

1 **Microbiome interaction networks and community structure from lab-  
2 reared and field-collected *Aedes aegypti*, *Aedes albopictus*, and *Culex  
3 quinquefasciatus* mosquito vectors.**

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5 Shivanand Hegde<sup>1\*</sup>, Kamil Khanipov<sup>2\*</sup>, Levent Albayrak<sup>3</sup>, George Golovko<sup>3</sup>, Maria  
6 Pimenova<sup>3</sup>, Miguel A. Saldaña<sup>4</sup>, Mark M Rojas<sup>3</sup>, Emily A. Hornett<sup>5</sup>, Greg C. Motl<sup>6</sup>, Chris  
7 L. Fredregill<sup>6</sup>, James A. Dennett<sup>6</sup>, Mustapha Debboun<sup>6</sup>, Yuriy Fofanov<sup>3</sup>, Grant L.  
8 Hughes<sup>7#</sup>

9  
10 \* The authors contributed equally

11  
12 <sup>1</sup> Department of Pathology, University of Texas Medical Branch, Galveston, TX, USA.  
13 <sup>2</sup> Department of Pharmacology and Toxicology, Sealy Center for  
14 Structural Biology and Molecular Biophysics. University of Texas Medical Branch,  
15 Galveston, TX, USA. Department of Computer Science, University of Houston, Houston,  
16 TX, USA.

17 <sup>3</sup> Department of Pharmacology and Toxicology, Sealy Center for  
18 Structural Biology. University of Texas Medical Branch, Galveston, TX, USA.

19 <sup>4</sup> Department of Microbiology and Immunology, University of Texas Medical Branch,  
20 Galveston, TX, USA,

21 <sup>5</sup> Department of Zoology, University of Cambridge, Cambridge, UK.

22 <sup>6</sup> Harris County Public Health, Mosquito & Vector Control Division, Houston, TX, USA

23 <sup>7</sup> Department of Pathology, Institute for Human Infections and Immunity, Center for  
24 Tropical Diseases, Center for Biodefense and Emerging Infectious Disease. University  
25 of Texas Medical Branch, Galveston, TX, USA.

26 <sup>#</sup>Corresponding author. Grant Hughes: [glhughes@utmb.edu](mailto:glhughes@utmb.edu)

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28

29 Running head: Microbial interactions in mosquitoes.

30

31 **Abstract.**

32 Microbial interactions are an underappreciated force in shaping insect microbiome  
33 communities. Although pairwise patterns of symbiont interactions have been identified,  
34 we have a poor understanding regarding the scale and the nature of co-occurrence and  
35 co-exclusion interactions within the microbiome. To characterize these patterns in  
36 mosquitoes, we sequenced the bacterial microbiome of *Aedes aegypti*, *Ae. albopictus*,  
37 and *Culex quinquefasciatus* caught in the field or reared in the laboratory and used  
38 these data to generate interaction networks. For collections, we used traps that attracted  
39 host-seeking or ovipositing female mosquitoes to determine how physiological state  
40 affects the microbiome under field conditions. Interestingly, we saw few differences in  
41 species richness or microbiome community structure in mosquitoes caught in either trap.  
42 Co-occurrence and co-exclusion analysis identified 116 pairwise interactions  
43 substantially increasing the list of bacterial interactions observed in mosquitoes.  
44 Networks generated from the microbiome of *Ae. aegypti* often included highly  
45 interconnected hub bacteria. There were several instances where co-occurring bacteria  
46 co-excluded a third taxa, suggesting the existence of tripartite relationships. Several  
47 associations were observed in multiple species or in field and laboratory-reared  
48 mosquitoes indicating these associations are robust and not influenced by  
49 environmental or host factors. To demonstrate that microbial interactions can influence  
50 colonization of the host, we administered symbionts to *Ae. aegypti* larvae that either  
51 possessed or lacked their resident microbiota. We found that the presence of resident

52 microbiota can inhibit colonization of particular bacterial taxa. Our results highlight that

53 microbial interactions in mosquitoes are complex and influence microbiome composition.

54

55 **Keywords.** Interaction networks, Microbe-microbe interactions, Gnotobiotic, Gut

56 symbiont, *Wolbachia*.

57

58

59 **Introduction.**

60 The microbiome of mosquitoes can be highly variable, both within and between species,  
61 and is often dominated by relatively few genera (Boissière et al., 2012; Buck et al., 2016;  
62 Muturi et al., 2017; Osei-Poku et al., 2012; Wang et al., 2011). Understanding the factors  
63 that influence this variation is important as microbes drastically alter host biology. For  
64 mosquitoes, bacteria can affect a diverse number of traits including immunity,  
65 reproduction, survival, and vector competence (Hegde et al., 2015; Jupatanakul et al.,  
66 2014). These phenotypes have ramifications for the vectorial capacity of pathogens, and  
67 as such, microbial-based vector control strategies are under investigation to reduce the  
68 burden of arthropod-borne diseases (Bourtzis et al., 2014; Dennison et al., 2014;  
69 Saldaña et al., 2017). While our understanding of the contributing factors that affect the  
70 composition and abundance of the microbiome is expanding, there are still many  
71 unanswered questions regarding microbiome assembly and maintenance within  
72 mosquito hosts.

73

74 Exposure to environmental microbes is undoubtedly a major influence on the mosquito  
75 microbiome. These effects are particularly pronounced at the aquatic stage as larvae  
76 and pupae are immersed in water and can acquire bacteria by filter feeding. Indeed,  
77 several studies have shown the larval stages possess a similar microbiome as their  
78 larval water environment (Duguma et al., 2013; Gimonneau et al., 2014; Vázquez-  
79 martínez et al., 2009), and exposure to bacteria at these immature stages has  
80 implications for adult traits (Dickson et al., 2017). Furthermore, the larval habitat can

81 influence the composition of the adult microbiome. Bacteria can be transstadially  
82 transmitted to the adult (Chen et al., 2015; Coon et al., 2014; Gonçalves et al., 2014;  
83 Jadin et al., 1966), and newly emerged adults are known to imbibe their larval water,  
84 which likely seeds the gut with microbiota (Lindh et al., 2008).

85

86 Host and bacterial genetics also contribute to microbiome composition and microbial  
87 abundance. Mosquitoes can maintain microbiome homeostasis by a variety of different  
88 mechanisms. Host pathways and processes known to influence microbiota in  
89 mosquitoes include immunity, amino acid metabolism, reactive oxygen species and  
90 calcium transport (Kumar et al., 2010; Pang et al., 2016; Short et al., 2017; Stathopoulos  
91 et al., 2014; Xiao et al., 2017; Zhao et al., 2017). Additionally, serial passaging of gut  
92 symbionts in mosquitoes selected for isolates that persist in the gut for longer periods of  
93 time (Dennison et al., 2016; Riehle et al., 2007), indicating that bacterial genetics is also  
94 important in shaping the microbiome.

95

96 Adult mosquito feeding behavior also has important implications for microbiome  
97 community structure. It is likely that bacteria can be acquired from the nectar of plants  
98 (Gusmão et al., 2007), and taking a blood meal alters the microbiome considerably. At  
99 24 hours post-blood meal, the bacterial load in the gut drastically increases while  
100 species diversity decreases (Kumar et al., 2010; Oliveira et al., 2011; Terenius et al.,  
101 2012; Wang et al., 2011). Culture based assays show that bacterial loads revert to pre-  
102 blood fed levels 2-3 days after the blood meal (Demaio et al., 1996; Oliveira et al., 2011;  
103 Pompuni et al., 1996), although other studies have seen high bacterial loads persist for

104 some time and species richness not reverting to the original composition seen prior to  
105 the blood meal (Gusmão et al., 2010; Wang et al., 2011). Most of these studies either  
106 used laboratory-reared mosquitoes to examine culturable bacterial load, or relocated  
107 field mosquitoes to the laboratory for experimentation, and as such, there are few  
108 studies examining the effect of blood feeding on the microbiome community structure in  
109 field populations.

110

111 Recently, it has become evident that a further force affects microbiome composition in  
112 mosquitoes – interactions between the microbes themselves. These interactions were  
113 first highlighted with the discovery that *Wolbachia* and *Asaia* are antagonistic to one  
114 another, thereby affecting the vertical transmission of *Wolbachia* in *Anopheles*  
115 mosquitoes (Hughes et al., 2014; Rossi et al., 2015). Further comparisons exploiting  
116 16S rRNA amplicon high throughput sequencing have identified interactions between  
117 *Wolbachia* and other microbes in both *Drosophila* and mosquitoes (Audsley et al.,  
118 2017b; Novakova et al., 2017; Simhadri et al., 2017; Ye et al., 2017; Zink et al., 2015). In  
119 addition to the specific interactions between *Wolbachia* and other bacterial taxa,  
120 pairwise negative and positive microbial interactions within bacteria or fungi, as well as  
121 cross-kingdom interactions (bacterial-fungal) have been reported in the La Crosse virus  
122 vectors, *Aedes triseriatus*, and *Ae. japonicus* (Muturi et al., 2016a). Taken together,  
123 these studies suggest that microbial interactions are important in dictating the  
124 composition and abundance of host-associated microbiota, yet it is unclear how  
125 ubiquitous and complex these interactions are within mosquitoes.

126

127 16S rRNA amplicon sequencing datasets have been analyzed to create microbial co-  
128 occurrence networks for several species and environments (Barberán et al., 2012;  
129 Chaffron et al., 2010; Faust and Raes, 2012; Faust et al., 2012; Goodrich et al., 2014),  
130 but these networks are lacking for mosquitoes and insects in general. These methods  
131 use presence/absence metrics, relative abundance, or both, to examine pairwise  
132 interactions to develop a network. Usually, interacting pairs of taxa are designated as  
133 having co-occurring or co-exclusionary relationships. Each method used for the  
134 identification of co-occurrence/co-exclusion networks has strengths and weaknesses in  
135 identifying particular patterns. CoNet (Faust and Raes, 2016), uses an ensemble  
136 approach that combines results from a collection of algorithms (Bray and Curtis, 1957;  
137 Cover and Thomas, 2012; Kullback and Leibler, 1951; Pearson, 1895; Sedgwick, 2014)  
138 using presence/absence and relative abundance data to identify statistically significant  
139 interactions. Interaction networks provide another methodology to examine the  
140 community structure of the microbiome of mosquitoes. Comparing microbiome networks  
141 generated from mosquitoes exposed to different conditions may provide insights into  
142 factors influencing microbiome structure in mosquitoes and identify pairwise interactions  
143 not affected by environmental conditions.

