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

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Abstract.

Microbial interactions are an underappreciated force in shaping insect microbiome communities. Although pairwise patterns of symbiont interactions have been identified, we have a poor understanding regarding the scale and the nature of co-occurrence and co-exclusion interactions within the microbiome. To characterize these patterns in mosquitoes, we sequenced the bacterial microbiome of *Aedes aegypti*, *Ae. albopictus,* and *Culex quinquefasciatus* caught in the field or reared in the laboratory and used these data to generate interaction networks. For collections, we used traps that attracted host-seeking or ovipositing female mosquitoes to determine how physiological state affects the microbiome under field conditions. Interestingly, we saw few differences in species richness or microbiome community structure in mosquitoes caught in either trap. Co-occurrence and co-exclusion analysis identified 116 pairwise interactions substantially increasing the list of bacterial interactions observed in mosquitoes. Networks generated from the microbiome of *Ae. aegypti* often included highly interconnected hub bacteria. There were several instances where co-occurring bacteria co-excluded a third taxa, suggesting the existence of tripartite relationships. Several associations were observed in multiple species or in field and laboratory-reared mosquitoes indicating these associations are robust and not influenced by environmental or host factors. To demonstrate that microbial interactions can influence colonization of the host, we administered symbionts to *Ae. aegypti* larvae that either 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
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56 symbiont, *Wolbachia*.
57
58

Introduction.

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

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

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

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

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

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

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

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

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

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

Material and Methods.

Mosquito collections and DNA extractions.

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

High throughput sequencing and bioinformatics analysis.

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

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

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

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

Detection of complex interaction patterns.

OTUs with read counts below 0.1% of total number of reads in all samples were excluded from analysis. The remaining OTUs were combined based on lowest common taxonomy assignments down to genus level, and relative abundance tables were generated by normalizing read counts against total number of reads in the original data. The resulting number of unique entries identified in samples was 33. Interactions (such as co-occurrence and co-exclusion) among these were identified using CoNet app (Faust and Raes, 2016) in Cytoscape (Shannon et al., 2003) using the following ensemble of methods: Pearson correlation (Pearson, 1895), Spearman correlation (Sedgwick, 2014), mutual information (Cover and Thomas, 2012), Bray-Curtis dissimilarity (Bray and Curtis, 1957) and Kullback-Leibler divergence (Kullback and Leibler, 1951). Statistical significance of each pair was tested using the row-shuffle randomization option and interactions that scored at the top and bottom 1% of 100 bootstraps were reported. Resulting statistically significant interactions were categorized by the software into three groups: co-presence, co-exclusion and unknown. Unknown interactions represent statistically significant patterns that cannot be clearly categorized 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 $1x10^7$

CFU/ml of transgenic symbionts possessing the plasmid the pRAM18dRGA-mCherry that was derived from pRAM18dRGA (Burkhardt et al., 2011). Bacterial cultures were quantified with a spectrophotometer (DeNovix DS-11, DeNovix) and validated by plating to determine CFU. For conventional rearing of mosquitoes, eggs (non-sterilized) were vacuum hatched and grown under non-aseptic conditions in a T75 tissue culture flask supplemented with transgenic symbionts at the concentration of $1x10^7$ CFU/ml. To feed mosquitoes, ground fish food pellets were sterilized by autoclaving, and mixed with sterile water. The equivalent of 6 mg of fish food was fed to both gnotobiotic and conventionally reared mosquitoes every second day. To quantify the bacterial load of symbionts, surface sterilized L4 larvae were homogenized and plated on a selective media (50 µg/ml Kanamycin) on which only transgenic symbionts grew (pRAM18dRGA-mCherry induces resistance to Kanamycin). After incubation at 30˚C or 37˚C (depending on symbiont) for 2-3 days, colonies (expressing mCherry fluorescent protein) were counted. All colonies observed on the kanamycin plate were confirmed to have mCherry fluorescence.

