Culex pipiens and Culex restuans egg rafts harbor diverse bacterial communities compared to their midgut tissues

Elijah O. Juma1*, Chang-Hyun Kim2, Christopher Dunlap3, Brian F. Allan1 and Chris M. Stone2

Abstract

Background: The bacterial communities associated with mosquito eggs are an essential component of the mosquito microbiota, yet there are few studies characterizing and comparing the microbiota of mosquito eggs to other host tissues.

Methods: We sampled gravid female Culex pipiens L. and Culex restuans Theobald from the field, allowed them to oviposit in the laboratory, and characterized the bacterial communities associated with their egg rafts and midguts for comparison through MiSeq sequencing of the 16S rRNA gene.

Results: Bacterial richness was higher in egg rafts than in midguts for both species, and higher in Cx. pipiens than Cx. restuans. The midgut samples of Cx. pipiens and Cx. restuans were dominated by Providencia. Culex pipiens and Cx. restuans egg rafts samples were dominated by Ralstonia and Novosphingobium, respectively. NMDS ordination based on Bray-Curtis distance matrix revealed that egg-raft samples, or midgut tissues harbored similar bacterial communities regardless of the mosquito species. Within each mosquito species, there was a distinct clustering of bacterial communities between egg raft and midgut tissues.

Conclusion: These findings expand the list of described bacterial communities associated with Cx. pipiens and Cx. restuans and the additional characterization of the egg raft bacterial communities facilitates comparative analysis of mosquito host tissues, providing a basis for future studies seeking to understand any functional role of the bacterial communities in mosquito biology.

Keywords: Mosquito egg raft, Mosquito midgut, Culex pipiens L., Culex restuans, Bacterial communities

Introduction

Studies applying high throughput, culture-independent sequencing of the bacterial 16S rRNA gene have advanced understanding of the association between mosquitoes and their bacterial communities [1–4]. The bulk of studies characterizing mosquito-associated bacterial communities have focused on the mosquito gut. However, other mosquito organs or tissues, including the ovaries, the male reproductive system, the salivary glands, and eggs, are also known to harbor bacterial communities that may play essential roles in mosquito biology [5–11]. The research focus on the mosquito gut, especially in the adult stage, is underpinned by the understanding that the mosquito midgut environment typically is the first barrier that mosquito-borne pathogens must overcome to develop successfully within the mosquito host and be transmitted to the next susceptible host [12–15].
Mosquito egg bacterial communities likely also play important roles in mosquito ecology. Studies with Aedes aegypti and Ae. triseriatus have shown that bacterial density on the egg surface mediates egg hatch rates and time-to-hatch, while mosquito eggs subjected to heavy larval grazing in high larval density habitats can exhibit delayed time-to-hatch [16–18]. The presence of aerobic microorganisms on the egg surface and in the larval environment has been associated with reduced oxygen tension, providing the stimulus for mosquito egg hatching [19, 20]. To what extent the bacterial communities on the egg surface drive the hatching effect relative to the microbes in the water column remains unclear.

Previous studies characterizing and comparing the bacterial communities of mosquito eggs with those of their other host tissues (e.g. midguts) have been conducted with well-known Afro-tropical or Asian vectors, including Anopheles or Aedes species, but not important North American vector species such as Culex pipiens L. or Culex restuans Theobald [5–7]. Culex pipiens is an introduced European species that arrived in North America in the early 16th century through trade and has been naturalized in the United States north of 39° latitude [21–23]. It serves as both an amplifying and bridge vector for West Nile virus (WNV) and St Louis encephalitis due to its preference for feeding on birds [24]. Culex restuans, native to North America, is also an important vector of WNV and distributed in the northeast and Great Lakes regions of the USA [22, 24]. The two species are common in urban, residential neighborhoods and woodlots and are ecologically similar and spatially-overlapping throughout much of their ranges, primarily utilizing birds as blood-meal sources and artificial container habitats for juvenile development [25–28]. Comparative studies with Cx. pipiens and Cx. restuans are necessary to expand the known library of bacterial communities associated with mosquito host tissues and to facilitate further studies on the role of these bacterial communities in mosquito vector biology and ecology and potentially benefit mosquito-borne disease control.

