Inter-species microbiota transplantation recapitulates microbial acquisition and persistence in mosquitoes

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Abstract

Background

Mosquitoes harbor microbial communities that play important roles in their growth, survival, reproduction, and ability to transmit human pathogens. Microbiome transplantation approaches are often used to study host-microbe interactions and identify microbial taxa and assemblages associated with health or disease. However, no such approaches have been developed to manipulate the microbiota of mosquitoes.

Results

Here, we developed an approach to transfer entire microbial communities between mosquito cohorts. We undertook transfers between (*Culex quinquefasciatus* to *Aedes aegypti)* and within (*Ae. aegypti to Ae. aegypti*) species to validate the approach and determine the number of mosquitoes required to prepare donor microbiota. After the transfer, we monitored mosquito development and microbiota dynamics throughout the life cycle. Typical holometabolous lifestyle-related microbiota structures were observed, with higher dynamics of microbial structures in larval stages, including the larval water, and less diversity in adults. Microbiota diversity in recipient adults was also more similar to the microbiota diversity in donor adults.

Conclusions

This study provides the first evidence for successful microbiome transplantation in mosquitoes. Our results highlight the value of such methods for studying mosquito-microbe interactions and lay the foundation for future studies to elucidate the factors underlying microbiota acquisition, assembly, and function in mosquitoes under controlled conditions.

Keywords

Microbiota; microbiome; transplantation; *Aedes aegypti; Culex quinquefasciatus;* insect; mosquito; diversity

Background

A substantial body of evidence has emerged revealing the importance of microbiota to the biology of the animal hosts they associate with, which has stimulated broad interest in understanding the assembly of these communities. However, the daunting complexity of the microbiota present in many higher eukaryotes and the lack of conventional microbiology techniques to culture these microbes has limited our ability to address important questions in the field. As such, the mechanisms facilitating host-microbe interactions and the functional role of the microbiome as a holistic unit are poorly elucidated. Microbiota transplantation approaches are one promising technique for basic research and therapeutics, but studies employing these techniques have mainly been undertaken in mammalian systems [1-6] and there has been little attempt to transfer these approaches to medically or agriculturally important insects.

Microbiome research has expanded in mosquitoes given their medical relevance and interesting biology. As holometabolous insects, mosquitoes have distinct aquatic and terrestrial life stages, including larvae that molt through four aquatic instars before pupating on the water's surface and emerging as terrestrial adults [7, 8]. All of these stages harbor gut microbial communities dominated by bacteria that can vary tremendously in diversity and abundance over time and space [9]. Larvae acquire their gut microbiota from their aquatic environment during feeding [10-14], after which bacteria experience periods of extreme turnover as parts of the larval gut are continuously shed and replaced during feeding, molting, and metamorphosis to the adult stage [15, 16]. The adult gut, in contrast, is initially seeded by bacteria from larvae and/or the larval environment but thereafter may be modulated by adult sugar and blood feeding activities [9], the latter of which underlies the ability of adult female mosquitoes to acquire and transmit disease-causing pathogens to humans [17]. While most of the larval and adult mosquito microbiota is thought to be restricted to the midgut [18-21], bacteria and other microbes are also known to colonize other mosquito tissues [22-25]. These include the common bacterial endosymbiont

Wolbachia, which infects the germline of *Culex quinquefasciatus,* but not *Aedes aegypti*, and is transovarially transmitted to offspring each generation [26].

Altogether, the microbiota associated with the mosquito gut and other tissues can have profound impacts on mosquito biology by modulating larval growth and development [10, 12, 27-29] as well as adult survival [30-32], reproduction [27, 33], and the competency of adult female mosquitoes to transmit human pathogens [34]. As such, there is a growing interest in exploiting microbes for vector control [35-37]. However, available tools to manipulate the microbiota in mosquitoes are limited, lagging behind research in other systems [38-41]. Antibiotic treatment has been commonly used to perturb microbiota for experiments investigating tripartite interactions between mosquitoes, their microbiota, and human pathogens. While these experiments have provided insights into the role of bacteria in mosquito vector competence [34, 42-44], the use of antibiotics has its limitations. For example, some bacteria may not be susceptible to the antibiotics, meaning these approaches alter the microbiota rather than eliminate all microbiota [12, 45]. Furthermore, antibiotics can have off-target effects on host physiology and can affect mitochondria [46-49]. Alternatively, introduction of specific bacterial taxa into mosquito larvae or adults can be achieved by spiking the larval water or sugar solution, respectively [50-52]. However, while these approaches are effective at transferring specific bacterial taxa to mosquitoes, they likely do not reflect acquisition processes that occur in the field and can only be undertaken with culturable microbes.

We previously developed an approach to generate and maintain gnotobiotic mosquitoes colonized by individual bacterial taxa by sterilizing the surface of mosquito eggs and inoculating water containing bacteria-free (axenic) larvae hatched from surface-sterilized eggs under sterile conditions [10]. Similarly, the generation of axenic adult mosquitoes that can thereafter be inoculated with individual bacterial taxa via a sugar meal has been undertaken using heat-killed bacteria, supplementation of axenic larval cultures with eukaryotes, or reversible colonization [28, 53-55]. While these approaches have been used to broadly examine the biology of mosquito-microbe interactions and decipher the role of

bacterial microbiota on host biology [10, 12, 21, 27, 28, 56-58], expanding these techniques to transfer complete or tailored microbial communities would be highly desirable.

Here, we developed an approach to transfer entire microbial communities between mosquito cohorts. We undertook transfers between (*Cx. quinquefasciatus* to *Ae. aegypti)* and within (*Ae. aegypti to Ae. aegypti*) species to validate the approach and determine the number of mosquitoes required to prepare donor microbiota. After the transfer, we monitored mosquito development and microbiota dynamics throughout the life cycle. Typical holometabolous lifestyle-related microbiota structures were observed, with higher dynamics of microbial structures in larval stages, including the larval water, and less diversity in adults. Furthermore, the diversity of microbiota present in recipient adults was more similar to the microbiota present in donor adults. Altogether, our results effectively demonstrate the transfer of a complete microbiota from one mosquito cohort to another and lay the foundation for future studies to examine microbiota assembly and function in mosquitoes colonized by defined microbiomes with potential for exploitation for mosquito and disease control.

Methods

Donor sample collection and preparation of donor pools for microbiota transplantation

Ae. aegypti (Galveston) and *Cx. quinquefasciatus* (Houston) mosquitoes used as donors in this study were reared under conventional conditions as described previously [57]. In order to characterize microbiota diversity in both donor species, 150 individual three-to-four-day-old sugar-fed adult females of each species were collected, surface sterilized by immersing in 70% ethanol for five minutes followed by three five minute washes in sterile 1X PBS, and stored at -20°C for downstream sequencing. Adult mosquitoes from both species were also used to generate donor pools for microbiota transplantation. In brief, cohorts of 10, 20, 40, or 80 three-to-four-day-old adult female mosquitoes were collected, surface-sterilized in 70% ethanol for five minutes, rinsed three times in sterile 1X PBS for five minutes, and transferred individually to sterile 2 ml safe lock tubes (Eppendorf, Hamburg, Germany)

containing 5 mm steel beads and 500 µl of sterile 1X PBS (Fig. 1). Tubes were then homogenized at a frequency of 26 Hz/sec for one minute, briefly centrifuged to collect debris, and 50 µl of the homogenate from each tube was pooled (Fig. 1). Pooled homogenates were subsequently centrifuged at 5000 x g for five minutes to pellet any remaining debris, and the resulting supernatant was passed through a 5 µm filter to produce a final filtrate for transplantation. Filtrates, which ranged in volume from 500 µl (pool of 10) to 4 ml (pool of 80), were finally adjusted to a total volume of 50 ml using sterile water, and we repeated the entire process a total of three times to produce a total of 150 ml of each donor pool prior to use in downstream experiments (Fig. 1). A 200 µl aliquot of each pool was also retained and immediately subjected to genomic DNA isolation using a NucleoSpin Tissue Kit (Machery-Nagel, Düren, Germany). The resulting DNA was then stored at -20°C for downstream sequencing.