Results

Microbiome Diversity.

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

When examining the sequencing data at the genus level, the microbiomes of *Cx. 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; Tourlousse 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

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

Factors that influence microbiome community structure

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

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

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

Common and differentially abundant bacterial between and within mosquito species.

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

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

Several studies have reported that blood feeding alters the species richness in mosquito guts (Kumar et al., 2010; Oliveira et al., 2011; Terenius et al., 2012; Wang et al., 2011). 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.,

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

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

Current evidence of microbial interactions within mosquitoes is mainly limited to interactions between *Wolbachia* and other microbiota (Audsley et al., 2017a; Hughes et al., 2014; Rossi et al., 2015; Zink et al., 2015), or between vertically transmitted symbionts in other arthropod systems (Goto et al., 2006; Kondo et al., 2005; Macaluso et al., 2002; Rock et al., 2017). As such, our understanding is generally restricted to inherited symbionts and we have a poor understanding of the scale of interactions 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 *Enterobacteriacaea*
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

possessed their resident microbiota, to 100% infection when infected into axenic larvae. This indicated that the poor infection rates were not related to host or bacterial genetics, but to microbial incompatibility. In our networks, *Serratia* and *Cedecea* have several co-exclusionary relationships with dominant bacterial taxa such as *Asaia*, *Pseudomonas,* and *Enterobacter*, which may explain these results, however specific examination of these interactions in adult mosquitoes is required. Importantly, not all of the bacterial taxa artificially infected into larvae increased in prevalence and density suggesting these enhanced colonization effects are not simply due to mosquitoes lacking their microbiota, but rather are specific for each taxa, likely due to specific microbial interactions. Similar to our findings, it has been reported that antibiotic treatment prior to bacterial supplementation in a sugar meal can increase the prevalence of infection of gut microbes in female *Anopheles* and *Aedes* mosquitoes (Ramirez et al., 2014), indicating that resident gut bacteria that are susceptible to antibiotics are antagonistic to the supplemented bacterium. It is important to note that our reinfection study, which exploited the gnotobiotic rearing system, examined interactions in larvae, not adults, and that differences in the gut morphology and function between these two life stages may alter microbial interactions (Engel and Moran, 2013). However, our findings combined with the work of Ramirez *et al*. (2014), suggest that microbial interactions between gut-associated bacteria occur within mosquitoes and influence symbiont colonization in aquatic and adult life stages, which likely affects microbiome species richness and evenness. These colonization traits and co-exclusionary associations could offer a 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.*
696 *aegypti*, *Ae. albopictus,* and *Cx. quinquefasciatus.* While the overall microbiome
697 structure between host-seeking or ovipositing females was similar, we identified specific
698 bacteria that changed in abundance between mosquito species. Our analysis identified a
699 suite of pairwise interactions used for generating microbial interaction networks, and
700 together with re-infection studies we have demonstrated that microbial interactions affect
701 microbiome composition and abundance of specific bacterial taxa. These findings add to
702 our understanding of microbiome community structure of mosquitoes and factors that
703 influence microbiome acquisition and maintenance in these important disease vectors.

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716

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Data accessibility

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

Author Contributions

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

Figure legends.

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

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

Figure 3. Common and differentially abundant bacteria (genus level) within mosquitoes. Venn diagram showing number of common bacterial genera between mosquito groups and species (A). Pairwise comparison of bacterial density between each mosquito species within each group (B). Pairwise comparison of bacterial density for mosquito caught in the G trap compared the BG trap (*Ae. aegypti* – *Aeg*, *Ae. albopictus* – *Alb*, *Cx. quinquefasciatus* - *Cq*.) (C). Log_2 values indicated fold change in bacterial density. List of common taxa for each species and group are presented in Table S3.