We used Illumina MiSeq sequencing of the V3-V4 hypervariable regions of the 16S rRNA gene to characterize the bacterial communities associated with egg rafts and midguts of Cx. pipiens and Cx. restuans, to gain insights into bacterial community structure and diversity, and to observe how they compare between the two host tissues. We tested the hypothesis that, within each mosquito species, egg-raft- and the midgut samples will harbor distinct bacterial communities given that the external egg surface and the mosquito midgut represent physiologically distinct environments, and thus are likely to support distinct bacterial communities. We also hypothesized that similar tissues across species (e.g. eggs or midguts for both Cx. pipiens and Cx. restuans) will harbor similar bacterial communities because similar tissues represent similar physiological environments. This study expands upon understanding of the bacterial communities associated with mosquito host tissues other than the mosquito gut and provides a basis for further studies focused on the role of mosquito egg-associated bacterial communities in mosquito biology and mosquito-borne disease control.

**Methods**

**Sampling and laboratory sample preparation**

Gravid traps for sampling of gravid Culex spp. mosquitoes were established in three woodland areas namely, Brownfield Woods (40°8′46.0716″N, 88°9′57.0852″W), South Farms (40°5′18.7692″N, 88°13′0.4188″W), and Trelease Woods (40°7′45.5412″N, 88°8′28.2696″W), and two residential neighborhoods with permission from property owners (40°4′57.0324″N, 88°15′25.7652″W; 40°5′25.8828″N, 88°15′36.4212″W) in Champaign County, Illinois. Brownfield Woods is a 26.14 ha “virgin” deciduous upland forest. It is primarily composed of mature oak (Quercus spp.), ash (Fraxinus spp.), and maple (Acer saccharinum L.) forest with a high, closed canopy and fairly open understory. Sugar maple is becoming the dominant tree species. South Farms is a 8.15 ha woodland composed of low canopy trees consisting mainly of sugar maple, sycamore (Platanus occidentalis L.), and pine (Pinus spp.), with oak and patchy grass undergrowth in some sections [27]. Approximately two years prior to this study, the invasive Amur honeysuckle (Lonicera maackii), that was the dominant shrub in this woodlot was removed. Trelease Woods is a 28.80 ha deciduous forest consisting mainly of mature oak, ash, hackberry (Celtis occidentalis) and maple species, with a high, closed canopy and moderately dense understory. The site includes two small seasonal ponds which provide suitable habitat for many aquatic and semi-aquatic invertebrates, including mosquitoes. At each sampling site, two CDC gravid traps baited with 3.8 l each of grass infusion [29] were deployed beginning on June 18, 2018 and sampling was conducted three times weekly up to July 20, 2018. Traps were placed in the evening just before dusk and the collection bags collected approximately 12 h later the next morning [30]. Individual gravid female Culex species mosquitoes were transferred separately to individual 270 ml paper cups to facilitate oviposition; each consisted of a 30 ml inner plastic oviposition cup filled to half capacity with distilled water. The mosquitoes were maintained in a walk-in environmental chamber at 27 ± 1°C and ±75 ± 5% relative humidity with a 16:8 (L:D) photoperiod. The paper cups were provisioned with cotton balls soaked in distilled water to provide additional
humidty and a source of water for the gravid females. Monitoring for egg rafts was conducted every 12 h. Following oviposition, the egg raft and the parous female were separately preserved at -80 °C for future bacterial DNA extraction.

Dissections and DNA extraction
Egg rafts and mosquito samples were thawed and adult mosquito samples surface sterilized in 70% ethanol for 5 min, transferred to 3% bleach solution for 3 min, transferred again to 70% ethanol for 5 min, and then rinsed 3 times in sterile water and 4 times in Dulbecco’s phosphate-buffered saline (DPBS) (Thermo Fisher Scientific, Waltham, MA, USA) [4]. Each sample was dissected in a small drop of sterile DPBS using a dissecting stereomicroscope and the midguts were transferred to PowerSoil bead tubes. Similarly, egg rafts were individually transferred to PowerSoil bead tubes. Due to their hydrophobicity and ease of disintegration during handling, egg rafts were processed for bacterial DNA without surface sterilization. Samples were homogenized using Retsch MM 300 TissueLyser (Retsch, Haan, Germany) and genomic DNA was extracted using MoBio PowerSoil DNA Isolation Kit (MoBio Laboratories, Inc., CA, USA) according to the manufacturer’s instructions. DNA was quantified using the Nanodrop 1000 (Thermo Fisher Scientific, Pittsburgh, PA, USA).