Microbiota transplantation and recipient sample collection

Axenic (microbe-free) *Ae. aegypti* (Galveston) L1 larvae served as the recipient host for all transplantations. In brief, axenic larvae were prepared by submerging eggs in 70% ethanol for 5 minutes, then transferring to a solution of 3% bleach and 0.01% Decon-Quat 200V (Ab Scientific Ltd) for 3 minutes, followed by a wash in 70% ethanol for 5 minutes, and finally rinsing three times in sterile water (Fig. 1). Eggs were then transferred to sterile water and vacuum hatched in sterile containers. Twenty axenic first instar larvae were then transferred to each of five T75 vented tissue culture flasks (Thermo Fisher Scientific, Waltham, MA USA) containing 50 ml suspension of a given donor pool (described above) and 60 µl of autoclaved powdered TetraMin pellets (Tetra, Melle, Germany) reconstituted with sterile water to a final concentration of 1 mg/ml (Fig. 1). In addition to the donor treatments, a negative control was done whereby larvae were transferred to flasks containing sterile water and diet only (*i.e.*, no microbes) and maintained alongside experimental flasks. All larvae (control and experimental) were provided sterilized diet every two days until the treatment groups pupated (control group did not pupate and died at L2 or L3) while water from control

168 flasks was used to screen for contamination throughout the experiment as described
169 previously [10]. Flasks were maintained at 70% humidity and 27°C.

170 In order to assess successful microbiome transplantation, four sets of recipient
171 samples were collected for sequencing: (*i*) 200 µl of larval water from each replicate flask,
172 collected when larvae had molted to the third instar, (*ii*) third instar larvae (pools of 5)
173 collected from the same flasks, and at least three individual sugar-fed adult females that had
174 emerged from pupae either (*iii*) three or (*iv*) six days prior to collection. Pupae produced from
175 replicate flasks containing the same donor pool were pooled and transferred to sterile water
176 in a sterile plastic chamber for adult emergence. Newly emerged adults were provided 10%
177 sucrose in sterile water *ad libitum* until collection as described above. Total genomic DNA
178 was extracted from all recipient samples using a NucleoSpin Tissue Kit (Machery-Nagel,
179 Düren, Germany). Larvae and adults were surface sterilized as described above before DNA
180 isolation.

181

182 *Amplicon library construction, sequencing, and data analysis*

183 Subsamples from the DNA extracts were used for amplifying the V3-V4 region of the
184 bacterial 16S rRNA gene using primers 341F (CCTACGGGNGGCWGCAG) and 805R
185 (GACTACHVGGGTATCTAATCC) [59], followed by PCR amplification for Illumina
186 sequencing. Samples were paired-end sequenced (2 x 250-bp) on an Illumina MiSeq.
187 Sequence reads were processed using the DADA2 pipeline in QIIME 2-2019.4 [60, 61]. In
188 brief, sequence reads were first filtered using DADA2's recommended parameters and an
189 expected error threshold of 0.5. Filtered reads were then de-replicated and denoised using
190 default parameters. After building the ASV table and removing chimeras and low-abundance
191 (<0.005%) ASVs, taxonomy was assigned using a Naive Bayes classifier natively
192 implemented in QIIME 2-2019.4 and pre-trained against the Greengenes reference
193 database (13.8) [62, 63]. A phylogenetic tree was built using FastTree (v2.1.3) [64] from a
194 multiple sequence alignment made with the MAFFT alignment tool [65] against the
195 Greengenes Core reference alignment [66]. Patterns of diversity within the ASV table were

196 analyzed using standard workflows in QIIME 2-2019.4, with a sampling depth of 1,000 reads
197 per sample and default parameters. Downstream statistical analyses were performed using
198 R (http://www.r-project.org/).
199
200 **Results**
201 *Adult mosquitoes harbor relatively complex bacterial communities that can be isolated for*
202 *transplantation*
203 For each donor species (*Ae. aegypti* and *Cx. quinquefasciatus*), we characterized the
204 microbiota of 40 individual adult mosquitoes by sequencing the V3-V4 regions of the 16S
205 rRNA gene. After filtering, denoising, merging, and removing chimeras, we obtained a total
206 of 2,544,303 reads with a median sequencing depth of 32,803 reads per sample (Additional
207 file 1). We obtained an unusually low number of reads (<1,000) for a single *Cx.*
208 *quinquefasciatus* sample (Additional file 1), which was removed from subsequent analyses.
209 We then assigned taxa and plotted relative abundance of taxa across samples.
210 We identified 103 and 120 ASVs across all of the *Ae. aegypti* and *Cx.*
211 *quinquefasciatus* individuals we sampled, respectively (Additional file 2). However, the vast
212 majority of our sequencing reads (>90%) were assigned to ASVs belonging to one of four
213 genera: *Serratia*, *Asaia, Cedecea* and another, unclassified genus within the
214 *Enterobacteriaceae* (Fig. 2a; Additional file 2). Considering both the presence/absence and
215 relative abundance of all of the ASVs we detected, bacterial communities present in both
216 donor species exhibited significant differences in both alpha diversity (as measured by
217 Shannon's H index and ASV richness) (Fig. 2b; Additional file 1) and beta diversity (as
218 measured by the Bray-Curtis dissimilarity index) (Fig. 2c), which were associated with shifts
219 in the relative abundance of specific community members detected in each species (Fig. 2a;
220 Additional file 2). As expected, these included the presence of *Wolbachia* in *Cx.*
221 *quinquefasciatus* donor individuals and the near complete absence of *Wolbachia* across the
222 *Ae. aegypti* donor individuals we sampled, which is consistent with no established natural
223 infection ever being observed in this species [67, 68]. *Cx. quinquefasciatus* donor individuals

224 further contained a notably greater percentage of taxa within the genus *Serratia*, while *Ae.*
225 *aegypti* donor individuals contained a greater percentage of *Asaia* spp. (Fig. 2a; Additional
226 file 2). Interestingly, we also detected significant negative correlations between the relative
227 abundance of ASVs belonging to the genus *Serratia* and ASVs belonging to other taxa
228 within the family *Enterobacteriaceae* across all *Ae. aegypti* and *Cx. quinquefasciatus* donors
229 we sampled (Additional files 2 & 3), which is consistent with previous results in field-
230 collected mosquitoes of the same species and our recent experimental findings [52, 57].
231 In order to determine how many individuals were required to transfer a representative
232 microbiome to recipients, we generated homogenates using pools of 10, 20, 40, or 80 *Ae.*
233 *aegypti* or *Cx. quinquefasciatus* donor individuals and assessed the diversity of ASVs
234 recovered in each pool relative to ASV diversity across the entire donor species populations
235 using 16S rRNA gene amplicon sequencing. We obtained a total of 438,839 sequences
236 representing 68 and 64 ASVs across the four *Ae. aegypti* and four *Cx. quinquefasciatus*
237 donor pools we sequenced, respectively, and each pool was dominated by the same four
238 genera detected in the subset of individuals we sequenced from each of our donor species
239 populations (Fig. 3a; Additional files 1 & 2). Each pool also captured the majority of donor
240 ASV diversity (>96%), although recovery varied with respect to how common a given ASV
241 was across all of the donor individuals we sequenced (Fig. 3b; Additional file 2). The number
242 of individuals used to generate each pool had the greatest impact on recovery of rare ASVs
243 (*i.e.,* those present in <50% of the donor individuals we sequenced), with significantly fewer
244 rare ASVs being recovered in the pool generated using 10 individuals. However, there were
245 no significant differences in recovery of more common ASVs, which were present in ε50% of
246 donor individuals and constituted >93% of individual donor sequences, between any of the
247 pools we generated (Fig. 3b; Additional file 2).
248
249 *Microbiota transplantation recapitulates donor microbial diversity in recipient individuals*
250 To assess our ability to transplant microbiota between different mosquito species, we
251 introduced each of our *Ae. aegypti* and *Cx. quinquefasciatus* donor pools into the water of