144  
145 To expand our understanding of the forces that shape the bacterial microbiome of  
146 mosquitoes, we examined the microbial composition and community structure from three  
147 major mosquito arboviral vectors, *Ae. aegypti*, *Ae. albopictus*, and *Cx. quinquefasciatus*,  
148 collected from the field or reared under uniform insectary conditions. For the field  
149 collections, we utilized two trapping methods that primarily attract mosquitoes in different

150 physiological states: host- or oviposition-seeking (Dennett et al., 2007; Figuerola et al.,  
151 2012; Maciel-de-Freitas et al., 2006; Reiter et al., 1986). Our sampling regime allowed  
152 us to examine how factors such as host species, environment, and physiological state in  
153 the field influenced the composition of the mosquito microbiome. We also compared the  
154 microbiome of mosquitoes that were naturally infected (*Ae. albopictus* and *Cx.*  
155 *quinquefasciatus*) and uninfected (*Ae. aegypti*) with *Wolbachia*. Furthermore, we  
156 developed microbial interaction networks to explore the complexity and nature of  
157 microbial interactions in mosquitoes. To demonstrate that microbial interactions  
158 influence host colonization, we infected *Ae. aegypti* larvae either possessing or lacking  
159 their native microbiota with a range of bacterial symbionts. Our results highlight the  
160 complexities of microbial networks in field collected *Ae. aegypti* mosquitoes and indicate  
161 the native microbiome induces colonization resistance to certain gut microbes.

162 **Material and Methods.**

163 **Mosquito collections and DNA extractions.**

164 Female *Ae. albopictus* and *Cx. quinquefasciatus* were collected from an abandoned tire  
165 repository in south-eastern Harris County, Houston, Texas, USA, while female *Ae.*  
166 *aegypti* were collected from a separate site in Houston (Figure S1). Further details  
167 describing the tire repository location were previously reported (Dennett et al., 2004). All  
168 mosquitoes were trapped over a 24 hour period with either the Biogents Sentinel (BG) or  
169 Harris County gravid (G) traps, which selectively collect host-seeking or gravid female  
170 mosquitoes, respectively (Dennett et al., 2007; Figuerola et al., 2012; Maciel-de-Freitas  
171 et al., 2006). Mosquito species were identified using morphological characteristics,  
172 surface sterilized (5 min in 70% ethanol followed by 3 washes in 1X PBS each for 5  
173 min), and stored in ethanol at -20°C while awaiting DNA extraction. 5-7 day old adult  
174 sugar fed laboratory-colonized mosquitoes (*Ae. aegypti*; Galveston strain, *Ae.*  
175 *albopictus*; Galveston strain, and *Cx. quinquefasciatus*; Houston strain) were reared  
176 under conventional conditions and then processed in the same manner as field samples.  
177 All laboratory-reared mosquitoes were reared in the insectary at the University of Texas  
178 Medical Branch.

179

180 **High throughput sequencing and bioinformatics analysis.**

181 High-throughput sequencing of the bacterial 16S ribosomal RNA gene was performed  
182 using gDNA isolated from each sample. Sequencing libraries for each isolate were  
183 generated using universal 16S rRNA V3-V4 region primers (Klindworth et al., 2012) in

184 accordance with Illumina 16S rRNA metagenomic sequencing library protocols. The  
185 samples were barcoded for multiplexing using Nextera XT Index Kit v2. Sequencing was  
186 performed on an Illumina MiSeq instrument using a MiSeq Reagent Kit v2 (500-cycles).  
187 The NCBI Bioproject accession number for the raw sequencing data reported here is  
188 PRJNA422599.

189  
190 To identify the presence of known bacteria, sequences were analyzed using the CLC  
191 Genomics Workbench 8.0.1 Microbial Genomics Module (<http://www.clcbio.com>). Reads  
192 containing nucleotides below the quality threshold of 0.05 (using the modified Richard  
193 Mott algorithm) and those with two or more unknown nucleotides or sequencing  
194 adapters were trimmed out. All reads were trimmed to 264 bases for subsequent  
195 operational taxonomic unit (OTU) classification. Reference based OTU picking was  
196 performed using the SILVA SSU v119 97% database (Quast et al., 2013). Sequences  
197 present in more than one copy but not clustered to the database were placed into de  
198 novo OTUs (97% similarity) and aligned against the reference database with 80%  
199 similarity threshold to assign the “closest” taxonomical name where possible. Chimeras  
200 were removed from the dataset if the absolute crossover cost was 3 using a k-mer size  
201 of 6. Alpha diversity was measured using Shannon entropy (OTU level), rarefaction  
202 sampling without replacement, and with 100,000 replicates at each point. Beta diversity  
203 was calculated using the Bray-Curtis diversity measure (OTU level). PERmutational  
204 Multivariate ANalysis Of VAriance (PERMANOVA) analysis was used to measure effect  
205 size and significance on beta diversity for grouping variables (Anderson, 2014). The  
206 significance is obtained by a permutation test. For each assessment, a permutation of

207 99,999 was chosen. Differentially abundant bacteria (genus level, >0.1%) were identified  
208 using analysis of composition of microbiomes (ANCOM) (Mandal et al., 2015) with a  
209 significance level of  $P > 0.05$ , while values quantifying fold change were obtained using  
210 the log2 fold change formula (Quackenbush, 2002).

211  
212 Detection of complex interaction patterns.

213 OTUs with read counts below 0.1% of total number of reads in all samples were  
214 excluded from analysis. The remaining OTUs were combined based on lowest common  
215 taxonomy assignments down to genus level, and relative abundance tables were  
216 generated by normalizing read counts against total number of reads in the original data.  
217 The resulting number of unique entries identified in samples was 33. Interactions (such  
218 as co-occurrence and co-exclusion) among these were identified using CoNet app  
219 (Faust and Raes, 2016) in Cytoscape (Shannon et al., 2003) using the following  
220 ensemble of methods: Pearson correlation (Pearson, 1895), Spearman correlation  
221 (Sedgwick, 2014), mutual information (Cover and Thomas, 2012), Bray-Curtis  
222 dissimilarity (Bray and Curtis, 1957) and Kullback-Leibler divergence (Kullback and  
223 Leibler, 1951). Statistical significance of each pair was tested using the row-shuffle  
224 randomization option and interactions that scored at the top and bottom 1% of 100  
225 bootstraps were reported. Resulting statistically significant interactions were categorized  
226 by the software into three groups: co-presence, co-exclusion and unknown. Unknown  
227 interactions represent statistically significant patterns that cannot be clearly categorized  
228 as co-exclusion or co-occurrence. Since we could not ascribe an interaction pattern, the

229 unknown interactions were excluded from the network. Resulting interaction networks  
230 were visualized using Cytoscape software (Shannon et al., 2003).

231

232 **Estimation of microbial load and screening for *Wolbachia* by PCR**  
233 Total bacterial load within each mosquito species or group was assessed by qPCR  
234 using gDNA as a template. qPCR was conducted using universal bacterial primers  
235 (Kumar et al., 2010) that amplified the bacterial 16S rRNA gene or primers that  
236 specifically amplified *Wolbachia* (Rao et al., 2006; Walker et al., 2009). Relative  
237 abundance was calculated by comparing the load of all bacteria or *Wolbachia* to a single  
238 copy mosquito gene (Calvitti et al., 2015; Isoe et al., 2011; Xia and Zwiebel, 2006).  
239 PCRs amplifying the 16S rRNA and *wsp* genes of *Wolbachia* were used to screen for  
240 *Wolbachia* in field caught *Ae. aegypti* (Baldo et al., 2006; O'Neill et al., 1992; Werren  
241 and Windsor, 2000; Zhou et al., 1998), and nematode specific primers were used to  
242 screen for nematode infections (Casiraghi et al., 2004).

243

244 **Re-infection of bacteria into mosquito larvae.**

245 *Aedes aegypti* gnotobiotic larvae were generated as previously described (Coon et al.,  
246 2014). To synchronize hatching, sterile eggs were transferred to a conical flask and  
247 placed under a vacuum for 45 min. To verify sterility, larval water was plated on non-  
248 selective LB agar plates and reared under sterile conditions. L1 larvae grown without  
249 bacteria have slow growth rates and do not reach pupation (Coon et al., 2014). Forty-  
250 five L1 larvae were transferred to a T75 tissue culture flask and inoculated with  $1 \times 10^7$

251 CFU/ml of transgenic symbionts possessing the plasmid the pRAM18dRGA-mCherry  
252 that was derived from pRAM18dRGA (Burkhardt et al., 2011). Bacterial cultures were  
253 quantified with a spectrophotometer (DeNovix DS-11, DeNovix) and validated by plating  
254 to determine CFU. For conventional rearing of mosquitoes, eggs (non-sterilized) were  
255 vacuum hatched and grown under non-aseptic conditions in a T75 tissue culture flask  
256 supplemented with transgenic symbionts at the concentration of  $1 \times 10^7$  CFU/ml. To feed  
257 mosquitoes, ground fish food pellets were sterilized by autoclaving, and mixed with  
258 sterile water. The equivalent of 6 mg of fish food was fed to both gnotobiotic and  
259 conventionally reared mosquitoes every second day. To quantify the bacterial load of  
260 symbionts, surface sterilized L4 larvae were homogenized and plated on a selective  
261 media (50  $\mu$ g/ml Kanamycin) on which only transgenic symbionts grew (pRAM18dRGA-  
262 mCherry induces resistance to Kanamycin). After incubation at 30°C or 37°C (depending  
263 on symbiont) for 2-3 days, colonies (expressing mCherry fluorescent protein) were  
264 counted. All colonies observed on the kanamycin plate were confirmed to have mCherry  
265 fluorescence.