Sequencing was performed at the National Center for Agricultural Utilization Research, Peoria, IL. The V3-V4 hypervariable region of bacterial 16S rRNA gene was PCR-amplified using previously published universal primers 341f and 806r [31, 32]. The V3-V4 hypervariable region has been shown to have higher sensitivity in bacterial phylogenetic analysis compared to the rest of the hypervariable regions of the 16S rRNA gene [1]. The following primer set specific for the V3-V4 region of the 16S rRNA gene was used: forward (5’-CCT ACG GGN GGC WGC AG-3’); reverse (5’-GAC TAC HVG GGT ATC TAA TCC-3’). The primers were incorporated into fusion primers for dual indexing and incorporation of adapters prior to genome sequencing using Illumina MiSeq (Illumina Inc., San Diego, CA, USA) to attach dual indices and Illumina sequencing adapters. Index PCR was conducted in 45 µl reactions containing 25 µl of 2× KAPA HiFi HotStart ReadyMix, 5 µl each of index 1 and index 2 combinations, and 10 µl of PCR grade water. Thermocycling conditions were 95 °C for 3 min; 8 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; 72 °C for 5 min; hold at 4 °C. A negative control sample made up of DNA extracted from molecular biology grade water was sequenced with the same protocol to allow detection of the contamination background. PCR amplicons were cleaned and normalized using a SequlSmart normalization plate (Thermo Fisher Scientific, Waltham, MA, USA). The pooled library was mixed with Phix control spike-in of 5% as a sequencing control. The samples were sequenced on Illumina MiSeq system with a MiSeq V3 2 × 300 bp sequencing kit. The demultiplexed reads were quality-trimmed to Q30 using CLC genomics workbench v12.0 (Qiagen Inc., Valencia, CA, USA). Read pairing, fixed-length trimming and OTU clustering were done using CLC Bio Microbial Genomics module (Qiagen Inc., Valencia, CA, USA) utilizing the reference sequences from the Greengenes ribosomal RNA gene database [34]. The operational taxonomic unit (OTU) assignment was done at 97% sequence similarity, which is considered adequate for bacterial identification to the genus level [35].

Species identification
A duplex real-time TaqMan PCR assay [36] was used for molecular identification of Cx. pipiens and Cx. restuans using primers and probes targeting the acetylcholinesterase gene (Ace2) adopted from [36]. Primers and probes for Cx. pipiens consisted of: CxPip-F1 (5’-GGT GGA AAC GCA TGA CCA CAA CAT A-3’); CxPip-R1 (5’-TGG AAT AAA GAG GTG GCC ACG-3’); and probe (FAM/AGC CAC GAA CAA CTA AAT CAT CAC AAG CAC AGC/3BHQ). Culex restuans primers and probes were as follows: CxRest-F1 (5’-ATC GGT CTG GCT TCC TTT CAG AT-3’); CxRest-R1 (5’-TGA TTA GTA AAG TTA ACT GCC CTA CAT CCT A-3’), and the probe (JOE/AGC AAA CTG GCC GTC GTC CAC CGA TAT AA AT/3BHQQ1). The target DNA used as a template was taken from DNA samples extracted from midgut samples for bacterial DNA analysis. Separate DNA samples were extracted from Cx. pipiens and Cx. restuans adults initially identified from larval stages and used as positive controls. Additionally, a reaction mixture consisting of Ae. albopictus DNA template minus reverse transcriptase was used as a negative control to rule out any chance of contamination in the PCR reaction. Each PCR sample was assayed in 25 µl reaction mixture consisting of 5 µl of

primer-dimer species. A second PCR was conducted using the Nextera XT Index Kit (Illumina, San Diego, CA, USA) to attach dual indices and Illumina sequencing adapters. Index PCR was conducted in 45 µl reactions containing 25 µl of 2× KAPA HiFi HotStart ReadyMix, 5 µl each of index 1 and index 2 combinations, and 10 µl of PCR grade water. Thermocycling conditions were 95 °C for 3 min; 8 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; 72 °C for 5 min; hold at 4 °C. A negative control sample made up of DNA extracted from molecular biology grade water was sequenced with the same protocol to allow detection of the contamination background. PCR amplicons were cleaned and normalized using a SequlSmart normalization plate (Thermo Fisher Scientific, Waltham, MA, USA). The pooled library was mixed with Phix control spike-in of 5% as a sequencing control. The samples were sequenced on Illumina MiSeq system with a MiSeq V3 2 × 300 bp sequencing kit. The demultiplexed reads were quality-trimmed to Q30 using CLC genomics workbench v12.0 (Qiagen Inc., Valencia, CA, USA). Read pairing, fixed-length trimming and OTU clustering were done using CLC Bio Microbial Genomics module (Qiagen Inc., Valencia, CA, USA) utilizing the reference sequences from the Greengenes ribosomal RNA gene database [34]. The operational taxonomic unit (OTU) assignment was done at 97% sequence similarity, which is considered adequate for bacterial identification to the genus level [35].