252 cultures containing axenic *Ae. aegypti* larvae. These cultures were subsequently maintained
253 under standard rearing conditions, and 16S rRNA gene amplicon sequencing was used to
254 assess bacterial diversity in replicate samples of the aquatic habitat (water in rearing flasks),
255 larvae (collected as third instars), and adults from each culture (collected 3- and 6-days
256 post-emergence). A total of 50, 40, and 108 water, larval, and adult samples were
257 sequenced, respectively, with a total of five samples being discarded prior to downstream
258 analyses due to low sequencing depth (<1,000 reads) (Additional file 1).

259 The resulting dataset, which contained a total of 4,540,617 sequences across all of
260 the recipient water, larval, and adult samples we sequenced (Additional file 1), revealed that
261 64 and 61 of the 68 and 64 ASVs found in *Ae. aegypti* and *Cx. quinquefasciatus* donor pool
262 communities, respectively, representing >99% of all donor pool sequences, were detected in
263 recipient samples (Additional file 2). The majority of these ASVs (>70%) also persisted in
264 recipient communities over time, although there were dramatic shifts in both alpha and beta
265 diversity across the different life stages and water samples we sequenced (Fig. 4 & Fig. 5;
266 Additional files 1, 2; 4 & 5). Alpha diversity (as measured by Shannon's H index and ASV
267 richness) was overall highest in water and larvae, while adult recipient individuals harbored
268 communities that did not significantly differ in alpha diversity from input donor communities
269 (Fig. 4b; Additional file 1), regardless of the size of the input donor pool (Additional files 1, 4
270 & 5). Differences in beta diversity, measured as average Bray-Curtis dissimilarity, were also
271 overall higher between input donor communities and the recipient water and larval samples
272 we collected than between input donor communities and recipient adult samples (Fig. 5),
273 although the degree of similarity between recipient adult and input donor communities varied
274 over time post emergence and as a function of the size of the input donor pool (Additional
275 file 6). More specifically, recipient water and larval communities displayed striking
276 amplification of taxa within the Chitinophagaceae, Microbacteriaceae (*Leucobacter* and
277 *Microbacterium* spp.), and Flavobacteriaceae (*Flavobacterium*, *Chryseobacterium*, and
278 *Elizabethkingia* spp.) that were less abundant in input donor communities (Fig. 4a; Additional
279 file 2). In contrast, adult recipient communities displayed striking amplification of taxa within

the genera *Serratia, Asaia*, *Cedecea*, and an unclassified genus within the *Enterobacteriaceae*, consistent with input donor communities (Fig. 4a; Additional file 2).

Both host and environmental factors impact microbiota transplantation efficacy

To assess whether microbiota transplantation recapitulated observed differences in microbiota diversity between donor species in recipient individuals, we last compared alpha and beta diversity of donor *Ae. aegypti* and *Cx. quinquefasciatus* individuals to recipient *Ae. aegypti* adults emerging from cultures inoculated with pools generated from each donor species. Consistent with our previous results comparing bacterial communities in donor individuals of each species (Fig. 2), communities in recipient adults that emerged from cultures inoculated with pools generated from each donor species exhibited significant differences in both alpha diversity (as measured by Shannon's H index and ASV richness) (Fig. 6b) and beta diversity (as measured by the Bray-Curtis dissimilarity index) (Fig. 6c). However, these differences were associated with shifts in the relative abundance of community members that were relatively rare in input donor communities (*e.g.*, taxa within the genera *Elizabethkingia*, *Acinetobacter*, *Pseudomonas*, and *Stenotophomonas*) (Fig. 2 & Fig. 6a; Additional file 2). More specifically, the predominance of *Asaia* in *Ae. aegypti* donor communities was not observed in recipient individuals, likely owing to the inability of this bacterium to reliably persist in the water of larval cultures (Fig. 6a & Additional file 2). *Wolbachia* from *Cx. quinquefasciatus* donor communities did not infect recipient individuals (Fig. 6a & Additional file 2), although this was expected given that transfections of *Wolbachia* into mosquitoes requires access through the germline. While these patterns were generally similar between groups of recipient individuals inoculated with input donor pools generated using different quantities of mosquitoes, adult recipients inoculated with pools generated using fewer individuals exhibited more variable bacterial communities overall, due to the stochastic proliferation of rare donor taxa and/or antagonism of *Serratia* by *Enterobacteriaceae,* consistent with our previous observations across donor individuals (Fig. 6a; Additional files 2 & 7).

Discussion

Numerous studies describing the diversity and function of mosquito-associated microbial taxa and assemblages have been published recently, leading to the discovery of potential interactions between mosquitoes and their microbiota that could be manipulated to enhance or reduce mosquito fitness and/or vector competency (reviewed in [36]). However, questions remain about the reproducibility, applicability, and physiological relevance of these data owing to discrepancies in experimental technique, lack of standardization, and the use of laboratory-colonized mosquito strains and species that harbor microbiota that vary substantially within and between different laboratories and that differ substantially from naturally occurring mosquitoes in the field [12, 13, 69-74]. In this study, we developed a simple approach to successfully isolate donor microbial communities from adult mosquitoes. We then used this approach to transfer microbiota within (*i.e.*, *Ae. aegypti* donors to *Ae. aegypti* recipients) and between (*i.e.*, *Cx. quinquefasciatus* donors to *Ae. aegypti* recipients) different donor and recipient species in order to assess our ability to recapitulate donor microbial diversity in adult recipients. We selected these donor and recipient species for two reasons. First, both *Ae. aegypti* and *Cx. quinquefasciatus* are biomedically relevant vector species that are commonly studied in the laboratory [7]. Previous studies have also carefully characterized microbiota acquisition and assembly across *Ae. aegypti* life history [10]. Second, while *Cx. quinquefasciatus* mosquitoes are readily infected by *Wolbachia* in the laboratory and commonly exhibit natural infections in the field [75 76], *Ae. aegypti* mosquitoes are generally recalcitrant to infection and are not known to harbor natural infections [67, 68]. By introducing microbiota derived from both *Ae. aegypti* and *Cx. quinquefasciatus* donors into *Ae. aegypti* recipients, we could therefore also establish whether our transplantation approach (*i*) recapitulated patterns of microbiota assembly and persistence previously observed in *Ae. aegypti* (positive control), and (*ii*) supported the expectation that we would not be able to successfully transfer *Wolbachia* from *Cx. quinquefasciatus* donors to *Ae. aegypti* recipients (negative control).