266

267 **Results**

268 **Microbiome Diversity.**

269 We sequenced amplicons of the V3/V4 region of the 16S rRNA gene from whole  
270 individual adult female mosquitoes either collected from the field or reared in the  
271 laboratory. In total, we sequenced 130 adult mosquitoes obtaining 10,668,291 reads  
272 (sample size per group and species is reported in Table S1). After quality filtering,  
273 7,051,256 reads were assigned to OTUs at 97% identity threshold and on average,  
274 there were 54,240 reads per mosquito sample. Rarefaction curve analysis indicated that  
275 our sequencing depth was sufficient to observe all OTUs in mosquito samples (Figure  
276 S2). We identified a total of 4,419 bacterial OTUs in the three mosquito species, but only  
277 58 were present at an infection frequency of over 0.1% within the dataset (Table S2).  
278 When abundant microbes were classified at higher taxonomic levels, our analysis found  
279 22 families, with Enterobacteriaceae being the most common when disregarding  
280 *Wolbachia*. Bacteria found in mosquitoes were classified into five phyla with bacteria in  
281 the phylum Proteobacteria most prevalent in the microbiome, which is consistent with  
282 previous studies (Audsley et al., 2017a; David et al., 2016; Muturi et al., 2016b; Osei-  
283 Poku et al., 2012), although other reports indicated Bacteroidetes or Acinetobacter phyla  
284 can be a major component of the microbiome (Coon et al., 2014; 2016b; Minard et al.,  
285 2014; 2015).

286

287 When examining the sequencing data at the genus level, the microbiomes of *Cx.*  
288 *quinquefasciatus* and *Ae. albopictus* were dominated by the endosymbiont *Wolbachia*

289 with 87 and 81 % of total reads, respectively (Figure S3, Table S2). While *Wolbachia*  
290 accounted for many of the reads, rarefaction analysis indicated our sampling depth was  
291 sufficient to identify rare OTUs. Other highly abundant genera in field-collected  
292 mosquitoes included *Halomonas*, *Shewanella*, and *Asaia* in *Cx. quinquefasciatus*,  
293 *Halomonas*, *Pseudomonas*, and *Zymobacter* in *Ae. albopictus*, and *Pseudomonas*,  
294 *Zymobacter*, *Tatumella*, and *Enterobacter* in *Ae. aegypti* (Figure S3, Table S2). Similar  
295 to a recent finding (Coon et al., 2016b), we found a small number of *Wolbachia* reads in  
296 a few *Ae. aegypti* individuals collected from the field in G traps. It is thought that *Ae.*  
297 *aegypti* are naturally uninfected by *Wolbachia* (Iturbe-Ormaetxe et al., 2011), although  
298 Coon et al. (2016) suggested that some populations may be infected. However, samples  
299 that contained *Wolbachia* reads from our sequencing data could not be independently  
300 validated by PCR using several *Wolbachia* genes (*wsp* and MLST genes) commonly  
301 used to screen for the bacterium, nor were they PCR positive for filarial nematode  
302 infection (that carry *Wolbachia*) when amplifying with primers that detect nematode  
303 DNA.

304  
305 Given the above finding and since microbiome sequencing can be susceptible to  
306 contamination (Pollock et al., 2018; Tournousse et al., 2017), we examined our data for  
307 other possible contamination signatures. While we could not find conclusive evidence of  
308 contamination, in this process we observed a possible batch effect for a specific  
309 bacterium in field-collected *Ae. aegypti*. These samples were extracted in two batches  
310 (see Table S1) and the field-collected samples from the latter extraction were found to

311 have higher loads of *Burkholderia* compared to those extracted in the first batch.  
312 However, all laboratory-reared mosquitoes extracted during this second extraction did  
313 not contain high levels of *Burkholderia*, indicating the prevalence of this microbe in the  
314 field-collected samples was not likely due to laboratory contamination. Despite this  
315 occurrence, the dataset is suitable for constructing networks as this analysis examines  
316 pairwise interactions, and as such, other interacting pairs will not be influenced by  
317 *Burkholderia*. Future studies should consider the use of reagent-only controls and spike  
318 controls to help determine if cross-contamination of samples occurs in the sequencing  
319 process (Pollock et al., 2018; Tourlousse et al., 2017), although it is questionable if  
320 these controls would have been of assistance in this case given that laboratory-reared  
321 mosquitoes did not have elevated *Burkholderia* reads.

322  
323 The Shannon diversity index was used to estimate the species richness in mosquitoes  
324 (Shannon, 2001). There were significant differences in diversity between the microbial  
325 communities of *Aedes* mosquitoes compared to *Cx. quinquefasciatus* when compared at  
326 the OTU level (Figure 1; Kruskal-Wallis  $P<0.0001$ ). When comparing groups within *Ae.*  
327 *aegypti*, field collected mosquitoes from either the BG or G trap had a significantly  
328 greater Shannon diversity index compared to the laboratory mosquitoes. No differences  
329 were seen between groups in *Ae. albopictus*, while *Cx. quinquefasciatus* caught in the G  
330 trap had a significantly lower Shannon diversity index compared to laboratory-reared  
331 mosquitoes. Across all species, we found no significant differences in species richness  
332 between mosquitoes caught in the BG and G traps.

333

334 Since both *Ae. albopictus* and *Cx. quinquefasciatus* were heavily infected with  
335 *Wolbachia*, we examined alpha diversity (OTU level) in these mosquitoes when this  
336 endosymbiont was computationally excluded from the dataset (Figure S4). In all cases,  
337 we observed an increase in Shannon diversity when *Wolbachia* was excluded. This was  
338 significant when analyzed by species (*Ae. albopictus*  $P < 0.0001$ ; *Cx. quinquefasciatus*  $P$   
339  $< 0.0001$ ), and for all groups with the exception of *Ae. albopictus* caught in the G traps  
340 (*Ae. albopictus*: BG  $P < 0.005$ , Lab  $P < 0.02$ ; *Cx. quinquefasciatus*: BG  $P < 0.04$ , G  $P <$   
341 0.001, Laboratory  $P < 0.01$ ). No significant differences were seen when comparing the  
342 BG to G groups after removal of *Wolbachia* (*Ae. albopictus*:  $P = 0.23$ ; *Cx.*  
343 *quinquefasciatus*:  $P = 0.57$ ).

344

#### 345 Factors that influence microbiome community structure

346 We examined how the environmental, physiological state in the field, and host species  
347 affected the bacterial community structure using beta diversity analysis by comparing  
348 the microbiomes of the three mosquito species or groups. Within each group, distinct  
349 microbiome clustering patterns were observed between the three mosquito species and  
350 all pairwise comparisons were significantly different (Figure 2; PERMANOVA with Bray-  
351 Curtis distance comparison  $P < 0.05$ ). In general, the microbiome of *Ae. aegypti* was  
352 more divergent compared to the microbiomes of *Cx. quinquefasciatus* and *Ae.*  
353 *albopictus* regardless of origin (Figure 2A). When *Wolbachia* was computationally  
354 excluded from the analysis (Figure 2A), the microbiomes of *Cx. quinquefasciatus* and  
355 *Ae. albopictus* became more divergent in the laboratory, but were not significantly

356 different when considering mosquitoes caught in the BG trap ( $P=0.24$ ). Interestingly,  
357 there was tight clustering of samples from *Cx. quinquefasciatus* and *Ae. albopictus*  
358 caught in the G trap (Figures 2A), although these two groups were significantly different  
359 ( $P=0.00001$ ). When *Wolbachia* was removed, this clustering became more divergent, yet  
360 still was significantly different ( $P=0.0052$ ) (Figure 2A).

361  
362 To determine how the environment and physiological state influences microbial  
363 composition we compared the microbiome of laboratory-reared and field-caught (BG or  
364 G) individuals within each mosquito species. For all species, laboratory-reared  
365 mosquitoes had a significantly distinct microbiota compared to their field counterparts  
366 (Figure 2B). This was most pronounced in *Ae. aegypti* but less distinct for *Ae. albopictus*  
367 and *Cx. quinquefasciatus*. No significant differences were observed in the microbiome  
368 community structure of mosquitoes caught in the BG or G traps for any of the three  
369 mosquito species. No differences were observed when these microbiomes were  
370 analyzed with *Wolbachia* computationally excluded (Figure 2B).

371  
372 Common and differentially abundant bacterial between and within mosquito  
373 species.

374 We examined our data for bacterial genera that were unique to or shared between  
375 mosquito species. The majority of bacteria were common between species with the  
376 notable exception that *Ae. aegypti* caught in the G trap possessed 12 genera not  
377 present in the other two mosquito species (Figure 3A, Table S3). Similarly, when

378 comparing within a species between groups, most bacteria were common to all groups  
379 (Laboratory, BG, G). To detect bacteria that may contribute to the observed differences  
380 in microbiome community structure of a particular mosquito species, we completed  
381 pairwise comparisons to identify bacteria that were differentially abundant (Figure 3B).  
382 These comparisons were completed within each group (Laboratory, BG or G). The  
383 largest differences in the microbiome were seen when comparing *Ae. aegypti* to the  
384 other two species, which agreed with our beta diversity analysis findings. Several  
385 bacterial taxa were differentially abundant between species regardless of group  
386 (Laboratory, BG and G traps), suggesting environmental factors are not greatly  
387 influential on these specific host-microbe associations. For example, *Aeromonas*,  
388 *Serratia*, *Shewanella* and *Wolbachia* were less abundant in *Ae. aegypti* compared to  
389 both *Ae. albopictus* and *Cx. quinquefasciatus* regardless of environmental conditions.  
390 When comparing differential abundance within a group, we were able to find infection  
391 gradients of specific microbes across species. The two examples of these infection  
392 clines were for *Serratia* and *Aeromonas* in laboratory-reared mosquitoes. These bacteria  
393 heavily infected *Cx. quinquefasciatus*, had moderate infection densities in *Ae. albopictus*  
394 and poorly infected or were absent from *Ae. aegypti*, despite the fact these three  
395 mosquito species were reared under common environmental laboratory conditions  
396 (Figure 3B and Figure S5).