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

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

Figure 6. Infection density and prevalence of bacteria inoculated into conventionally 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

Supplementary figures

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

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

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

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

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

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

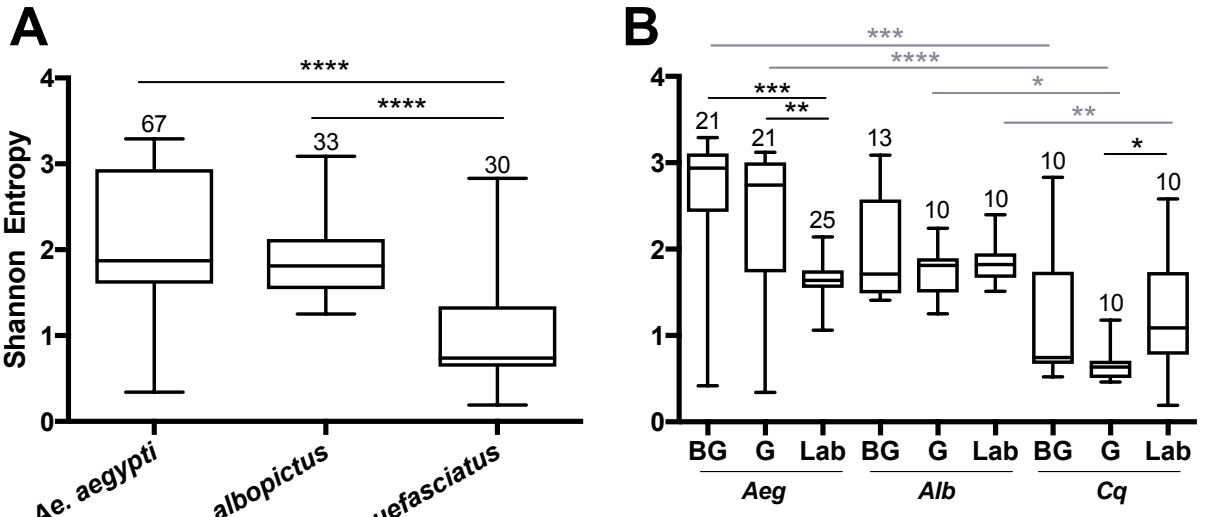
Supplemental tables.