Our results demonstrate the successful transfer of bacteria from donor to recipient populations of mosquitoes, with recipient adult individuals retaining the vast majority of donor bacterial diversity. Our results also demonstrate that relatively small pools of donor microbiota (*i.e.*, derived from ≥10 donor individuals) are sufficient to capture taxa that are highly abundant and/or common in a given donor mosquito population. This suggests that our methods could readily be applied to study microbiota isolated from field populations, even in cases where large-scale mosquito collections are difficult. However, future work will be necessary to confirm that the patterns observed using our approach are indeed the same for donor microbiota generated from field mosquitoes that harbor microbial communities that are more diverse than those present in laboratory mosquitoes and that are not adapted to the laboratory environment [12, 13, 69-74]. Recently developed methods to generate axenic adults [28, 53-55] also strongly position us to examine the potential to adapt our protocol to introduce field-derived donor microbiota pools directly into adults via a sugar meal and therefore avoiding selective pressures exerted on introduced microbes by standard laboratory larval diets and larval growth and molting.

These results also highlight the value of our approach for studying the underlying bases of mosquito-microbe interactions, as has been demonstrated in other animals. For example, microbiome transplantation approaches have been employed in *Nasonia* wasps to assess the selective pressures contributing to observed patterns of phylosymbiosis in the system [41] as well as *Drosophila melanogaster* flies and *Bombus terrestris* bumblebees to identify relationships between host microbiota composition and different thermotolerance or immunity phenotypes [39, 40]. Reciprocal microbiome transplantations have also been performed across different vertebrate animal species. For example, reciprocal microbiota transplantations between zebrafish and mice have revealed factors specific to the host gut habitat that shape microbial community structure in each species [38]. Indeed, our results point to both host and environmental factors in shaping microbiota acquisition and persistence in mosquitoes. That the microbiota in recipient adult individuals looked most similar to the microbiota of donor individuals supports previous studies in *Ae. aegypti*

364 identifying life stage as a dominant factor shaping mosquito microbiota [10, 11, 14, 69, 70,
365 77-82]. Additionally, there were differences in microbiota diversity between our donor
366 species in recipient adults which supports previous findings indicating that while community
367 membership may be largely driven by the environment and life stage, community features
368 such as total and taxon-specific abundances may also be shaped by host genetics [83, 84].
369 Future studies could employ microbiome transplantation to improve our understanding of the
370 factors shaping microbiome acquisition and assembly in mosquitoes and the mechanisms by
371 which specific microbial taxa and assemblages contribute to different mosquito traits under
372 controlled conditions.

373 Future work is also warranted to determine if the approach developed herein is
374 relevant for other microbes such as fungi or viruses, which are also known to impact
375 mosquito biology [85, 86]. The applicability of this approach for other microbes likely
376 depends on their biology, and in particular, their mode of transmission. For example,
377 extracellular members of the mosquito gut microbiota that are commonly detected in
378 environments where mosquitoes persist in the laboratory are likely to be transferred, while
379 those that are intracellular may not be. In line with this, our results supported the expectation
380 that the intracellular bacterium *Wolbachia*, which was present in the *Cx. quinquefasciatus*
381 donors used in this study, would not be successfully transferred to *Ae. aegypti* recipients.
382 We also appreciate that tissue localization of bacteria and other microbes may be pertinent
383 for the transplantation process. Here, we prepared donor microbiota from whole-body
384 mosquitoes and similarly assessed microbiota assembly and persistence in whole-body
385 recipient individuals. Additional work will be necessary to establish patterns of donor
386 microbiota colonization across recipient host tissues and/or to validate methods for isolation
387 of microbiota from specific donor tissues. Methods for long-term preservation of donor
388 microbiota will also be necessary to facilitate long-term studies and intra- and inter-
389 laboratory comparisons of microbiota assembly across different host strains. Nevertheless,
390 our results provide a critical first step toward the standardization of microbiome studies in the
391 field of vector biology to include mosquito hosts colonized by defined microbiota that can be

replicated within and between labs. They also provide a critical first step toward our ability to recapitulate and study field-derived microbiota in laboratory settings.

Conclusions

We have successfully isolated and transplanted microbiomes from donor to recipient mosquitoes. This approach lays the foundation for future work to facilitate standardized studies of mosquito-microbe interactions, examine host-microbe interactions, and to identify effective strategies for manipulating mosquito microbiota to control mosquito populations and mosquito-borne diseases.

List of Abbreviations

Not applicable.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Raw Illumina reads are available in the NCBI Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra) under BioProject ID PRJNA767109.

Competing interests

The authors declare that they have no competing interests.

Funding

GLH and KLC were supported by an NIH grant (R21AI138074) to conduct these studies. Additional support to KLC was provided by the NSF (2019368), the USDA NIFA (2018-67012-28009), and the University of Wisconsin-Madison. Additional support to GLH was provided by the NIH (R21AI129507), the BBSRC (BB/T001240/1 and V011278/1), the UKRI (20197 and 85336), the EPSRC (V043811/1) a Royal Society Wolfson Fellowship (RSWF\R1\180013), and the NIHR (NIHR2000907). SH was supported by a James W. McLaughlin postdoctoral fellowship from the University of Texas Medical Branch and a Liverpool School of Tropical Medicine Director's Catalyst Fund award. The views expressed herein are those of the author(s) and do not necessarily reflect those of any agency of the US or UK government.

Authors' contributions

KLC, SH, and GLH conceived and designed the experiments. SH performed the experiments. KLC carried out the data analysis. KLC wrote the initial manuscript, and SH and GLH contributed to revisions.

Acknowledgements

We thank George Golovko, Kamil Khanipov, and Maria Pimenova of the Department of Pharmacology and Toxicology, Sealy Center for Structural Biology, University of Texas Medical Branch (Galveston, TX USA) for assistance with sequencing. We also thank the insectary staff at the University of Texas Medical Branch for assistance in rearing mosquitoes.

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692

Figure legends

Figure 1. Overview of methodology used to perform inter- and intra-species microbiota transplantations in mosquitoes. (Left) Preparation of *Ae. aegypti* and *Cx. quinquefasciatus* donor pools. Individual 3-4-day-old sugar-fed adult females from our standard laboratory colonies were collected, surface-sterilized, and homogenized (1). Individual homogenates were then pooled and centrifuged to collect debris prior to filtering of the resulting supernatant to produce a final filtrate (2). Filtrates, which ranged in volume from 500 µl (Pool 10) to 4 ml (Pool 80), were then adjusted to a total volume of 50 ml using sterile water prior to transplantation (2). (Right) Transplantation of donor pools into a focal host species (*Ae. aegypti*). Eggs laid by blood-fed adult females from the standard laboratory colony were surface-sterilized and hatched in sterile water to produce axenic larvae (3). Larvae were then transferred to replicate flasks (*n*=5) containing a 50 ml suspension of a given donor pool and provided sterilized diet every other day until pupation (4). Pupae produced from replicate flasks containing the same donor pool were finally pooled in water from the larval rearing flasks and transferred to a sterile plastic chamber for adult emergence (5). Donor and recipient samples collected for sequencing are indicated in bold. See *Methods* for more information. Created with BioRender.com.