397  
398 Several studies have reported that blood feeding alters the species richness in mosquito  
399 guts (Kumar et al., 2010; Oliveira et al., 2011; Terenius et al., 2012; Wang et al., 2011).  
400 While at the community level, the microbiomes of G and BG were not significantly

401 different (Figure 2), we did find specific bacteria that were differentially abundant  
402 between these groups (Figure 3C). These changes were mainly seen in *Cx.*  
403 *quinquefasciatus*. Of the known bacteria, the greatest changes were seen in  
404 *Acinetobacter*, *Tatumella*, and *Pantoea*, with the former two being more abundant in the  
405 mosquitoes caught in BG traps, while the latter was more abundant in mosquitoes  
406 caught in the G trap.

407

#### 408 Total bacterial load in mosquitoes.

409 While high throughput sequencing allows characterization of the composition of the  
410 microbiota, it only provides a relative measure of bacterial density (Gloor et al., 2017).  
411 Therefore, to obtain an estimate of the total bacterial load in each vector species, we  
412 completed qPCR on mosquitoes with universal eubacterial primers that broadly amplify  
413 bacterial species (Kumar et al., 2010). *Culex* mosquitoes were seen to have a higher  
414 total bacterial load when compared to either of the two *Aedes* species (Figure 4; Kruskal  
415 Wallis P<0.0001). When comparing within a species between groups, we found that  
416 laboratory-reared *Ae. aegypti* had significantly greater load than those caught in the field  
417 (Kruskal Wallis P<0.0001). This was also the case for *Cx. quinquefasciatus* (Kruskal  
418 Wallis P<0.0001) while no significant differences were seen between *Ae. albopictus*  
419 groups. As both *Ae. albopictus* and *Cx. quinquefasciatus* are infected by *Wolbachia*, we  
420 also quantified *Wolbachia* by qPCR to determine its relative density in proportion to the  
421 total bacterial load of mosquitoes. While it is inappropriate to statistically compare  
422 amplicons that have different amplification efficiencies, it is evident that *Wolbachia*

423 comprises a major component of the microbiome in *Ae. albopictus* and *Cx.*  
424 *quinquefasciatus*, which corroborates the high throughput sequencing data.

425

426 Microbial interaction networks within mosquitoes.

427 The 16S rRNA sequencing data can be analyzed to create microbial interaction  
428 networks, providing information on potential interaction patterns of microbes such as co-  
429 occurrence and co-exclusion. We created network maps of bacterial interactions using a  
430 variety of models that use presence/absence and relative abundance data to identify  
431 pairwise relationships (Faust and Raes, 2016). In general, we saw that interaction  
432 networks from *Ae. aegypti* were complex, in that they had more nodes and connections  
433 compared to networks from *Ae. albopictus* and *Cx. quinquefasciatus* (Figure 5, Table  
434 S4). For all mosquito species, both co-occurrence and co-exclusion interaction patterns  
435 were observed in all networks (examples of these patterns are shown in Figure S6). We  
436 were able to identify taxa, or groups of bacteria, within these interactions that appear to  
437 be important to the overall structure of the network. For example, in the *Ae. aegypti*  
438 networks from field collected mosquitoes, *Enterobacter* and *Pseudomonas* are highly  
439 interconnected species having between 6 and 15 interactions in these networks. In the  
440 laboratory-reared *Ae. aegypti*, an *Enterobacteriaceae* had several interactions with other  
441 bacteria. Three-way interactions were also seen in many of the networks, and often  
442 these involved *Pseudomonas* and *Enterobacter*. Examples of tripartite co-occurrences  
443 interactions in *Ae. aegypti* networks include *Pseudomonas*, *Asaia* and a *Clostridium*  
444 isolate in laboratory-reared mosquitoes, *Pseudomonas*, *Serratia*, and *Enterobacter* and

445 *Pseudomonas*, *Acidovorax*, and *Enterobacter* in the G and BG groups, respectively.

446 Common co-exclusionary interactions were found in the networks generated from field-

447 collected mosquitoes such as *Pseudomonas* co-excluding *Pantoea* and *Tatumella*.

448 *Burkholderia* co-excluded *Enterobacter*, *Acidovorax* and *Escherichia-Shigella*, however,

449 these *Burkholderia* interactions could possibly be an artifact due to extraction batch

450 variation.

451

452 *Wolbachia* was a highly interconnected taxon in the interaction networks generated from

453 *Ae. albopictus* and *Cx. quinquefasciatus* mosquitoes, and often had co-exclusionary

454 relationship with other bacteria. In BG-collected *Ae. albopictus*, *Wolbachia* co-excluded

455 six other bacteria including *Asaia* and *Pseudomonas*. In other groups, *Wolbachia* was

456 seen to repeatedly exclude *Aeromonas*, *Serratia* and *Shewanella*. The three-way

457 interaction of *Wolbachia* co-excluding the co-occurring *Aeromonas* and *Serratia* was

458 observed in both *Ae. albopictus* and *Cx. quinquefasciatus*. The *Aeromonas* and *Serratia*

459 co-occurrence pattern appears highly robust and independent of environmental factors

460 as this interaction was observed in five of the six *Ae. albopictus* and *Cx.*

461 *quinquefasciatus* groups.

462

463 Artificial infection of symbionts in germ-free or septic mosquitoes

464 The microbial interaction networks highlight the multifarious interactions that occur within

465 mosquito systems. We undertook preliminary validation experiments using *Ae. aegypti*

466 larvae to further demonstrate that microbial interactions can influence microbiome

467 composition and abundance. To this end, we exploited the recently developed  
468 gnotobiotic rearing approach where mosquito larvae can be infected with a single  
469 bacterial taxon (Coon et al., 2014). We compared the density and prevalence of  
470 artificially infected symbionts in gnotobiotic lines (infected with the single symbiont)  
471 compared to conventionally reared septic mosquitoes (that possessed their resident  
472 microbiota) (Figure 6). We completed this with six bacteria isolated from *Aedes*  
473 mosquitoes. Three of the bacteria were isolated from *Ae. aegypti* (*Pantoea*, *Cedecea*  
474 [*Cedecea-aeg*], *Asaia*) while another three were isolated from *Ae. albopictus* (*Serratia*,  
475 *Cedecea* [*Cedecea-alb*], and *Enterobacter*). Three bacteria (*Serratia*; Mann Whitney  
476 P<0.0001, *Cedecea-alb*; Mann Whitney P<0.0001, and *Cedecea-aeg*; Mann Whitney  
477 P<0.0001) were observed to significantly infect *Ae. aegypti* mosquitoes at higher  
478 densities when inoculated into axenic rather than non-axenic mosquitoes. The  
479 prevalence (number of individuals infected) of *Serratia* (Fisher's exact test, P<0.0001)  
480 and *Cedecea-alb* (Fisher's exact test, P<0.0002) was also significantly higher in  
481 gnotobiotic compared to conventionally reared mosquitoes. No change in either the  
482 prevalence or density of infection was seen for *Enterobacter*, *Pantoea* or *Asaia*. Taken  
483 together, these data indicate that microbial interactions influence colonization and  
484 infection dynamics of specific bacterial species within mosquitoes.

485 **Discussion.**

486 It is clear that complex factors combine to shape the microbiome of an organism. To  
487 further increase our understanding of factors that affect the microbiome of mosquitoes,  
488 we sequenced the microbiome of laboratory-reared and field-caught adult mosquitoes  
489 exploiting traps that attract host- or oviposition-seeking individuals. Anautogenous  
490 mosquitoes require a blood meal to provide the necessary nutrition for egg development  
491 and this dramatic influx of blood has been shown to substantially alter the microbiome of  
492 laboratory-reared mosquitoes or those caught in the field and blood-fed in the laboratory  
493 (Kumar et al., 2010; Oliveira et al., 2011; Terenius et al., 2012; Wang et al., 2011).  
494 Intriguingly, we saw few differences in the microbiome between host- and oviposition-  
495 seeking mosquitoes. Across all species, there were no significant differences in alpha or  
496 beta diversity for mosquitoes caught in either trap. Similarly, the total bacterial load was  
497 similar between mosquitoes caught in the BG and G traps, and this was consistent  
498 across all three species. However, when comparing beta diversity between traps, we did  
499 see less variation in the groupings of individuals from both *Ae. albopictus* and *Cx.*  
500 *quinquefasciatus* in the G trap compared to the BG trap. Overall, our results are in  
501 contrast to studies using lab-reared mosquitoes or mosquitoes caught in the field and  
502 blood-fed in the laboratory that indicate increased bacterial load, but decreased diversity  
503 over a 24-48 hour window following a blood meal (Kumar et al., 2010; Oliveira et al.,  
504 2011; Terenius et al., 2012; Wang et al., 2011).

505

506 Several factors could explain the differences between our results examining field-  
507 collected samples and those of previous studies (Kumar et al., 2010; Oliveira et al.,

508 2011; Terenius et al., 2012; Wang et al., 2011). First, mosquitoes often take multiple  
509 blood meals, particularly *Ae. aegypti* (Scott et al., 1993). In our collections, the post-  
510 blood feeding history of mosquitoes is unknown, and it is possible that mosquitoes  
511 caught in either trap may have had a blood meal (or several) prior to being caught. The  
512 body of work examining the influence of blood feeding on the microbiome has only  
513 examined the effect of a single blood meal, not several, and while it appears the  
514 microbiome reverts to a pre-blood fed microbiome several days post-blood meal, it is  
515 unknown how quickly this occurs in the field. In field collected *Anopheles* mosquitoes  
516 reared in the laboratory, reversion to a Bacteroidetes-dominated microbiome, which was  
517 the dominant phylum seen in sugar fed mosquitoes, was seen 4 days post-blood meal  
518 (Wang et al. 2011). In our samples collected from G traps, females may have gone  
519 several days without a blood meal before finding a suitable oviposition site (i.e., the  
520 trap), possibly even relying on nectar-based food sources for sustenance. It is also  
521 possible that mosquitoes in search of an oviposition site may have never taken a blood  
522 meal. While most mosquitoes usually require a blood meal to develop eggs, autogeny  
523 has been reported in these species (Ariani et al., 2015; Chambers and Klowden, 1994;  
524 Olejnícek and Gelbic, 2000). Autogeny rates, which are variable and depend on  
525 temperature and nutrition, have been reported to range from 3 – 34% for *Ae. aegypti*,  
526 around 5% for *Ae. albopictus*, and up to 87% in *Culex* mosquitoes (Ariani et al., 2015;  
527 Chambers and Klowden, 1994; Olejnícek and Gelbic, 2000; Trpis, 1977). Little is known  
528 regarding the influence of microbiota and autogeny, although in the autogenous  
529 mosquito *Ae. atropalpus*, specific gut taxa have been shown to influence egg production  
530 (Coon et al., 2016a). Finally, while reports indicate that BG and G traps preferential

531 catch mosquitoes in different physiological states (Dennett et al., 2007; Figuerola et al.,  
532 2012; Maciel-de-Freitas et al., 2006; Reiter et al., 1986), we did not explicitly examine if  
533 females were gravid or not. While we contemplated dissecting mosquitoes to examine  
534 their parity, we chose not to, as it would increase the potential for contamination of the  
535 samples. Although in our work we did not see dramatic differences in the mosquitoes  
536 caught in BG or G traps, the earlier points highlight the challenges in undertaking studies  
537 on field-derived samples and could explain the disparity between our results and those  
538 from studies undertaken in laboratory settings.