Species identification
A duplex real-time TaqMan PCR assay [36] was used for molecular identification of Cx. pipiens and Cx. restuans using primers and probes targeting the acetylcholinesterase gene (Ace2) adopted from [36]. Primers and probes for Cx. pipiens consisted of: CxPip-F1 (5’-GGT GGA AAC GCA TGA CCA CAA CAT A-3’); CxPip-R1 (5’-TGG AAT AAA GAG GTG GCC ACG-3’); and probe (FAM/AGC CAC GAA CAA CTA AAT CAT CAC AAG CAC AGC/3BHQ). Culex restuans primers and probes were as follows: CxRest-F1 (5’-ATC GGT CTG GCT TCC TTT CAG AT-3’); CxRest-R1 (5’-TGA TTA GTA AAG TTA ACT GCC CTA CAT CCT A-3’), and the probe (JOE/AGC AAA CTG GCC GTC GTC CAC CGA TAT AA AT/3BHQQ1). The target DNA used as a template was taken from DNA samples extracted from midgut samples for bacterial DNA analysis. Separate DNA samples were extracted from Cx. pipiens and Cx. restuans adults initially identified from larval stages and used as positive controls. Additionally, a reaction mixture consisting of Ae. albopictus DNA template minus reverse transcriptase was used as a negative control to rule out any chance of contamination in the PCR reaction. Each PCR sample was assayed in 25 µl reaction mixture consisting of 5 µl of
the target DNA, 12.5 µl SensiFAST™ Probe Hi-ROX Kit master mix (Bioline, Tauton, MA, USA), 1.25 µl each of *Cx. pipiens* forward and reverse primer; 0.625 µl *Cx. pipiens* probe (Fam); 1.25 µl each of *Cx. restuans* forward and reverse primer; 0.625 µl *Cx. restuans* probe (Fam) and 1.25 µl of nuclease-free water. Thermocycling was performed on an ABI 7300 HT sequence detection system (Applied Biosystems, Foster City, CA, USA) using the following reaction conditions: 95 °C for 5 min followed by 40 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 60 s [36].

**Statistical analysis**

All analyses were conducted using R version 3.6.1 [37] within the Rstudio environment version 1.2.1335 [38] and PAST version 3.15 [39]. OTUs accounting for < 0.005% of the total number of sequences were removed prior to analysis to eliminate spurious OTUs [40]. The OTU sequence numbers varied markedly within samples (mean ± SE = 7621.28±547.17 per sample). Bacterial sequences were rarefied to an even depth of 1007 reads per sample to standardize the sampling coverage [41, 42]. From an initial sample size of 188 samples, 44 samples had < 1007 reads and were excluded from further analysis. To estimate sample coverage, rarefaction curves were fitted on the un rarefied data using the *phyloseq* package version 1.24.0 in R [41–43]. Alpha diversity metrics, including Shannon diversity index, observed species, and Chao1, were generated in QIIME 2 [44]. The means and 95% confidence intervals were calculated in R to test for significant differences in the alpha diversity indices between treatments. The Kruskal-Wallis test was used to test for differences in means between egg raft samples and midgut samples and the Wilcoxon rank sum test with Bonferroni correction was performed pairwise to separate significant treatments. Beta-diversity measures were estimated using the Bray-Curtis dissimilarity index using the *phyloseq* package and non-metric multidimensional scaling (NMDS) ordination plots were generated to visualize the results. The non-parametric Analysis of Similarity (ANOSIM) test with Bonferroni adjustments was performed in PAST version 3.26 [39] to determine degree of dissimilarity in bacterial composition between treatment groups. Similarity percentage (SIMPER) analysis was performed in PAST to identify the bacterial species characterizing each treatment group. Venn diagrams were generated using the R package *limma* [45] version 3.40.2 to visualize OTUs that were shared between egg rafts and midgut samples of *Culex pipiens* and *Culex restuans*. Using the function chisq.test from the R package *RVaideMemoire* version 0.9.7-4, differences in bacterial OTU per sample were tested and pairwise multiple comparison test with Bonferroni correction was applied.

**Results**

**Sequence processing and alpha diversity analysis**

Sequencing of the V3-V4 regions of the 16S rRNA gene from 188 samples (66 *Cx. pipiens* egg rafts; 66 *Cx. pipiens* midguts; 28 *Cx. restuans* egg rafts; and 28 *Cx. restuans* midguts) generated 1,432,800 raw sequences (mean ± SE = 7621.28 ± 547.17 per sample). After quality-filtering to remove chimeric sequences, other non-bacterial sequences, and bacterial OTUs constituting < 0.005% of the total sequences and rarefying the reads to an even depth of 1007 sequences per sample to standardize sampling effort, a total of 144 samples were retained (59 *Cx. pipiens* egg rafts; 39 *Cx. pipiens* midguts; 27 *Cx. restuans* egg rafts; and 19 *Cx. restuans* midguts). This sample size constituted a total of 1,422,059 sequences (mean ± SE = 9875.41 ± 598.19 per sample) clustered into 153 bacterial OTUs and assigned taxonomic identity at 97% sequence similarity.