Figure 2. a Relative abundance of bacterial genera in individual adult mosquitoes sampled from conventionally maintained populations of each of the donor species used in the study (*i.e.*, *Ae. aegypti* and *Cx. quinquefasciatus*). Adults were provided 10% sucrose in water (wt/vol) *ad libitum* prior to being sampled 3-4 days post-emergence. Each bar represents an individual mosquito. Low abundance genera (<1%) are represented by the 'Other' category. **b & c** Alpha and beta diversity of *Ae. aegypti* (*Aa*) and *Cx. quinquefasciatus* (*Cq*) donor individuals. Panel **b** shows the difference in alpha diversity between *Ae. aegypti* and *Cx. quinquefasciatus* donor individuals as measured by Shannon's H index (left) and ASV richness (right). Box-and-whisker plots show high, low, and median values, with lower and upper edges of each box denoting first and third quartiles, respectively. Asterisks (***)

721 indicate significant differences between *Ae. aegypti* and *Cx. quinquefasciatus* donor
722 individuals (Kruskal-Wallis test, $P < 0.05$). Panel **c** shows the difference in beta diversity
723 between *Ae. aegypti* and *Cx. quinquefasciatus* donor individuals. Principal coordinates
724 analysis using the Bray-Curtis dissimilarity index identified significant clustering by donor
725 species (PERMANOVA, $P = 0.001$).
726
727 **Figure 3. a** Relative abundance of bacterial genera in *Ae. aegypti* and *Cx. quinquefasciatus*
728 donor pools. Low abundance genera (<1%) are represented by the 'Other' category. **b**
729 Proportion of rare ASVs (left) and common ASVs (right) found in at least one donor
730 individual that were present in a given donor pool. An ASV was considered "rare" if it was
731 detected in <50% of donor individuals, while an ASV was considered "common" if it was
732 detected in ≥50% of donor individuals. Pools that do not share a letter above the bars had
733 significantly different results as determined by paired Fisher's Exact tests ($P < 0.05$); ns,
734 pools not significantly different ($P > 0.05$).
735
736 **Figure 4. a** Relative abundance of bacterial genera in donor pools and recipient samples.
737 Biological replicates were pooled for the bar graphs presented. Low abundance genera
738 (<1%) are represented by the 'Other' category. **b** Alpha diversity of donor pools and recipient
739 samples, as measured by Shannon's H index and ASV richness. Box-and-whisker plots
740 show high, low, and median values, with lower and upper edges of each box denoted first
741 and third quartiles, respectively. Asterisks (***) indicate samples that significantly differed
742 from the donor pools (Dunn's test with Bonferroni correction, $P < 0.0125$).
743
744 **Figure 5. a** Principal coordinates analysis using the Bray-Curtis dissimilarity index. Circles
745 are colored by sample source (donor pools: orange, water: purple, larvae: green, 3-day old
746 adults: blue, 6-day old adults: red). **b** Average Bray-Curtis dissimilarity between (b/w) donor
747 pools versus between a given donor pool and recipient samples. Mean values ± standard

errors are shown. Asterisks (***) indicate comparisons for which the average dissimilarity between a given donor pool and group of recipient samples was significantly higher than that expected as a result of the transplantation procedure itself (*i.e.*, between donor pools) (Dunn's test with Bonferroni correction, $P < 0.0125$).

Figure 6. a Relative abundance of bacterial genera in 3-day and 6-day old *Ae. aegypti* recipient adults that emerged from cultures inoculated with donor pools generated from either *Ae. aegypti* or *Cx. quinquefasciatus* individuals. Each bar represents an individual mosquito. Genera representing >1% of reads from any one donor individual are listed in the legend; all other taxa are grouped together under 'Other'. **b & c** Alpha and beta diversity of recipient *Ae. aegypti* adults emerging from cultures inoculated with pools generated from *Ae. aegypti* (*Aa*) or *Cx. quinquefasciatus* (*Cq*) donor individuals. Panel **b** shows the difference in alpha diversity between *Aa* and *Cq* recipient adults as measured by Shannon's H index (left) and ASV richness (right). Box-and-whisker plots show high, low, and median values, with lower and upper edges of each box denoting first and third quartiles, respectively. Asterisks (***) indicate significant differences between *Aa* and *Cq* recipient adults (Kruskal-Wallis test, $P < 0.05$). Panel **c** shows the difference in beta diversity between *Aa* and *Cq* recipient adults. Principal coordinates analysis using the Bray-Curtis dissimilarity index identified significant clustering by donor species (PERMANOVA, $P = 0.001$).

775 **Additional files**

776 **Additional file 1: Supplementary Table 1**. Sequencing and diversity statistics for 16S
777 rRNA gene amplicon libraries prepared from donor and recipient samples.

778

779 **Additional file 2: Supplementary Table 2**. Taxonomic assignments and prevalence of
780 each ASV in each sample, along with whether a particular ASV was considered 'rare' or
781 'common' among individuals of a particular donor species.

782

783 **Additional file 3: Supplementary Figure 1**. Significantly negative correlation between
784 relative abundance of ASVs belonging to the bacterial family Enterobacteriaceae and ASVs
785 belonging to the genus *Serratia* across all *Ae. aegypti* and *Cx. quinquefasciatus* donor
786 individuals (Spearman's rank test, $P < 0.05$).

787

788 **Additional file 4: Supplementary Figure 2**. (Left) Relative abundance of bacterial genera
789 in *Ae. aegypti* donor pools and recipient samples. Biological replicates were pooled for the
790 bar graphs presented. Low abundance genera (<1%) are represented by the 'Other'
791 category. (Right) Alpha diversity of donor pools and recipient samples, as measured by
792 Shannon's H index and ASV richness. Box-and-whisker plots show high, low, and median
793 values, with lower and upper edges of each box denoting first and third quartiles,
794 respectively. Asterisks (***) indicate samples that significantly differed from the donor pools
795 (Dunn's test with Bonferroni correction, $P < 0.0125$).

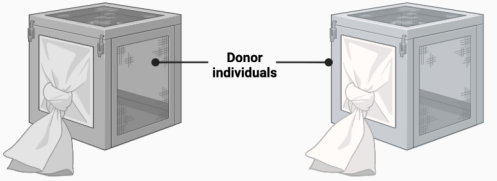
796

797 **Additional file 5: Supplementary Figure 3.** (Left) Relative abundance of bacterial genera
798 in *Cx. quinquefasciatus* donor pools and recipient samples. Biological replicates were pooled
799 for the bar graphs presented. Low abundance genera (<1%) are represented by the 'Other'
800 category. (Right) Alpha diversity of donor pools and recipient samples, as measured by
801 Shannon's H index and ASV richness. Box-and-whisker plots show high, low, and median
802 values, with lower and upper edges of each box denoting first and third quartiles,

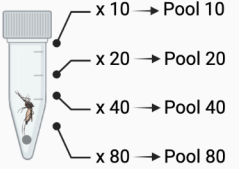
803 respectively. Asterisks (***) indicate samples that significantly differed from the donor pools
804 (Dunn's test with Bonferroni correction, $P < 0.0125$).
805
806 **Additional file 6: Supplementary Figure 4**. Average Bray-Curtis dissimilarity between
807 (b/w) donor pools versus between a given donor pool and recipient samples. Mean values ±
808 standard errors are shown. Asterisks (***) indicate comparisons for which the average
809 dissimilarity between a given donor pool and group of recipient samples was significantly
810 higher than that expected as a result of the transplantation procedure itself (*i.e.*, between
811 donor pools) (Dunn's test with Bonferroni correction, $P < 0.0125$).
812
813 **Additional file 7: Supplementary Figure 5**. Significantly negative correlation between
814 relative abundance of ASVs belonging to the bacterial family Enterobacteriaceae and ASVs
815 belonging to the genus *Serratia* across all recipient *Ae. aegypti* adults (Spearman's rank
816 test, $P < 0.05$).
817