539  
540 Since both *Ae. albopictus* and *Cx. quinquefasciatus* are heavily infected with *Wolbachia*,  
541 we analyzed our data with and without this endosymbiont to garner a better  
542 understanding of the other bacterial microbiota in these mosquitoes. Our high  
543 throughput sequencing and qPCR results demonstrated that *Wolbachia* was the most  
544 abundant bacterium in *Ae. albopictus* and *Cx. quinquefasciatus*. In spite of this, we  
545 obtained sufficient sequencing depth to identify other bacterial taxa – a common  
546 challenge when characterizing *Wolbachia*-infected species using amplicon sequencing  
547 (Minard et al., 2014). Removing *Wolbachia* from our analysis increased the Shannon  
548 diversity index, indicating the remaining microbiota within these mosquitoes is relatively  
549 even. For beta diversity, we found that our results were mixed and dependent on group.  
550 When comparing within a group, removing *Wolbachia* made the microbiomes of  
551 laboratory-reared *Ae. albopictus* and *Cx. quinquefasciatus* more divergent while the  
552 microbiomes of field mosquitoes tended to be less different. These findings are  
553 consistent with a study by Novakova et al (2016) that found the removal of *Wolbachia*

554 from their analysis led to less distinct differences for field-collected mosquitoes. When  
555 comparing groups within a species, exclusion of *Wolbachia* made the comparison of  
556 field and laboratory mosquitoes more distinct, likely due to differences in the gut-  
557 associated microbiota. Similar to a recently published study (Coon et al., 2016b), we  
558 found a small number of *Wolbachia* reads in *Ae. aegypti* mosquitoes collected in G  
559 traps. However, we could not confirm the presence of the bacteria with conventional  
560 PCR-based approaches, suggesting these results could be a sequencing or laboratory  
561 artifact. Should populations of *Ae. aegypti* be naturally infected with *Wolbachia*, this  
562 could have important ramifications for biological control strategies being implemented  
563 into the field (Bourtzis et al., 2014; Flores and O'Neill, 2018; Hughes and Rasgon, 2014),  
564 and as such, further research in this area is warranted.

565  
566 Similar to findings in other mosquito species, many bacteria were shared between  
567 different mosquito species, and laboratory-reared mosquitoes were seen to have a  
568 divergent microbiome from their field counterparts (Boissière et al., 2012; Coon et al.,  
569 2016b). These common taxa, particularly those that infect at high abundance, could be  
570 candidate bacteria for consideration in novel pan-mosquito microbial control strategies  
571 as they would likely be compatible for all three vector species (Saldaña et al., 2017).  
572 When focusing specifically on bacterial titers, few bacteria were seen to be significantly  
573 different between mosquitoes caught in either traps. In *Culex* mosquitoes, *Pantoea* was  
574 more abundant in individuals caught in the G trap. Similar to this finding, *Pantoea* has  
575 been found to increase in abundance after a blood meal in *Anopheles* mosquitoes  
576 (Wang et al., 2012). When considering pairwise comparisons between species, there

577 were more differentially abundant genera when comparing *Ae. aegypti* to the other two  
578 species, which explains the greater divergence of the *Ae. aegypti* microbiome to the  
579 microbiome of the other two species. The *Ae. aegypti* samples were collected from a  
580 different location than the other two species, thus, it is possible that environmental  
581 factors could explain these differences. However, we also saw common changes that  
582 were consistent regardless of groups (BG, G and Laboratory), such as *Aeromonas*,  
583 *Clostridium*, *Serratia*, *Shewanella* and *Wolbachia*, indicating these bacteria were not  
584 greatly influenced by the environment and that other factors affected their presence in  
585 the particular mosquito species. Notably, we found that in laboratory-reared mosquitoes,  
586 *Culex* harbored significantly higher titers of *Serratia* and *Aeromonas*, compared to *Ae.*  
587 *albopictus*, while these bacteria were at low abundance or absent from *Ae. aegypti*.  
588 These results suggest there are host and/or bacterial related factors that make this  
589 particular strain of *Ae. aegypti* (Galveston) inhospitable for *Serratia* and *Aeromonas* as  
590 all three mosquito species were subjected to similar uniform environmental conditions  
591 when reared in the insectary.

592

593 Current evidence of microbial interactions within mosquitoes is mainly limited to  
594 interactions between *Wolbachia* and other microbiota (Audsley et al., 2017a; Hughes et  
595 al., 2014; Rossi et al., 2015; Zink et al., 2015), or between vertically transmitted  
596 symbionts in other arthropod systems (Goto et al., 2006; Kondo et al., 2005; Macaluso  
597 et al., 2002; Rock et al., 2017). As such, our understanding is generally restricted to  
598 inherited symbionts and we have a poor understanding of the scale of interactions  
599 between microbes that are associated with insect guts. To address this, we created

600 microbial interaction networks to identify pairwise co-occurrence and co-exclusion  
601 patterns. To avoid spurious interactions, which could be due to the presence or absence  
602 of a microbe in one environmental condition but not another, we limited our network  
603 analysis to within a group for each particular species. Our analysis identified 116 co-  
604 occurrence or co-exclusion interactions, substantially increasing the number of bacterial  
605 interactions observed in mosquitoes. Bacterial interaction networks generated from *Ae.*  
606 *aegypti* mosquitoes were more complex than *Ae. albopictus* or *Cx. quinquefasciatus* in  
607 that they had more nodes and connections. Species richness may explain the  
608 differences observed in network structure, as in general, the more complex networks  
609 had a greater number of OTUs. Other factors that may have influenced the identification  
610 of interacting bacteria are the presence of *Wolbachia* in *Ae. albopictus* and *Cx.*  
611 *quinquefasciatus*, as well as the differences in sample size between mosquito species.  
612 Further studies are warranted to determine why some networks are highly  
613 interconnected while others are not.

614  
615 Interestingly, in the more complex networks, we saw evidence of hub microbial taxa that  
616 were highly interconnected. *Pseudomonas* and bacteria within the *Enterobacteriaceae*  
617 appear to be important hub taxa. Some of the interactions observed here have been  
618 previously reported in *Ae. triseriatus* and *Ae. japonicus* (Muturi et al., 2016a), including  
619 negative interactions between bacteria within the *Burkholderiaceae* and *Pseudomonas*  
620 and *Acinetobacter* as well as *Asaia-Enterobacter* and *Asaia-Ralstonia* interactions. The  
621 majority of interactions reported in *Ae. triseriatus* and *Ae. japonicus* were negative  
622 (Muturi et al., 2016a), whereas here we see a mix of both co-occurrence and co-

623 exclusion patterns. Specific hub microbes that are strongly interconnected have been  
624 found in plant microbiomes and these taxa have a profound effect on overall microbiome  
625 structure (Agler et al., 2016). In our data, we also saw three-way interactions. Often  
626 these were relationships were co-occurring, or were formed by two co-occurring bacteria  
627 both co-exclude another bacterium, suggesting the existence of multi-taxa interactions.  
628 Further work is required to determine the functionality of these multi-interacting partners  
629 and if these interactions represent keystone guilds (Banerjee et al., 2018). The  
630 identification of common interaction pairings across several groups indicates that these  
631 interactions, and the methods we employed to identify them, are robust and presumably  
632 not influenced by physiological state or other environmental conditions.

633  
634 It is important to highlight that these network maps represent patterns, and not direct  
635 interactions. Many of the observed interactions may be due to microbes sharing a similar  
636 ecological niche, and a substantial challenge, particularly for highly interconnected taxa,  
637 will be to investigate these interactions further. To undertake initial validation steps and  
638 to demonstrate that microbial interactions are an important factor influencing the  
639 colonization of gut-associated microbiota, we infected six culturable bacterial taxa into  
640 *Ae. aegypti* larvae that either possessed or lacked their resident microbiota. *Serratia* and  
641 *Cedecea*, which were isolated from *Ae. albopictus*, poorly infected *Ae. aegypti* when it  
642 possessed its native microbiome, however, when mosquitoes lacked their native  
643 microbiota, these bacteria infected at a higher titer. Even more striking was the effect on  
644 prevalence (number of individuals infected), which changed from 38% and 50% for  
645 *Serratia* and *Cedecea-alb*, respectively, in conventionally reared mosquitoes that

646 possessed their resident microbiota, to 100% infection when infected into axenic larvae.