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

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

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

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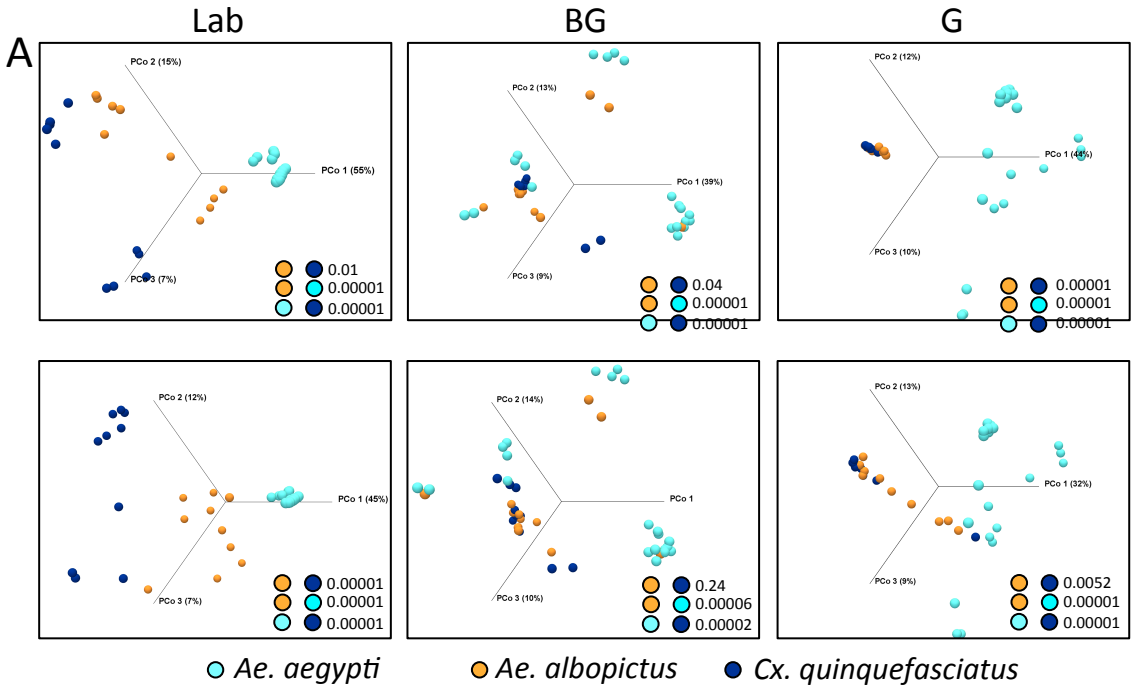
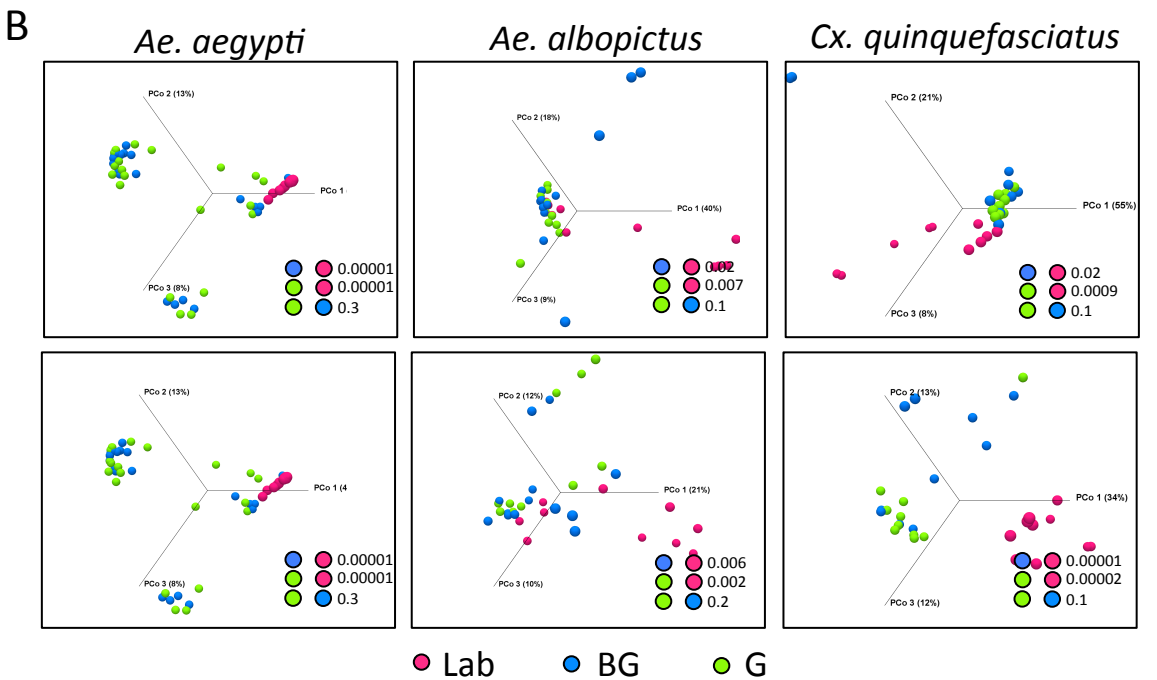
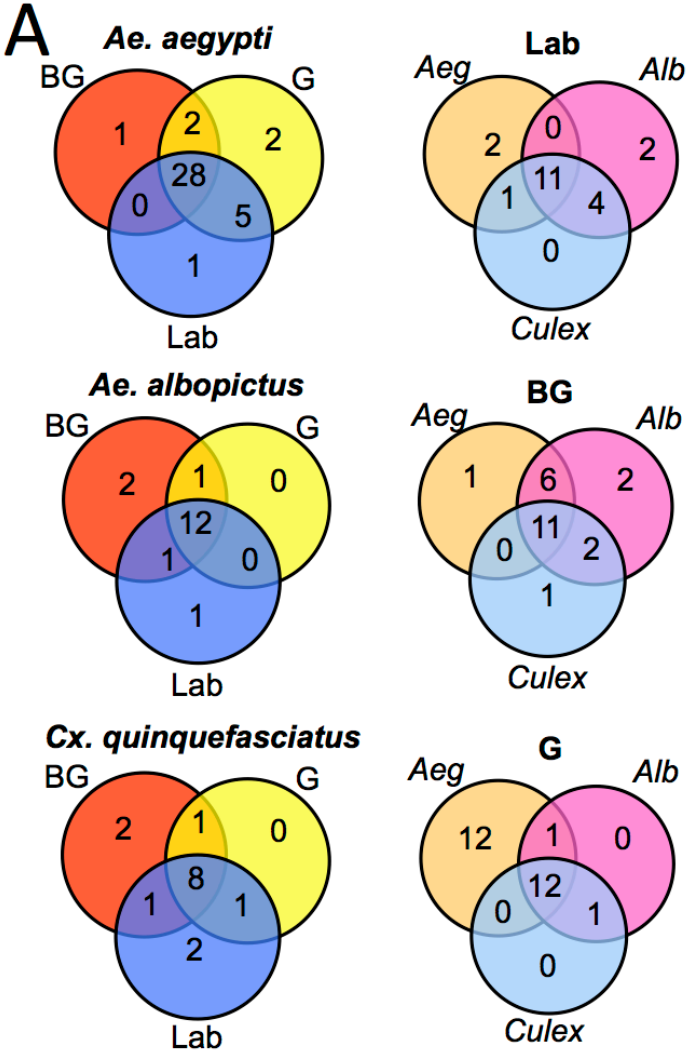
Alb
Cq