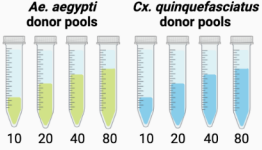
DONORS

Ae. aegypti *Cx. quinquefasciatus*



1. Surface-sterilize and homogenize individual mosquitoes

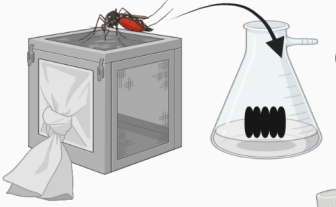




2. Pool homogenates, centrifuge to collect debris, filter supernatant, adjust to final volume of 50 ml

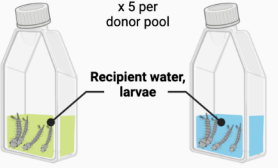
RECIPIENTS

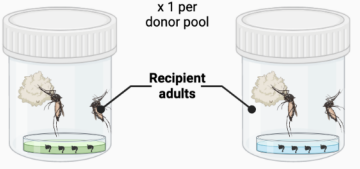
Ae. aegypti



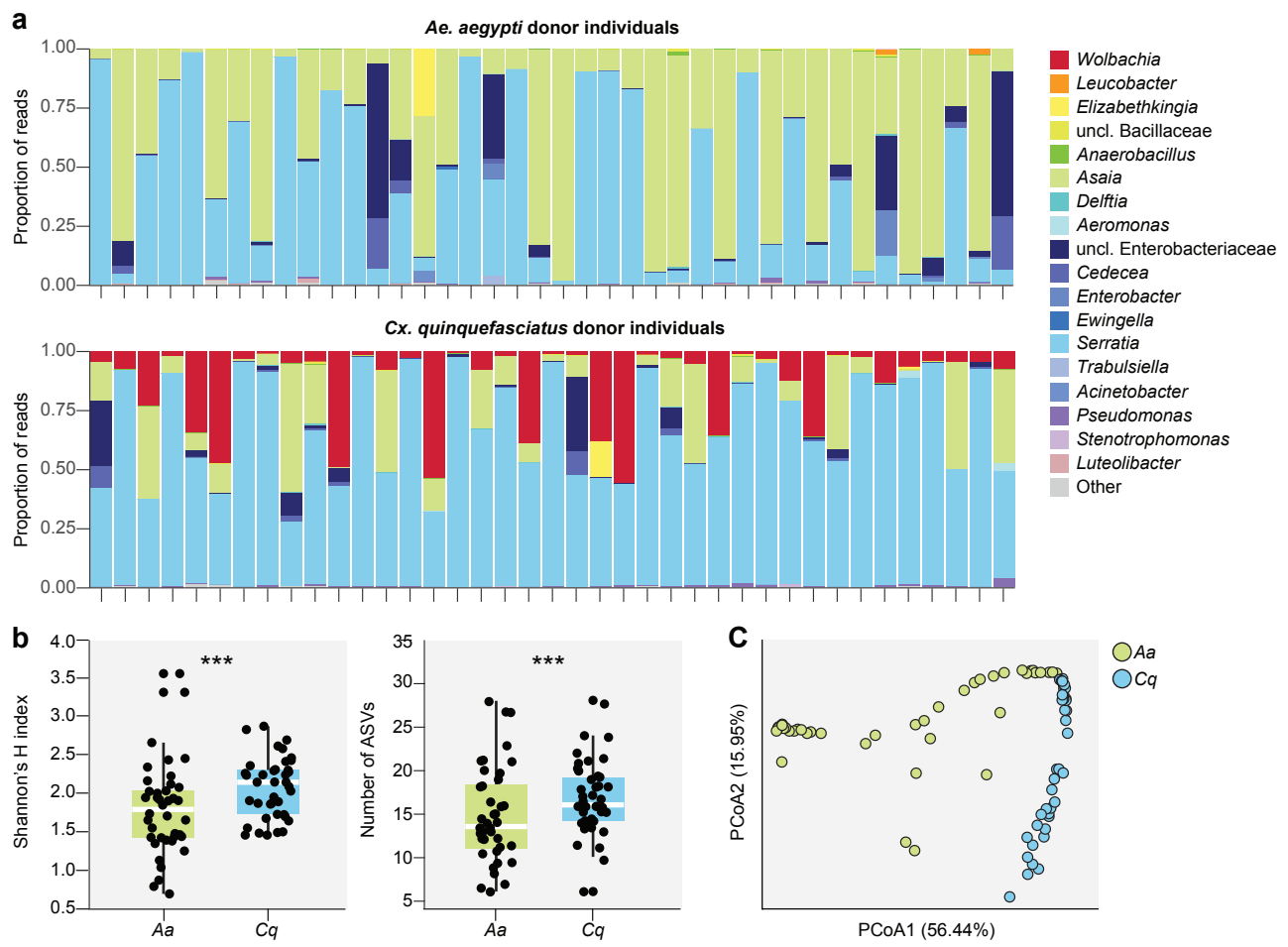
3. Surface-sterilize eggs, hatch in sterile water to produce axenic larvae

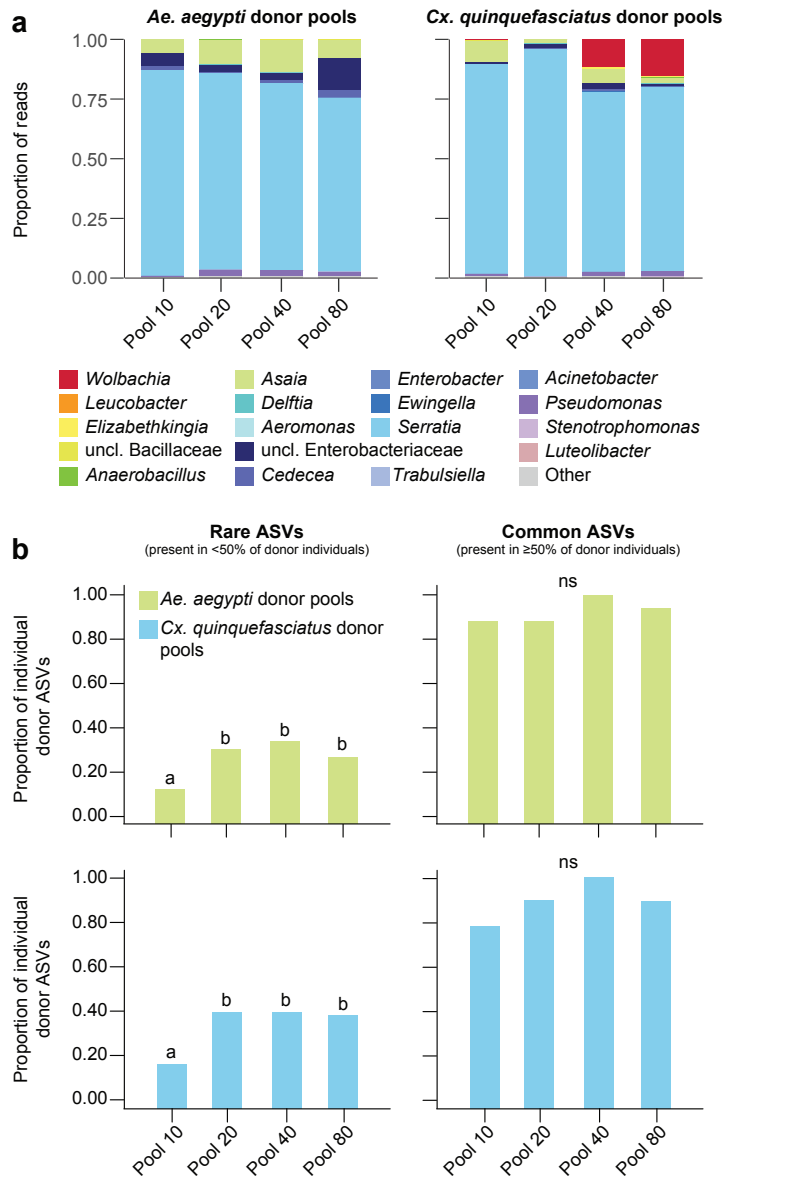
4. Transfer larvae to flasks containing donor pool, feed until pupation

