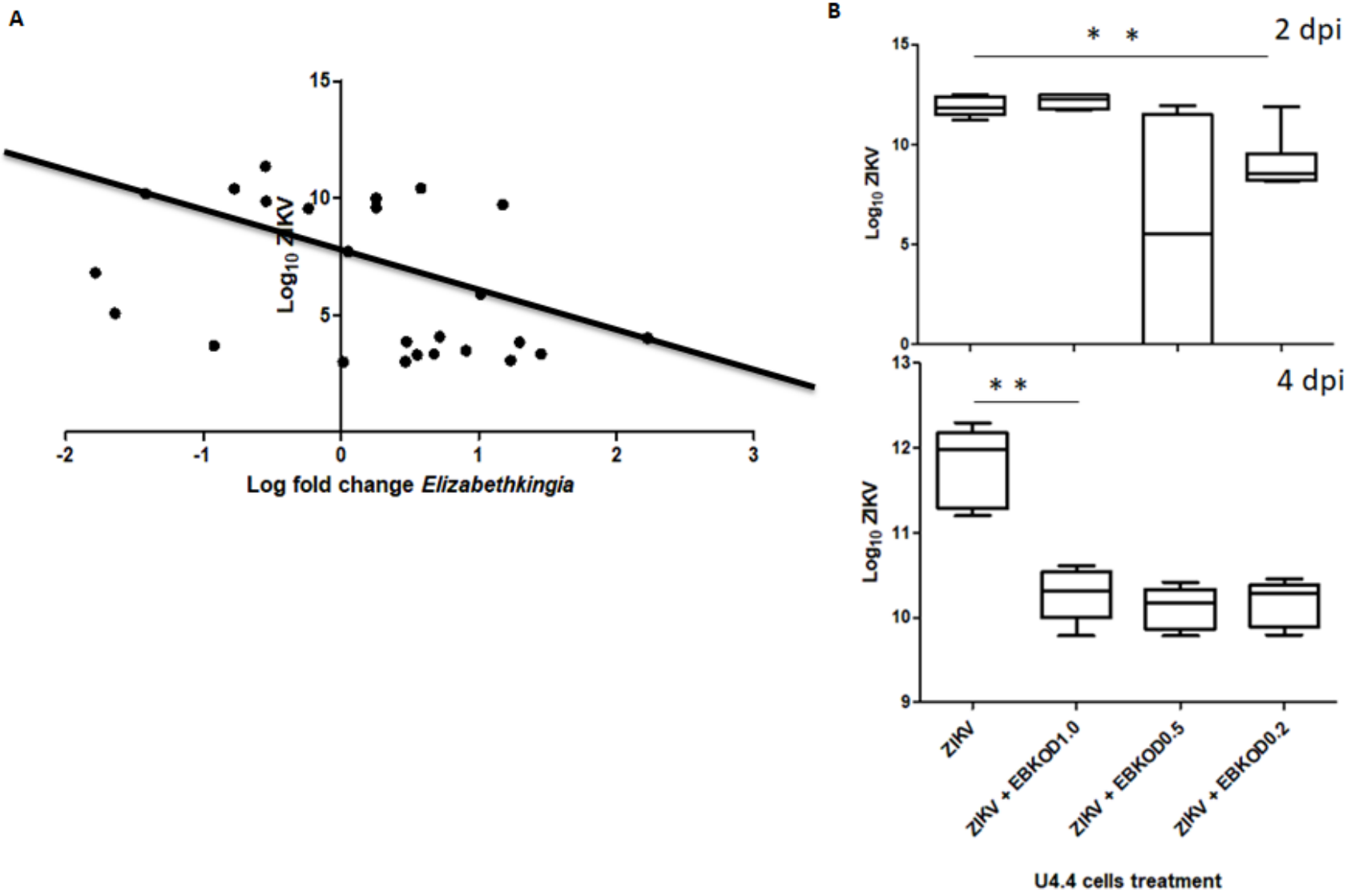
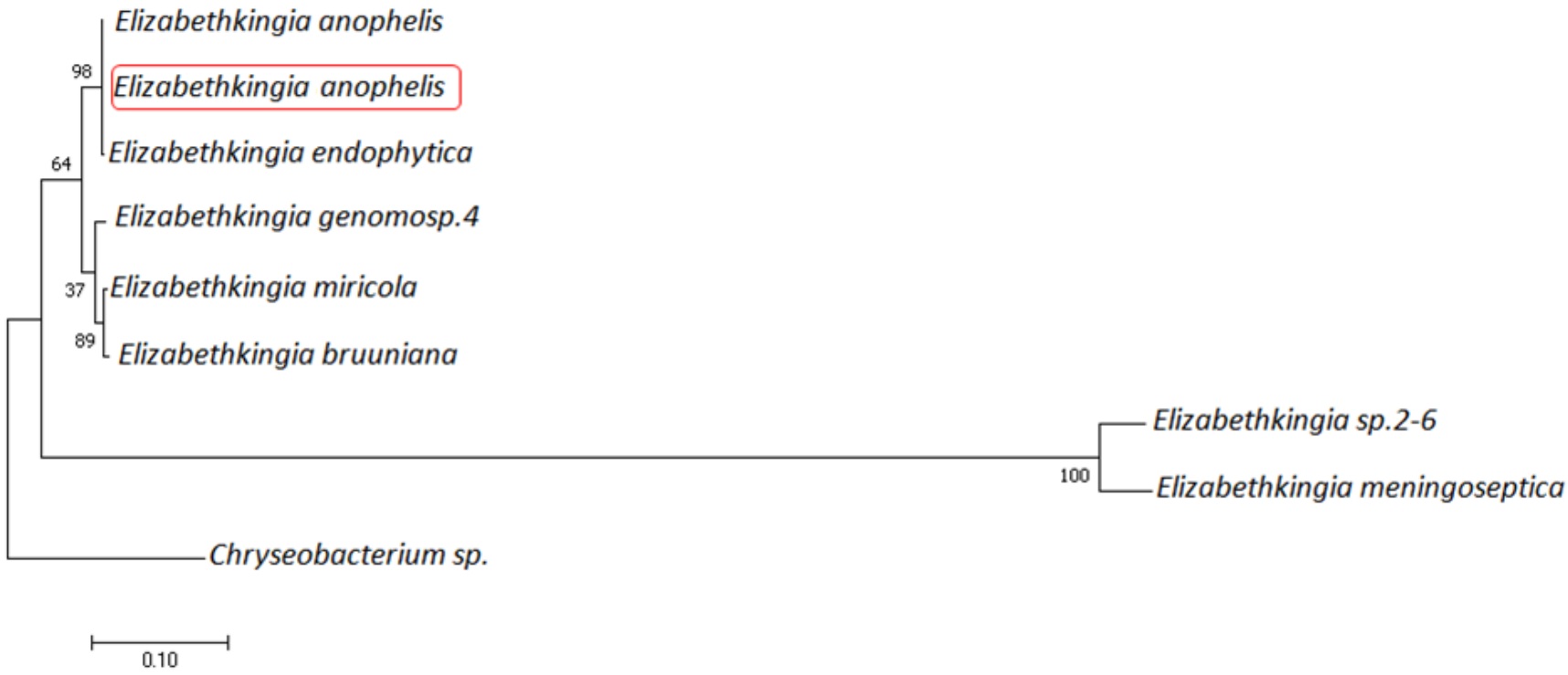

A
15
10
5
Log10 ZIKV
-2
-1
0
1
2
3
Log fold change Elizabethkingia
B
2 dpi
* *
15
10
5
0
Log10 ZIKV
4 dpi
* *
13
12
11
10
9
Log10 ZIKV
ZIKV
ZIKV + EBKOD1.0
ZIKV + EBKOD0.5
ZIKV + EBKOD0.2
U4.4 cells treatment

Figure 6.


Elizabethkingia anophelis
98
Elizabethkingia anophelis
Elizabethkingia endophytica
64
Elizabethkingia genomosp.4
37
Elizabethkingia miricola
89
Elizabethkingia bruuniana
Elizabethkingia sp.2-6
100
Elizabethkingia meningoseptica
Chryseobacterium sp.
0.10