647 This indicated that the poor infection rates were not related to host or bacterial genetics,

648 but to microbial incompatibility. In our networks, *Serratia* and *Cedecea* have several co-

649 exclusionary relationships with dominant bacterial taxa such as *Asaia*, *Pseudomonas*,

650 and *Enterobacter*, which may explain these results, however specific examination of

651 these interactions in adult mosquitoes is required. Importantly, not all of the bacterial

652 taxa artificially infected into larvae increased in prevalence and density suggesting these

653 enhanced colonization effects are not simply due to mosquitoes lacking their microbiota,

654 but rather are specific for each taxa, likely due to specific microbial interactions. Similar

655 to our findings, it has been reported that antibiotic treatment prior to bacterial

656 supplementation in a sugar meal can increase the prevalence of infection of gut

657 microbes in female *Anopheles* and *Aedes* mosquitoes (Ramirez et al., 2014), indicating

658 that resident gut bacteria that are susceptible to antibiotics are antagonistic to the

659 supplemented bacterium. It is important to note that our reinfection study, which

660 exploited the gnotobiotic rearing system, examined interactions in larvae, not adults, and

661 that differences in the gut morphology and function between these two life stages may

662 alter microbial interactions (Engel and Moran, 2013). However, our findings combined

663 with the work of Ramirez et al. (2014), suggest that microbial interactions between gut-

664 associated bacteria occur within mosquitoes and influence symbiont colonization in

665 aquatic and adult life stages, which likely affects microbiome species richness and

666 evenness. These colonization traits and co-exclusionary associations could offer a

667 possible explanation for the variability seen in the mosquito microbiome between

668 individuals, as bacteria that initially infect the gut may impede colonization by other  
669 microbes.

670

671 In this study, we compared microbial interaction networks from field and laboratory  
672 mosquitoes to examine the influence of the general environment but it is possible other  
673 factors may influence network structure. In particular, it would be interesting to  
674 determine if microbial networks differ between tissues within mosquitoes, such as the  
675 salivary glands, germline and the gut. For example, microbial network analysis from the  
676 human microbiome project found strong niche specialization in their networks, whereby  
677 different body sites had contrasting microbial networks (Faust et al., 2012). In *Anopheles*  
678 mosquitoes, salivary glands have a more diverse microbiome compared to the gut  
679 (Sharma et al., 2014), and it is conceivable that elevated species richness would allow  
680 for greater network interactions. Furthermore, the germlines of male and female  
681 *Anopheles* mosquitoes share some common taxa but there are also quantitative  
682 differences (Segata et al., 2016). These differing microbial niches could be exploited to  
683 determine the influence of species evenness on microbial networks. Performing network  
684 analysis on gut samples of *Ae. albopictus* and *Cx. quinquefasciatus* may also overcome  
685 any issue with *Wolbachia* sequestering the majority of the reads, as *Wolbachia* primarily  
686 resides within the germline in these mosquito species. Here, we assessed whole  
687 mosquitoes to give an initial overall picture of microbial interactions, but analysis of  
688 distinct tissues may identify interactions of bacteria that are proximal to one another, and  
689 these interactions are more likely to reflect true microbe-microbe interactions, rather than  
690 patterns associated with environmental exposure. Within a species we collected

691 mosquitoes from a single site, but future studies examining interaction networks should  
692 incorporate diverse sites. Common pairwise interactions identified across sites would  
693 indicate robust relationships not influenced by environmental factors.

694  
695 In summary, we examined the microbiome of three important mosquito vectors, *Ae. aegypti*, *Ae. albopictus*, and *Cx. quinquefasciatus*. While the overall microbiome  
696 structure between host-seeking or ovipositing females was similar, we identified specific  
697 bacteria that changed in abundance between mosquito species. Our analysis identified a  
698 suite of pairwise interactions used for generating microbial interaction networks, and  
699 together with re-infection studies we have demonstrated that microbial interactions affect  
700 microbiome composition and abundance of specific bacterial taxa. These findings add to  
701 our understanding of microbiome community structure of mosquitoes and factors that  
702 influence microbiome acquisition and maintenance in these important disease vectors.  
703

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716

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718

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1053

1054

1055 **Data accessibility**

1056

1057 The NCBI accession number for the raw sequencing data reported here is  
1058 PRJNA422599.

1059

1060

1061 **Author Contributions**

1062

1063 SH, KK, LA, JAD, EAH, YF, and GLH designed the experiments. GCM, CLF, JAD and  
1064 MD oversaw the fieldwork. SH and MP completed the experiments. KK, MMR, LA, SH,  
1065 EAH and GLH undertook analysis. SH, KK, JAD, MD, and GLH wrote and edited the  
1066 manuscript and all authors agreed to the final version. YF and GLH acquired funding  
1067 and supervised the work.

1068

1069 **Figure legends.**

1070

1071 **Figure 1.** Shannon diversity indices at the OTU level for all mosquito species (A) or for  
1072 each group within a species (B). A Kruskal-Wallis test with a Dunn's multiple comparison  
1073 test was used to determine significance (\*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  
1074  $p < 0.0001$ ) within a species (black) or group (grey). Abbreviations: Aeg - *Ae. aegypti*, Alb  
1075 - *Ae. albopictus*, Cq - *Cx. quinquefasciatus*. Bars on the box plots show maximum to  
1076 minimum range. Sample size for species and group is indicated by the numbers above  
1077 the box-plots and in Table S1.

1078

1079 **Figure 2.** Principal Coordinates Analysis (OTU level) using Bray-Curtis dissimilarity,  
1080 comparing identified OTUs within a group (A) or species (B). PERMANOVA significance  
1081 values for pairwise comparison are reported in the lower right corner for each analysis.  
1082 For A and B, upper plots include *Wolbachia* while *Wolbachia* has been computationally  
1083 excluded in the lower plots.

1084

1085 **Figure 3.** Common and differentially abundant bacteria (genus level) within mosquitoes.  
1086 Venn diagram showing number of common bacterial genera between mosquito groups  
1087 and species (A). Pairwise comparison of bacterial density between each mosquito  
1088 species within each group (B). Pairwise comparison of bacterial density for mosquito  
1089 caught in the G trap compared the BG trap (*Ae. aegypti* – Aeg, *Ae. albopictus* – Alb, *Cx.*  
1090 *quinquefasciatus* - Cq.) (C). Log<sub>2</sub> values indicated fold change in bacterial density. List of  
1091 common taxa for each species and group are presented in Table S3.

1092

1093 **Figure 4.** Total bacterial load of mosquitoes. Comparison of bacterial load for each  
1094 species (left) or within each group for each mosquito species (right). Bacterial load is  
1095 represented as a ratio between 16S rRNA gene copies to S7 copies (*Ae. aegypti* and  
1096 *Cx. quinquefasciatus*) or actin (*Ae. albopictus*) genes. The density of *Wolbachia* was  
1097 estimated for *Ae. albopictus* (*Wolbachia* 16s:Actin) and *Cx. quinquefasciatus* (ftsZ:S7). A  
1098 Kruskal-Wallis test with a Dunn's multiple comparison test was used to determine  
1099 significance (\*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $p < 0.0001$ ) of total bacterial loads  
1100 within a species. Bars on the box plots show maximum to minimum range. Sample size  
1101 for all species and groups is described in Table S1.

1102

1103 **Figure 5.** Microbial interaction networks for mosquitoes. Interaction networks were built  
1104 using CoNet. Node colors represent unique taxonomy identifiers. Red edges represent  
1105 co-exclusion/negative correlation, green edges represent co-occurrence/positive  
1106 correlation interactions between relative abundance profiles. Multiple edges connecting  
1107 the same nodes indicate significance from more than one metric (Bray-Curtis  
1108 dissimilarity, Kullback-Leibler divergence, mutual information, Spearman correlation, and  
1109 Pearson correlation). Undetermined interactions have been removed from the network.

1110

1111 **Figure 6.** Infection density and prevalence of bacteria inoculated into conventionally  
1112 reared or axenic mosquitoes. As such, gnotobiotic reared lines (GB) only possessed the

1113 inoculated bacteria while conventionally reared lines (C) possess their native microbiota  
1114 in addition to the inoculated strain. Inoculated bacteria possessed a plasmid expressing  
1115 antibiotic resistance and mCherry fluorescent protein. The bacterial load was quantified  
1116 by counting mCherry colonies on selective plates. *Serratia*, *Cedecea-alb* and  
1117 *Enterobacter* were isolated from *Ae. albopictus* (Galveston) while *Pantoae*, *Asaia* and  
1118 *Cedecea-aeg* were isolated from *Ae. aegypti* (Galveston). A Mann-Whitney test with a  
1119 Dunn's multiple comparison test was used to determine significance (\*\*\*\* is  $P<0.0001$ ).  
1120 Pie charts indicate prevalence of infection (Fisher's exact test, \*\*\*\*  $P<0.0001$ , \*\*\*  
1121  $P<0.0002$ ).  
1122

1123 **Supplementary figures**

1124

1125 **Figure S1.** Map of Houston, Texas, indicating the field collection sites.

1126

1127 **Figure S2.** Shannon entropy rarefied at intervals between 0 and 100,000 reads in each  
1128 sample from different groups (G, BG, Laboratory) in all three mosquito species.

1129

1130 **Figure S3.** Heat maps indicating bacterial relative abundance for the three mosquito  
1131 species. OTUs were grouped to genus level or higher ranks (when genus was  
1132 ambiguous) and the relative abundance indicated by color for each individual (column) is  
1133 shown. The upper heat map is with *Wolbachia* present while the lower has *Wolbachia*  
1134 excluded. The dendrogram/clustering of the bacteria is generated based on their relative  
1135 abundance correlation across samples.

1136

1137 **Figure S4.** Shannon diversity of *Ae. albopictus* and *Cx. quinquefasciatus* with and  
1138 without *Wolbachia*. For analysis of samples without the endosymbiont, *Wolbachia* reads  
1139 were computationally excluded from the analysis and then Shannon diversity was  
1140 recalculated.

1141

1142 **Figure S5.** Relative abundance of *Serratia* and *Aeromonas* from high-throughput  
1143 sequencing in *Cx. quinquefasciatus*, *Ae. albopictus* and *Ae. aegypti* mosquitoes reared  
1144 in the lab. Data were analyzed using a one-way ANOVA using Tukeys method for  
1145 pairwise comparisons (\*\*\*\* is P<0.0001).

1146

1147 **Figure S6.** Examples of co-occurrence and co-exclusion microbial pairs identified in the  
1148 interaction networks. Scatterplots of relative abundance profiles displaying statistically  
1149 significant co-occurrence and co-exclusion patterns in mosquito groups. Points  
1150 represent the relative abundance values of the pair in each sample.