A
Lab
BG
G
PCo 2 (15%)
PCo 1 (55%)
PCo 3 (7%)
0.01
0.00001
0.00001
PCo 2 (13%)
PCo 1 (39%)
PCo 3 (9%)
0.04
0.00001
0.00001
PCo 2 (12%)
PCo 1 (44%)
PCo 3 (10%)
0.00001
0.00001
0.00001
PCo 2 (12%)
PCo 1 (45%)
PCo 3 (7%)
0.00001
0.00001
0.00001
PCo 2 (14%)
PCo 1
PCo 3 (10%)
0.24
0.00006
0.00002
PCo 2 (13%)
PCo 1 (32%)
PCo 3 (9%)
0.0052
0.00001
0.00001
Ae. aegypti
Ae. albopictus
Cx. quinquefasciatus


B
Ae. aegypti
Ae. albopictus
Cx. quinquefasciatus
PCo 2 (13%)
PCo 1
PCo 3 (8%)
0.00001
0.00001
0.3
PCo 2 (18%)
PCo 1 (40%)
PCo 3 (9%)
0.02
0.007
0.1
PCo 2 (21%)
PCo 1 (55%)
PCo 3 (8%)
0.02
0.0009
0.1
PCo 2 (13%)
PCo 1 (4
PCo 3 (8%)
0.00001
0.00001
0.3
PCo 2 (12%)
PCo 1 (21%)
PCo 3 (10%)
0.006
0.002
0.2
PCo 2 (13%)
PCo 1 (34%)
PCo 3 (12%)
0.00001
0.00002
0.1
Lab
BG
G