Rarefaction analysis of the bacterial OTU samples revealed that the sequencing depth coverage sufficiently recovered most of the bacterial OTUs. Chao1 estimator revealed that up to 86.3 ± 0.07% (mean ± SE) of the bacterial OTUs were recovered. The highest bacterial OTU richness was reported in *Cx. pipiens* eggs, while *Cx. restuans* midgut had the lowest bacterial OTU richness (Additional file 1: Figure S1). *Culex pipiens* egg raft samples had significantly higher observed and expected (Chao1) bacterial OTU richness compared to *Cx. pipiens* midgut samples, or *Cx. restuans* egg raft and midgut samples. *Culex restuans* egg samples had significantly higher observed and expected (Chao1) bacterial OTU richness compared to *Cx. pipiens* midgut samples (Observed OTUs: Kruskal-Wallis $\chi^2 = 92.72$, df = 3, $P < 0.0001$; Chao1: Kruskal-Wallis $\chi^2 = 89.02$, df = 3, $P < 0.0001$; Shannon index: Kruskal-Wallis $\chi^2 = 85.82$, df = 3, $P < 0.0001$) (Table 1).

**Taxonomic classification and bacterial composition**

The 153 bacterial OTUs were classified into 7 phyla, 13 classes, 27 orders, 40 families, and 54 genera. The most dominant phyla were Proteobacteria (85.5%), consisting of Alphaproteobacteria (34.0%), Betaproteobacteria (18.4%), Gammaproteobacteria (33.0%) and Deltaproteobacteria (0.01%). Other phyla included Spirochaetes (9.3%), Bacteroidetes (3.3%) and Firmicutes (1.1%), and the rest were < 1% cumulatively (Fig. 1a). Alpha- and Betaproteobacteria were dominant in egg raft samples of either species, while Gammaproteobacteria was dominant in the midgut samples of both species. Spirochaetes was abundant in *Cx. restuans* midgut samples (Fig. 1a). The top five most abundant families accounted for 68.7% of all sequences. They included *Enterobacteriaceae* (25.8%), *Sphingomonadaceae* (15.9%), *Oxalobacteraceae*
Bray-Curtis distance matrix revealed bacterial communities from the midgut samples of both species, Spironema samples, and Cx. restuans in Cx. pipiens. One hundred and five bacterial OTUs were detected in Ralstonia Spironema (9.3%) and (10.3%), while 73 bacterial OTUs midgut samples of both species, Sphingomonadaceae in Cx. restuans egg samples, and Borrelliaceae in Cx. restuans midgut samples (Fig. 1b). At the genus level, the top five most abundant OTUs accounted for 58.1% of all sequences. They included Providencia (17.8%), Novosphingobium (13.6%), Ralstonia (10.3%), Spironema (9.3%) and Wolbachia (7.1%) (Fig. 1c). Providencia was the dominant taxon in the midgut samples of both species, Novosphingobium in Cx. restuans egg samples, Ralstonia in Cx. restuans egg samples, and Spironema in Cx. restuans. Overall, 64 (41.8%) bacterial OTUs were shared between all sample type combinations of mosquito species and host tissue (Additional file 1: Figure S2a). Seventy-two bacterial OTUs (61.5%) were shared between Cx. pipiens midgut samples and Cx. restuans midgut samples and 128 of the bacterial OTUs (91%) were shared between the egg samples of the two mosquito species. Ninety-three (62.8%) bacterial OTUs were shared between egg and midgut samples of Cx. pipiens, while 73 bacterial OTUs (51.4%) were shared between egg and midgut samples of Cx. restuans mosquitoes. Overall, there was higher bacterial OTU richness and diversity in egg rafts compared to midgut samples for both Cx. pipiens and Cx. restuans. One hundred and five bacterial OTUs were detected in Cx. pipiens midgut samples (CXP.MG) compared to 138 in Cx. pipiens egg-raft samples (CXP.ERG), and 83 and 132 in Cx. restuans midgut samples (CXR.MG) and Cx. restuans egg-raft samples (CXR.ERG), respectively. The differences in OTUs detected per sample were statistically significant ($\chi^2 = 17.0$, df = 3, $P < 0.001$). Multiple pairwise comparison with Bonferroni corrections revealed two statistically different sample groups (CXP.ERG vs CXP.MG, $P = 0.001$; CXR.MG vs CXP.ERG, $P = 0.005$). Non-metric multidimensional scaling (NMDS) using Bray-Curtis distance matrix revealed bacterial communities clustered by host tissue and are supported by results of ANOSIM pairwise comparisons: (CXP.ERG vs CXP.ERG; ANOSIM: $R = 0.19$, $P < 0.001$), (CXP.MG vs CXP.ERG (ANOSIM: $R = 0.30$, $P < 0.001$) (Fig. 2, Table 2). Culex restuans egg-raft and midgut samples formed distinct clusters on the NMDS plot indicating distinct bacterial community composition (CXR.ERG vs CXP.MG; ANOSIM: $R = 0.70$, $P < 0.001$), whereas there was moderate overlap in Cx. pipiens egg raft and midgut bacterial communities, but still formed distinct clusters (CXP.ERG vs CXP.MG; ANOSIM: $R = 0.51$, $P < 0.001$) (Fig. 3, Table 2). SIMPER analysis identified 9 bacterial OTUs that were responsible for 70% of the observed differences between groups, with Providencia (19.14%), Ralstonia (10.57%), Novosphingobium (10.34%), and Spironema (8.59%) constituting the largest variation (Additional file 1: Table S1). Providencia was the most dominant bacterial OTU in midgut samples of both Cx. pipiens and Cx. restuans. Ralstonia was the dominant bacterial OTU in Cx. pipiens egg-raft samples but was also present in high proportions in Cx. restuans egg-raft samples. Novosphingobium was dominant in egg-raft samples from both species. Spironema was the dominant bacterial taxon in Cx. restuans midgut samples (Fig. 1c).