1151

1152

1153     **Supplemental tables.**

1154

1155     **Table S1.** Number of samples used in the study and their division into groups by  
1156     metadata.

1157

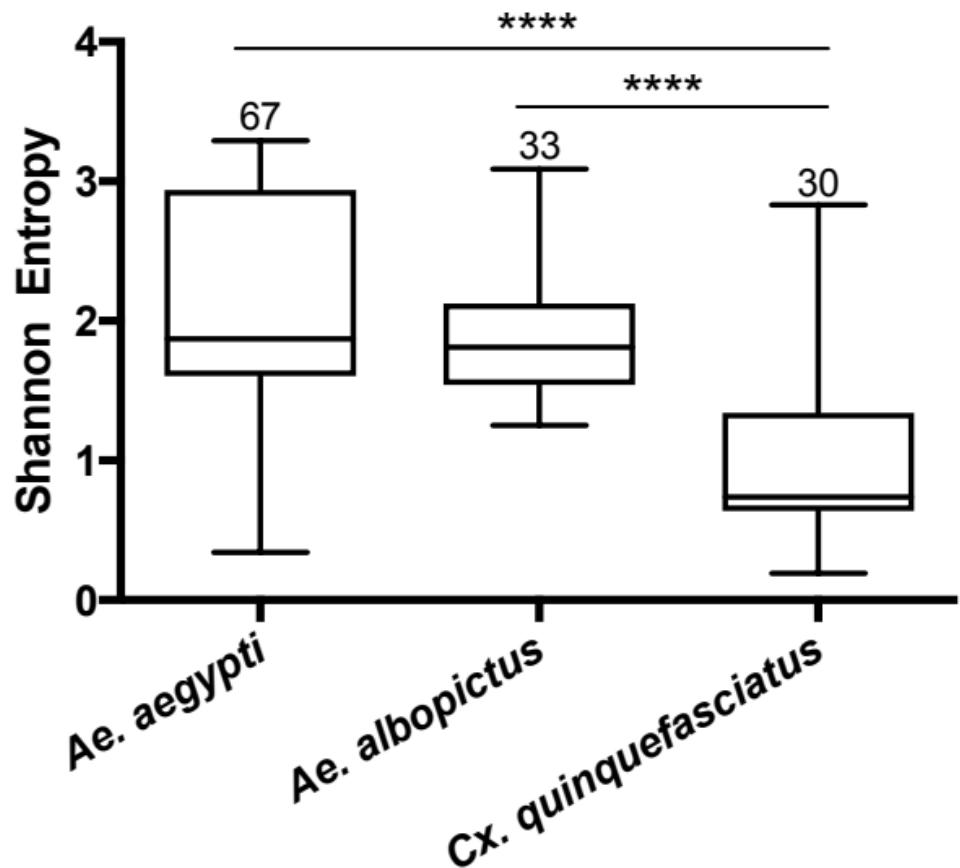
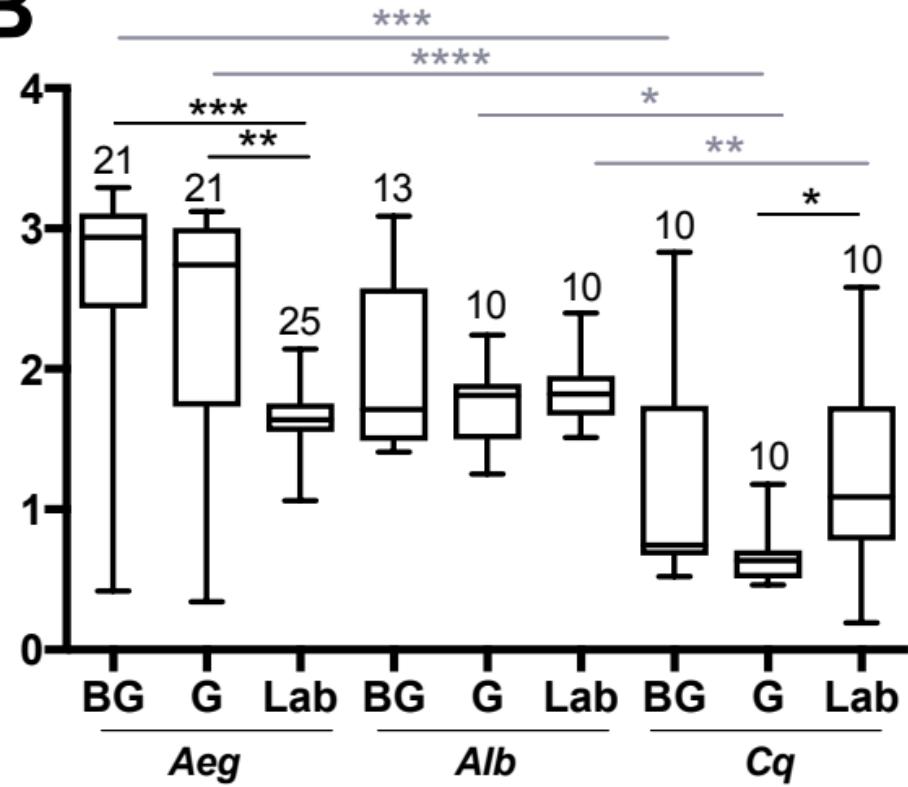
1158     **Table S2.** Complete and filtered to 0.1% OTU table with read counts from each  
1159     individual mosquito (*Ae. aegypti*, *Ae. albopictus* *Cx. quinquefasciatus*). Each library was  
1160     constructed from a single female mosquito.

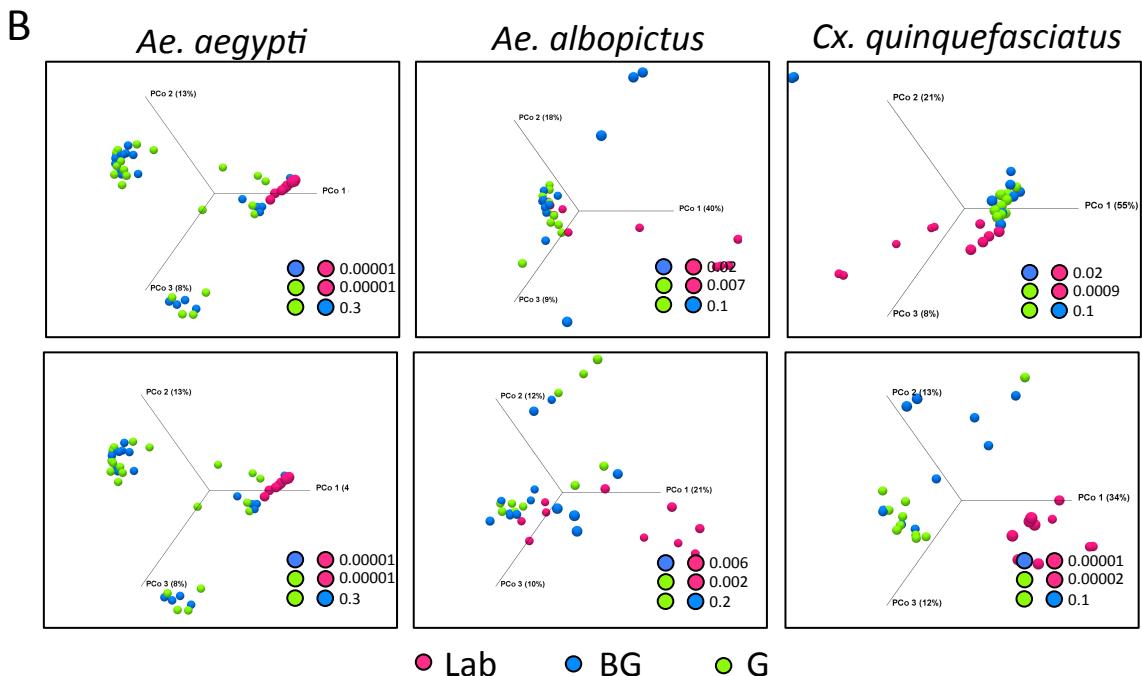
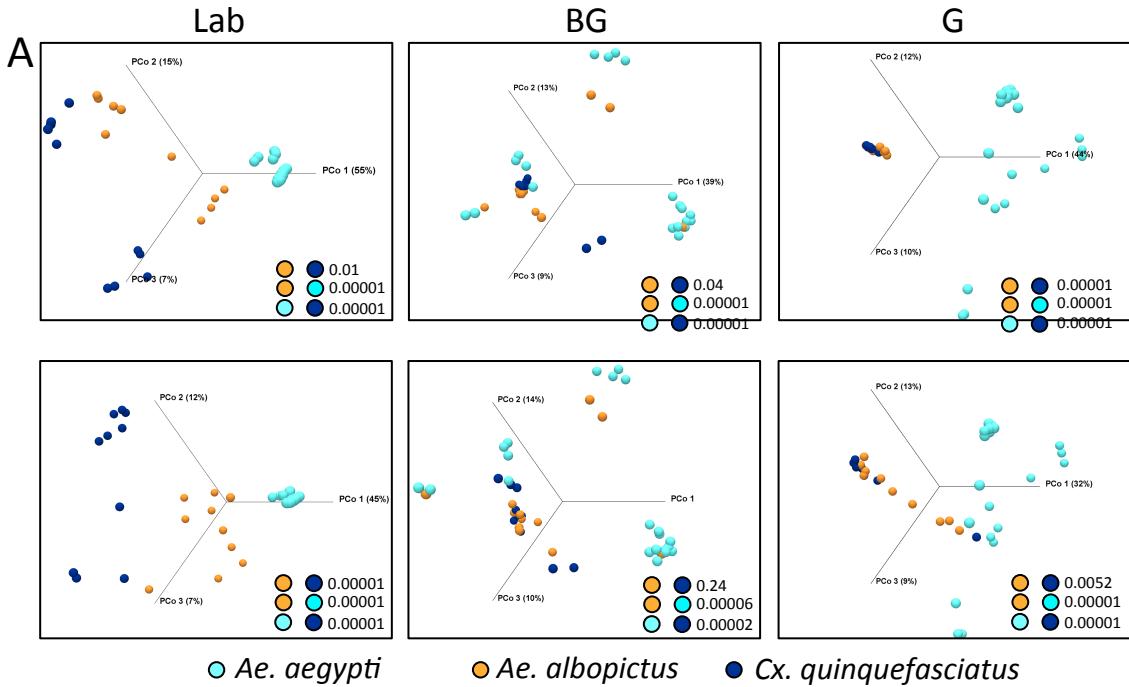
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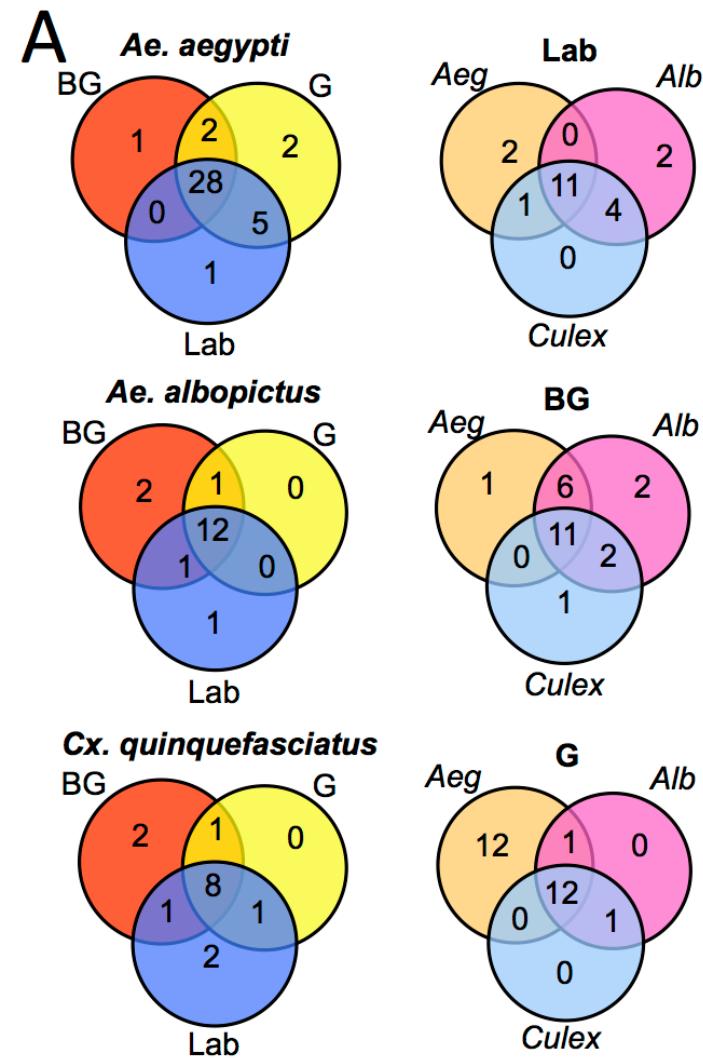
1162     **Table S3.** List of the bacterial taxa present or absent within each species and group  
1163     which was used to create Venn diagrams (Figure 3A).