A

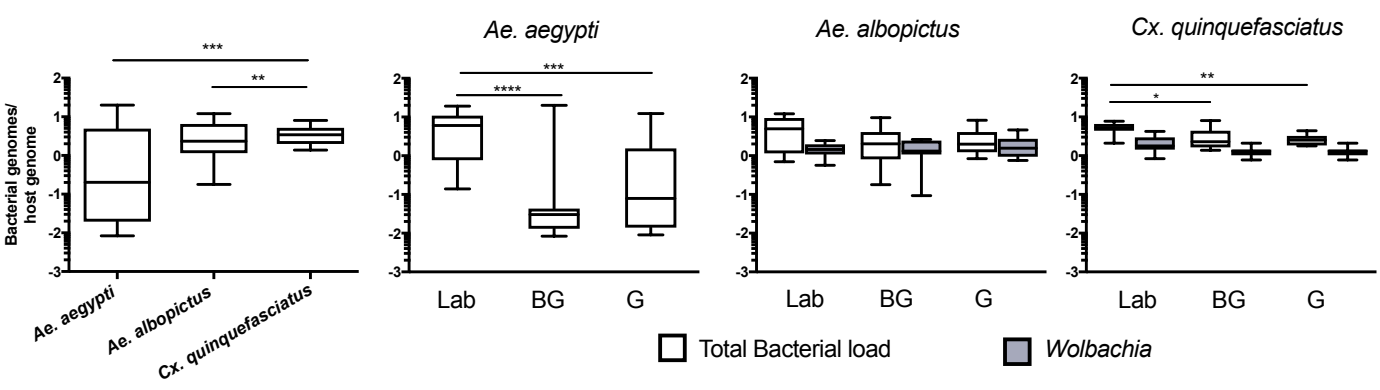


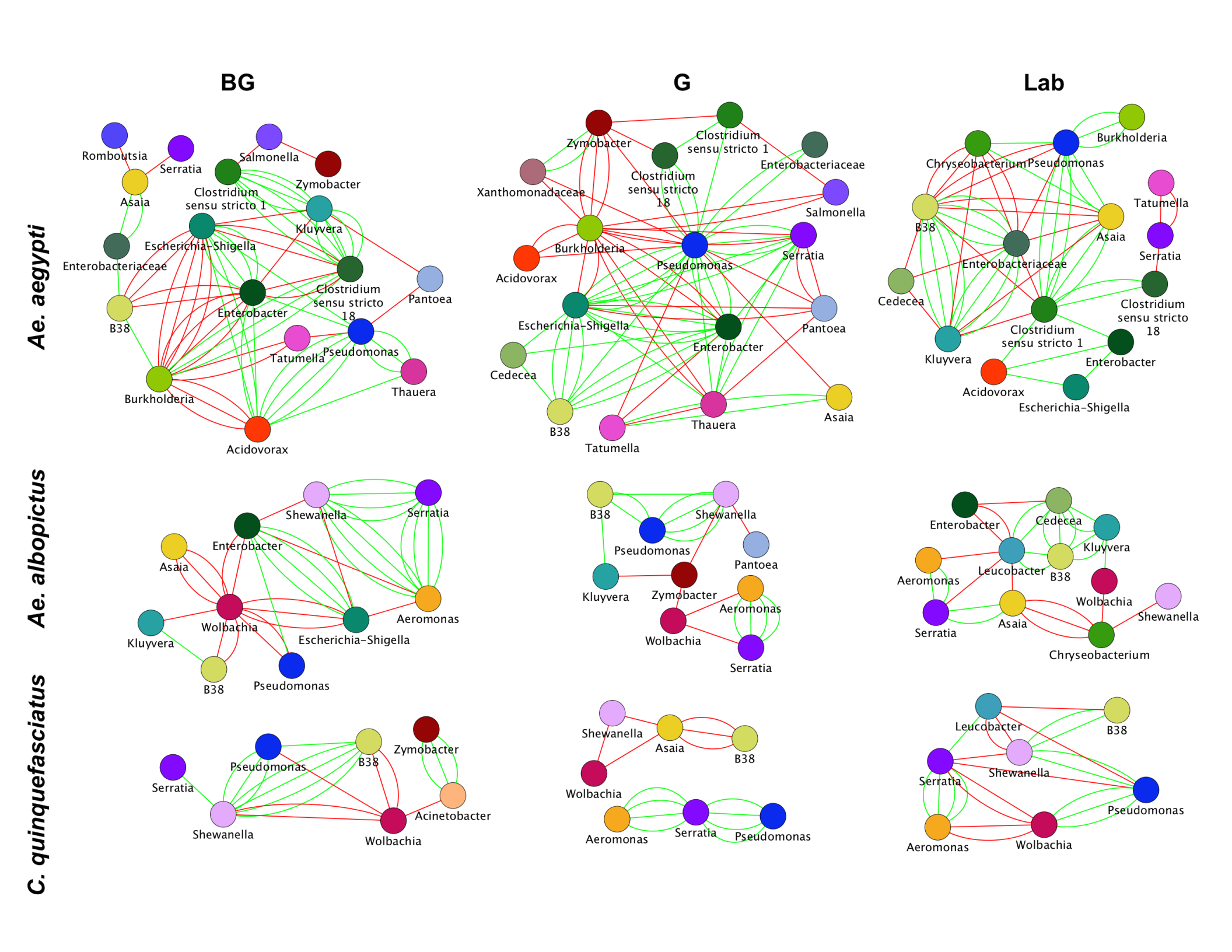
B

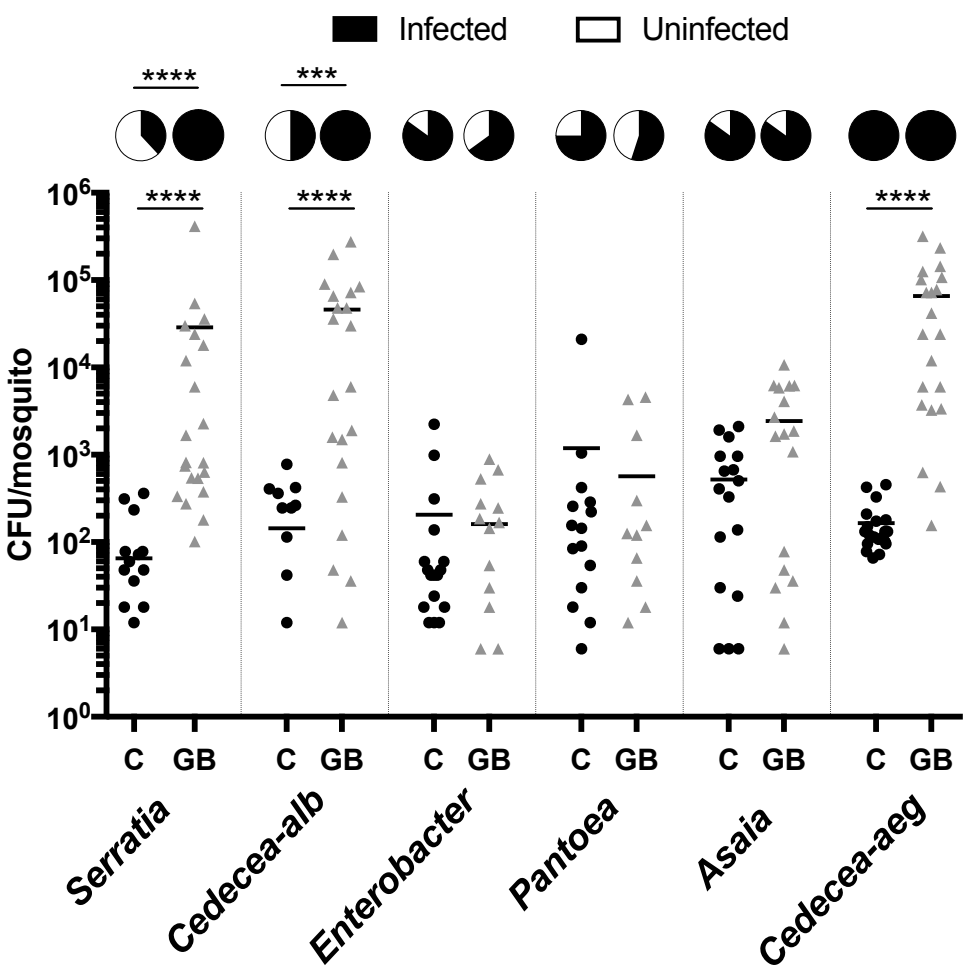
	Aeg vs *Alb*			*Aeg* vs *Cul*			*Cul* vs *Alb*		
Name	Lab	BG	G	Lab	BG	G	Lab	BG	G
Acinetobacter					15.3				
Aeromonas	12.9	11.5	12.4	15.8	10.3	10.7	2.9		
Asaia	-2.7			-6.6					
Burkholderia	-8.3			-8.3					
Cedecea	-7.2			-16.1					
Chryseobacterium	11.7								
Clostridium.sensu.stricto.1	-11.4	-3.5	-15.7	-11.4	-17.1	-15.7			
Clostridium.sensu.stricto.18	-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