### Discussion
In this study, we characterized the composition and diversity of the bacterial communities associated with egg rafts and midgut samples of Cx. pipiens and Cx. restuans. The egg-raft samples in both species were more diverse compared to midgut samples, with Cx. restuans midgut samples supporting the lowest bacterial diversity. Bacterial communities clustered by mosquito host tissue, such that the egg rafts from Cx. pipiens and Cx. restuans shared substantially similar bacterial communities and so did their midgut samples. However, both species had significantly different bacterial communities between their egg-raft and midgut tissues.

The bacterial communities associated with mosquito eggs are mostly localized on the external surfaces of the eggs. Previous studies have not been able to isolate bacterial communities from within the egg cytoplasm [4, 6, 7, 17, 46]. The egg-raft samples from both species in our study had highly overlapping bacterial communities with up to 91% of the bacterial OTUs shared between them. We presume, based on existing evidence, that these bacterial communities were mostly localized on the exterior of the egg rafts, thus representing the natural bacterial community diversity.
Fig. 1 Relative abundance of bacterial communities in samples of \textit{Cx. pennis} and \textit{Cx. restuans} midgut and egg samples. Taxa with sequence abundance < 1\% of total sequences were pooled together as "Other" in all the taxonomic ranks. \textit{CXP.EG} \textit{Cx. pennis} egg-raft samples, \textit{CXP.MG} \textit{Cx. pennis} midgut samples, \textit{CXR.EG} \textit{Cx. restuans} egg-raft samples, \textit{CXR.MG} \textit{Cx. restuans} midgut samples.
communities supported by the exterior egg-raft surface environment. In this study, female mosquito oviposition took place in deionized water, which is deficient in microorganisms [47], and the egg rafts were preserved at -80 °C within hours of oviposition. These measures limit the possibility that any significant level of bacterial colonization of the egg rafts may have occurred immediately following oviposition. We suspect that most of the bacterial communities colonizing the egg rafts were inherited maternally from the ovaries through egg-smearing or through a yet-to-be-described form of transovarial transmission method [6, 7]. However, this study did not characterize the bacterial communities associated with the ovarian tissues from either species to validate this

**Fig. 2** NMDS based on Bray-Curtis distance matrix of bacterial communities from *Cx. pipiens* and *Cx. restuans* egg and midgut samples. **a** Bacterial communities from *Cx. pipiens* and *Cx. restuans* samples presented together. **b** Bacterial communities from *Cx. pipiens* and *Cx. restuans* samples partitioned by life stage sampled.

**Table 2** Pairwise ANOSIM comparisons by mosquito species and life stage

| Pairwise comparison | Global $R$ | $P$-value |
|---------------------|------------|-----------|
| CXP vs CXP | 0.5195 | 0.0006 |
| CXP vs CXR | 0.1902 | 0.0054 |
| CXP vs CXRMG | 0.8894 | 0.0006 |
| CXP vs CXR | 0.782 | 0.0006 |
| CXP vs CXR | 0.2992 | 0.0006 |
| CXP vs CXRMG | 0.6989 | 0.0006 |

The significance values are Bonferroni-corrected for multiple comparisons.