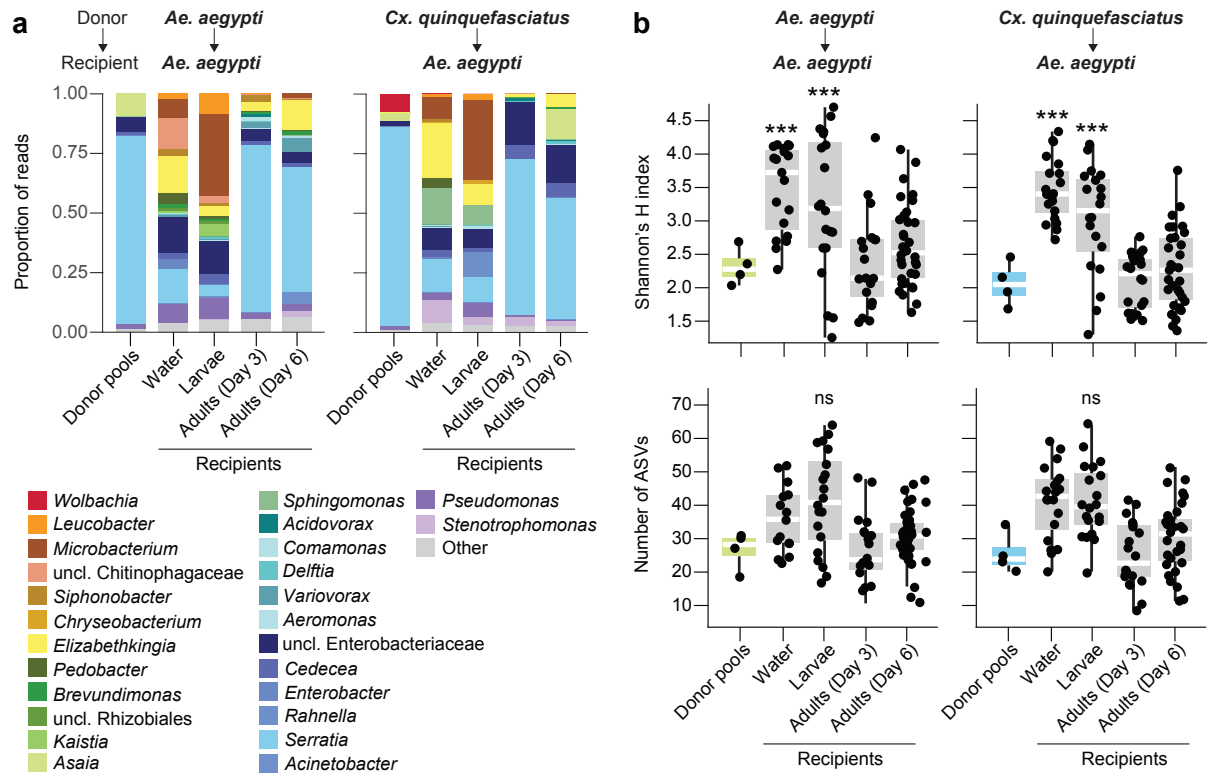


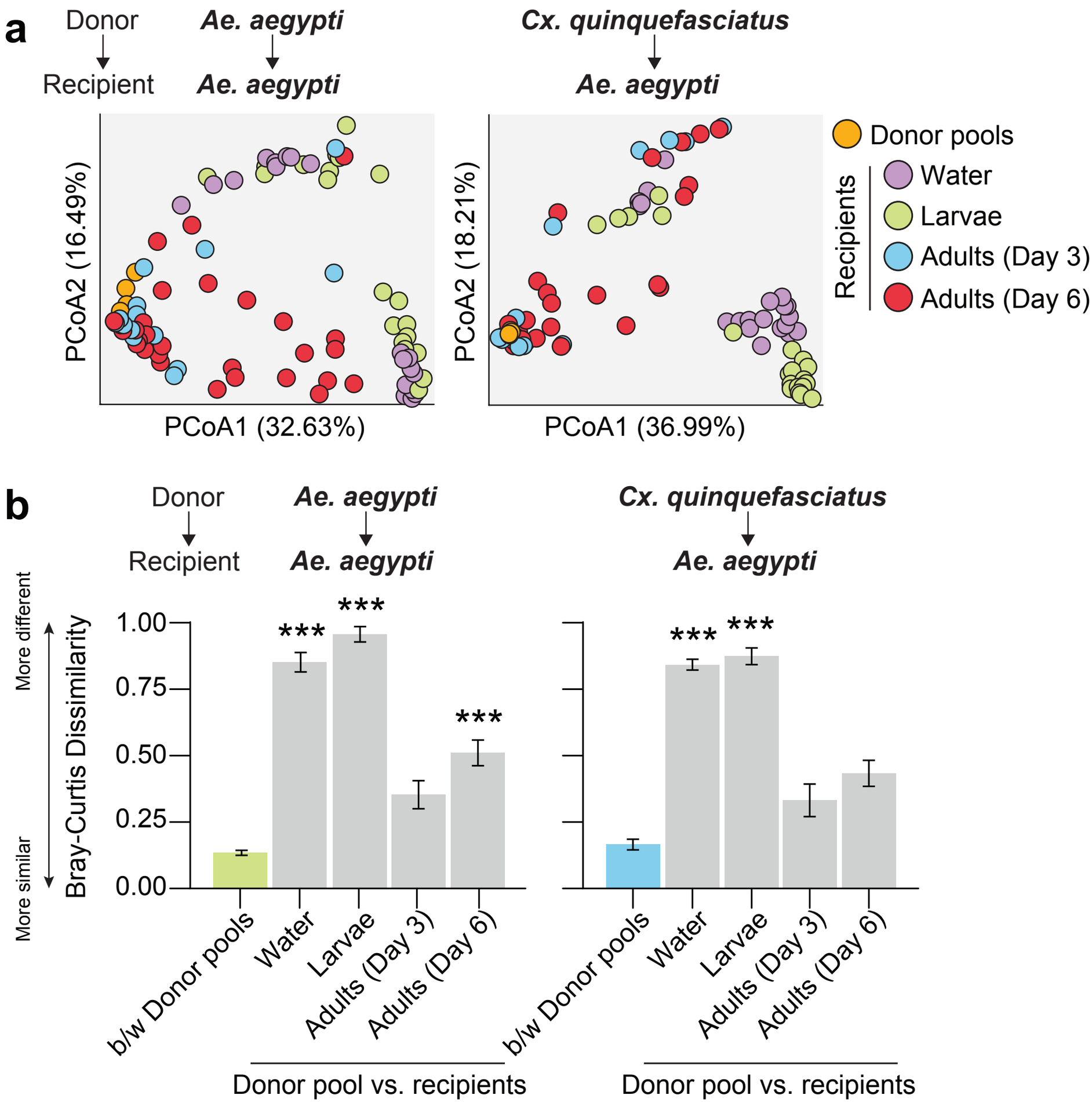


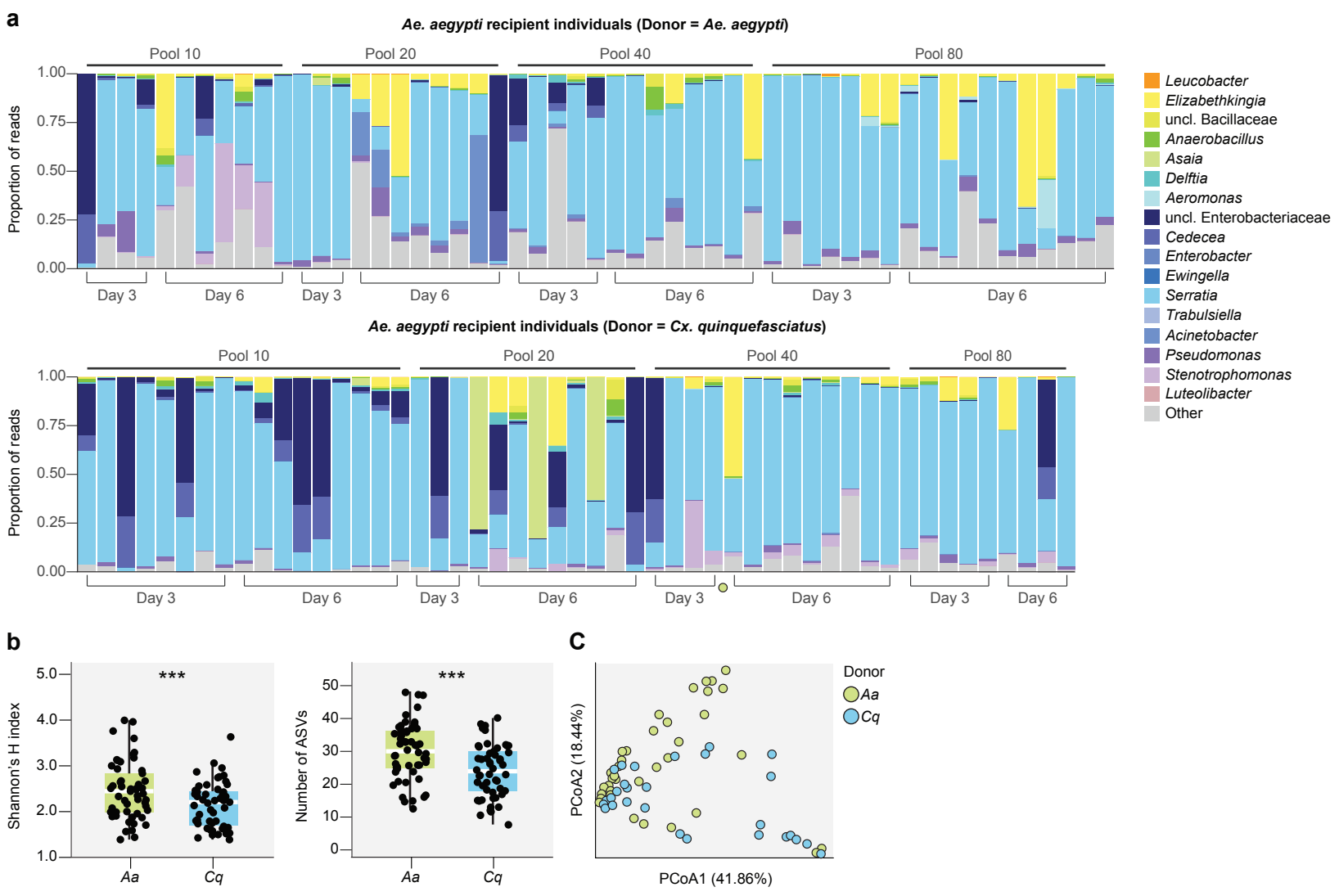
5. Combine pupae, transfer to sterile chamber for adult emergence


a
Ae. aegypti donor individuals
Cx. quinquefasciatus donor individuals
Proportion of reads
1.00
0.75
0.50
0.25
0.00
Wolbachia
Leucobacter
Elizabethkingia
uncl. Bacillaceae
Anaerobacillus
Asaia
Delftia
Aeromonas
uncl. Enterobacteriaceae
Cedecea
Enterobacter
Ewingella
Serratia
Trabulsiella
Acinetobacter
Pseudomonas
Stenotrophomonas
Luteolibacter
Other
b
Shannon's H index
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
***
Aa
Cq
Number of ASVs
35
30
25
20
15
10
5
***
Aa
Cq
C
Aa
Cq
PCoA2 (15.95%)
PCoA1 (56.44%)


a
Ae. aegypti donor pools
Cx. quinquefasciatus donor pools
Proportion of reads
1.00
0.75
0.50
0.25
0.00
Pool 10
Pool 20
Pool 40
Pool 80
Wolbachia
Asaia
Enterobacter
Acinetobacter
Leucobacter
Delftia
Ewingella
Pseudomonas
Elizabethkingia
Aeromonas
Serratia
Stenotrophomonas
uncl. Bacillaceae
uncl. Enterobacteriaceae
Luteolibacter
Anaerobacillus
Cedecea
Trabulsiella
Other
b
Rare ASVs
(present in <50% of donor individuals)
Common ASVs
(present in ≥50% of donor individuals)
Ae. aegypti donor pools
Cx. quinquefasciatus donor pools
Proportion of individual donor ASVs
1.00
0.80
0.60
0.40
0.20
0.00
a
b
b
b
ns
Pool 10
Pool 20
Pool 40
Pool 80


a
Donor
Recipient
Ae. aegypti
Ae. aegypti
Cx. quinquefasciatus
Ae. aegypti
Proportion of reads
1.00
0.75
0.50
0.25
0.00
Donor pools
Water
Larvae
Adults (Day 3)
Adults (Day 6)
Recipients
Wolbachia
Leucobacter
Microbacterium
uncl. Chitinophagaceae
Siphonobacter