	G vs BG		
Name	*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


Bacterial genomes/ host genome
***
**
Ae. aegypti
Ae. albopictus
Cx. quinquefasciatus
Ae. aegypti
***
****
Lab
BG
G
Ae. albopictus
Lab
BG
G
Cx. quinquefasciatus
**
*
Lab
BG
G
Total Bacterial load
Wolbachia


BG
G
Lab
Ae. aegypti
Ae. albopictus
C. quinquefasciatus
Romboutsia
Serratia
Asaia
Salmonella
Zymobacter
Clostridium sensu stricto 1
Kluyvera
Escherichia-Shigella
Enterobacteriaceae
Clostridium sensu stricto 18
Pantoea
Enterobacter
B38
Tatumella
Pseudomonas
Thauera
Burkholderia
Acidovorax
Xanthomonadaceae
Clostridium sensu stricto 1
Enterobacteriaceae
Clostridium sensu stricto 18
Salmonella
Burkholderia
Pseudomonas
Serratia
Acidovorax
Escherichia-Shigella
Pantoea
Enterobacter
Cedecea
B38
Thauera
Tatumella
Asaia
Chryseobacterium
Pseudomonas
Burkholderia
Tatumella
B38
Asaia
Serratia
Enterobacteriaceae
Cedecea
Clostridium sensu stricto 18
Clostridium sensu stricto 1
Kluyvera
Enterobacter
Acidovorax
Escherichia-Shigella
Shewanella
Serratia
Enterobacter
Asaia
Aeromonas
Wolbachia
Escherichia-Shigella
Kluyvera
B38
Pseudomonas
B38
Shewanella
Pseudomonas
Pantoea
Kluyvera
Zymobacter
Aeromonas
Wolbachia
Serratia
Enterobacter
Cedecea
Kluyvera
Aeromonas
Leucobacter
B38
Wolbachia
Serratia
Asaia
Shewanella
Chryseobacterium
Serratia
Pseudomonas
B38
Zymobacter
Acinetobacter
Shewanella
Wolbachia
Shewanella
Asaia
B38
Wolbachia
Aeromonas
Serratia
Pseudomonas
Leucobacter
B38
Serratia
Shewanella
Pseudomonas
Aeromonas
Wolbachia


Infected
Uninfected
****
***
****
****
****
CFU/mosquito
10^6
10^5
10^4
10^3
10^2
10^1
10^0
C
GB
Serratia
Cedecea-alb
Enterobacter
Pantoea
Asaia
Cedecea-aeg