*CXP Cx. pipiens, CXR Cx. restuans, EG egg samples, MG midgut samples.*

Permutations N: 9999; Mean rank within: 3400; Mean rank between: 5859; $R$: 0.4777; $P$ (same): 0.0001.
possibility. Additional future studies characterizing the bacterial communities of mosquito ovaries in addition to the egg rafts and midgut tissues would shed more light on this question. This study did not assess the potential bacterial composition of the deionized water used as oviposition substrate by the gravid females. While many studies assume deionized water to be microbe-free, this requires further investigation in future studies of the microbial composition of mosquito tissues.

The midgut samples of *Cx. pipiens* and *Cx. restuans* mostly shared similar bacterial community composition with over 61% of the bacterial OTUs shared between them. Previous studies with the adults of these two mosquito species have generated variable results, with one study showing that they harbor distinctly different bacterial communities [48], while another study did not report unique clustering in the bacterial communities of the two species [49]. Both studies were conducted with non-blood-fed adult mosquito samples whose parity status was not assessed, a factor that might have contributed to the observed differences. We can partly attribute the overlap in the bacterial OTUs of the adult midgut samples of the two species to the shared environment resulting in colonization by similar bacterial communities. Mosquitoes from different species sampled from common habitats have been shown to share more similar bacterial communities, a possible indication of horizontal acquisition from the surrounding environment [8, 49, 50]. The two species also overlap in their larval habitat use, which potentially predisposes them to acquisition of
similar bacterial communities that are then propagated transstadially all the way to adulthood [28]. Similarly, the blood-fed and gravid status of the females at the time of sampling may have contributed to similar internal gut oxidative environment subsequently supporting comparable bacterial communities [51, 52]. It may also be possible that the two mosquito species largely blood-fed on similar blood-meal sources, most likely birds, given their ecological and behavioral similarity, as well as spatial and seasonal overlap, although the two species also display some degree of seasonal separation [22, 25, 53]. Future experimental designs could incorporate blood-meal analysis and location data to refine other correlates of mosquito tissue bacterial composition and diversity.

The significant separation of the bacterial community composition between egg rafts and midguts within each mosquito species, is not surprising since the physiological environment in the mosquito gut is expected to differ substantially from that of the egg rafts, and thus is likely to facilitate colonization by very different consortia of bacterial communities. The disproportionate dominance of a few distinct bacterial taxa in the egg rafts compared to midgut samples for both species further indicates that egg and midgut environments were substantially different. However, studies comparing the bacterial communities of mosquito midguts and eggs are rare. One such study focusing on *Aedes aegypti* shows that mosquito guts share a significant proportion of their bacterial community composition with those of the eggs. Most of the taxa that have been reported to be shared between mosquito egg and midgut life stage are widespread in mosquito species and have been described in many other mosquito microbial studies [5].

There was high bacterial richness in the egg-raft samples compared to midgut samples for both *Cx. pipiens* and *Cx. restuans*. The midgut samples were from gravid females that possibly experienced a sudden and sharp decline in bacterial diversity due to their prior blood-meal diet. Wang et al. (2011) previously showed that mosquito gut bacterial diversity falls markedly in blood-fed compared to sugar-fed adults [51]. They hypothesized that this was mediated by the breakdown of the heme proteins from the blood-meal diet releasing reactive oxygen species that alter the physiological configuration of the gut environment, thus limiting the bacterial communities to those that can tolerate the high oxidative stress [51, 54]. However, more recent study by Muturi et al. [54] reported increased bacterial diversity in mosquitoes exposed to different blood-meal sources relative to sugar meal, indicating that blood-meal diet may produce variable effects on mosquito gut bacterial diversity, and that bacterial diversity is additionally determined by mosquito species, as well as the source of the blood meal [54]. The digestive process involving the movement of the blood bolus along the midgut endoperitrophic space may also have contributed to physical propulsion and excretion of a significant proportion of the midgut bacterial communities further reducing the midgut bacterial diversity [55]. The lower bacterial diversity in the guts of field-collected mosquitoes relative to their egg tissues may also be influenced by exposure to insecticides in the wild, or the insecticide resistance status of the female mosquitoes. The guts of insecticide-resistant mosquitoes may be enriched with bacterial communities with selective advantage over the pesticide constituents thus reducing their bacterial diversity [56, 57]. The gravid female adults used in this study were all field-collected. We did not determine their chronological (calendar ages) or biological age (number of gonotrophic cycles) as this was beyond the scope of this study. Future studies can explore how chronological age, biological age, and possibly insecticide resistance status of field-collected gravid mosquitoes may influence the bacterial communities of mosquito tissues such as the midguts and the egg stages.