Chryseobacterium
Elizabethkingia
Pedobacter
Brevundimonas
uncl. Rhizobiales
Kaistia
Asaia
Sphingomonas
Acidovorax
Comamonas
Delftia
Variovorax
Aeromonas
uncl. Enterobacteriaceae
Cedecea
Enterobacter
Rahnella
Serratia
Acinetobacter
Pseudomonas
Stenotrophomonas
Other
b
Ae. aegypti
Ae. aegypti
Cx. quinquefasciatus
Ae. aegypti
Shannon's H index
4.5
4.0
3.5
3.0
2.5
2.0
1.5
***
***
***
***
Number of ASVs
70
60
50
40
30
20
10
ns
ns
Donor pools
Water
Larvae
Adults (Day 3)
Adults (Day 6)
Recipients


a
Donor
Recipient
Ae. aegypti
Ae. aegypti
Cx. quinquefasciatus
Ae. aegypti
PCoA2 (16.49%)
PCoA1 (32.63%)
PCoA2 (18.21%)
PCoA1 (36.99%)
Donor pools
Recipients
Water
Larvae
Adults (Day 3)
Adults (Day 6)
b
Donor
Recipient
Ae. aegypti
Ae. aegypti
Cx. quinquefasciatus
Ae. aegypti
More different
More similar
Bray-Curtis Dissimilarity
1.00
0.75
0.50
0.25
0.00
***
***
***
***
***
b/w Donor pools
Water
Larvae
Adults (Day 3)
Adults (Day 6)
Donor pool vs. recipients
b/w Donor pools
Water
Larvae
Adults (Day 3)
Adults (Day 6)
Donor pool vs. recipients


a
Ae. aegypti recipient individuals (Donor = Ae. aegypti)
Pool 10
Pool 20
Pool 40
Pool 80
Proportion of reads
1.00
0.75
0.50
0.25
0.00
Day 3
Day 6
Ae. aegypti recipient individuals (Donor = Cx. quinquefasciatus)
Leucobacter
Elizabethkingia
uncl. Bacillaceae
Anaerobacillus
Asaia
Delftia
Aeromonas
uncl. Enterobacteriaceae
Cedecea
Enterobacter
Ewingella
Serratia
Trabulsiella
Acinetobacter
Pseudomonas
Stenotrophomonas
Luteolibacter
Other
b
Shannon's H index
5.0
4.0
3.0
2.0
1.0
***
Aa
Cq
Number of ASVs
50
40
30
20
10
0
C
PCoA2 (18.44%)
PCoA1 (41.86%)
Donor
Aa
Cq