1164

1165     **Table S4.** OTU tables (relative abundance profiles) used in CoNet analysis. Relative  
1166     abundance of each OTU in a sample was calculated by dividing number of reads by total  
1167     number of reads of the sample and OTUs with read counts below 0.1% across all  
1168     mosquito samples were excluded from analysis. OTU relative abundance profiles were  
1169     summed based on lowest common taxonomy, down to genus level. When building  
1170     networks for each mosquito group (*Ae. aegypti* BG, G or Lab, *Ae. albopictus* BG, G or  
1171     Lab, or *Cx. quinquefasciatus* BG, G or Lab), abundance profiles present (non-zero  
1172     abundance) in less than 5 samples were excluded from analysis.

**A****B**



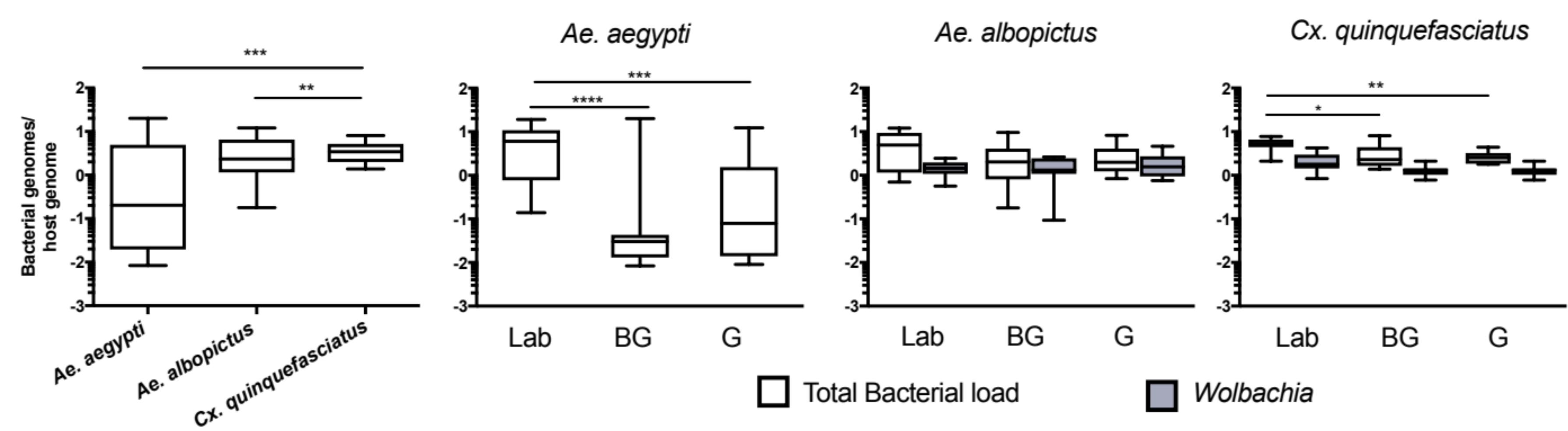


**B**

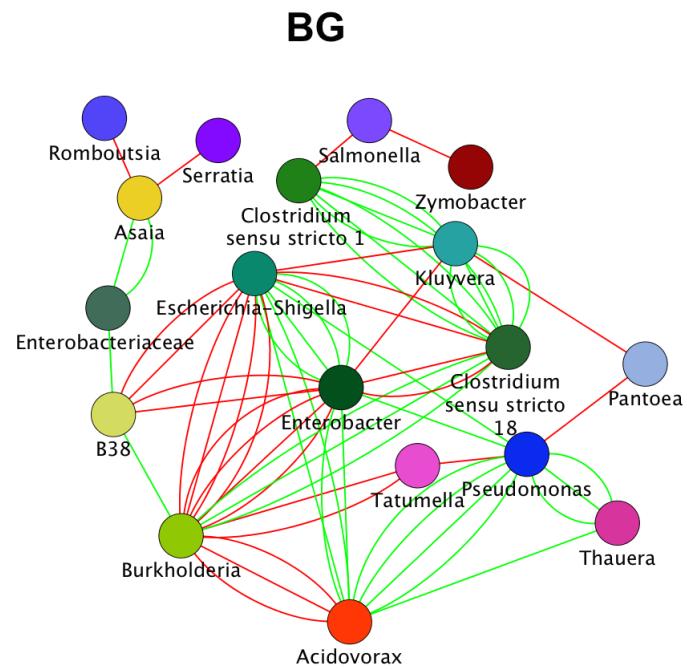
Name	Aeg vs Alb			Aeg vs Cul			Cul vs Alb		
	Lab	BG	G	Lab	BG	G	Lab	BG	G
Acinetobacter						15.3			
Aeromonas	12.9	11.5	12.4	15.8	10.3	10.7	2.9		
Asaia	-2.7			-6.6					
Burkholderia	-8.3			-8.3					
Cedecea	-7.2			-16.1					
Chryseobacterium	11.7								
<i>Clostridium.sensu.stricto.1</i>	-11.4	-3.5	-15.7	-11.4	-17.1	-15.7			
<i>Clostridium.sensu.stricto.18</i>	-11.4	-2.6	-15.4	-11.4	-16.3	-15.4			
Enterobacter				-11.1			-9.5	-12.0	
Escherichia/Shigella				-3.1	-11.3		-9.2	-12.6	
Kluyvera				-5.1		-8.4			
Leucobacter		14.5				15.0			
Pantoea									
Pseudomonas									
Romboutsia				-10.2		-10.2			
Serratia	6.3	4.1	2.8	9.1	4.2	0.6	2.8		
Shewanella	13.5	13.7	13.5	10.5	16.0	13.5			
Sphingobacterium					14.9			9.4	
Tatumella									
Thauera				-5.2					
uncultured							16.1		
uncultured.bacterium.1	-5.6	-1.3		-12.6					
uncultured.bacterium.2	-7.1			-15.5					
Wolbachia	14.7	10.1	5.4	14.7	10.4	5.5			0.1

**C**

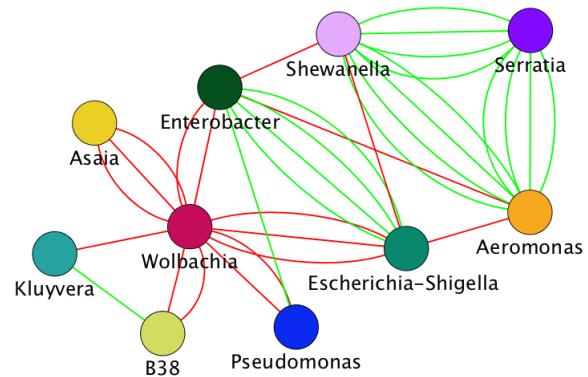
Name	G vs BG		
	Aeg	Alb	Cq
Acinetobacter			13.0
Pantoea			-12.9
Pseudomonas		3.3	3.3
Tatumella	1.2		11.1
uncultured			16.1
uncultured bacterium-1			1.6
uncultured bacterium-2			5.1
Wolbachia			-0.4



### *Ae. aegypti*



### *Ae. albopictus*



### *C. quinquefasciatus*