The dominance of *Ralstonia* and *Novosphingobium* in egg-raft samples of both species point to their possible adaptation to colonizing the mosquito egg raft stages. It also could be related to their potential role in mosquito ecology, such as inducing egg hatch, but this requires further research. Literature is scanty on the isolation and characterization of *Ralstonia* or *Novosphingobium* from mosquito egg stages, but this may be attributed to the dearth of studies characterizing mosquito egg bacterial communities. However, *Ralstonia* and *Novosphingobium* have been isolated in mosquito midgut samples from several mosquito species including *Aedes japonicus*, *Ae. aegypti*, *Ae. albopictus* and *Anopheles coluzzii*. They have also been described from the natural environment, including soil and aquatic sources, indicating that mosquitoes may acquire them horizontally [58–63]. Their isolation from egg rafts in this study shows that these taxa may be part of the common mosquito bacterial commensals shared between different mosquito life stages including the eggs. Additional studies characterizing the bacterial communities of *Culex* ovaries, in addition to egg rafts, would help shed light on microbial presence in the ovary tissues and whether they are passed maternally to the egg rafts through egg-smearing or other forms of transovarial transmission.

The midgut samples of *Cx. pipiens* and *Cx. restuans* were dominated by *Providencia*, while *Wolbachia* and *Spiromena* were the second most abundant bacterial taxa in *Cx. pipiens* and *Cx. restuans* midgut samples, respectively. We attribute the dominance of *Providencia* in both species potentially to the blood-meal diet prior to oviposition, and potentially from an avian blood-meal source,
has isolated the bacterial genus *Wolbachia* in *Cx. pipiens* or *Cx. restuans*. *Providencia* is a genus consisting of gram-negative rods with peritrichous flagella belonging to the family *Enterobacteriaceae*. It is an enteric bacterial pathogen commonly isolated from human intestines. It has also been isolated from other organisms, including birds and pigs, pointing to its ubiquity in the natural environment. However, its role in mosquito biology has not been described. The ease of culturing *Providencia* in bacteriological media, its ready availability in the natural environment, and its abundance in *Cx. pipiens* in this study points to its potential suitability as a candidate for manipulation for paratransgenesis for mosquito vector management [65]. The bacterial genus *Spironema* has been characterized in *Culicidae* [66], *Ae. aegypti* un-infected with *Wolbachia* [67], *Cx. pipiens* [68], and was also dominant in *Culex nigripalpus* [69]. This taxon has been isolated from soil as well as river water samples, providing evidence of its potential horizontal acquisition in the *Cx. restuans* in this study [70]. High abundance in *Cx. restuans* has not been reported previously and opens an avenue to conduct further studies on its potential role in mosquito biology and disease transmission. The presence of *Wolbachia* in *Cx. pipiens* was expected as *Cx. pipiens* naturally harbor *Wolbachia*, a maternally inherited endosymbiont common in many arthropods, where they mediate several reproductive manipulations in their hosts [71]. Our study did not report any trace of *Wolbachia* in *Cx. restuans* egg-raft samples. However, it was reported in the *Cx. restuans* midgut samples at < 0.1%, providing further evidence of its recent detection in *Cx. restuans* elsewhere [72]. The presence of *Wolbachia* in *Cx. restuans* has potential to alter the epidemiology of WNV infection especially in the regions of the US where WNV has become endemic. Transient infection of *Cx. tarsalis* with the wAlbB strain has been associated with enhanced replication of WNV in the host [73]. In other studies, transient infection of *Ae. aegypti* with WNV was associated with enhanced viral replication [14]. However, it is likely that the interaction between *Wolbachia* and WNV in the naturally infected *Culex* populations could manifest differently. This area requires further investigation to disentangle the role of *Wolbachia* in WNV incubation and subsequently the epidemiology of the disease in the endemic regions.

In conclusion, our study has shown that *Cx. pipiens* and *Cx. restuans* egg-raft samples are more diverse in their bacterial communities and the bacterial communities differ significantly between egg-raft and midgut tissues within each mosquito species. However, the bacterial communities of the egg-raft *versus* the midgut tissues of the two species are mostly similar in their community composition. Whereas previous studies with *Cx. pipiens* or *Cx. restuans* have prioritized characterizing the bacterial communities from adult midguts [48, 49, 74], the additional characterization of the egg raft bacterial communities in this study fills an important gap in our understanding of the bacterial communities associated with the egg rafts and how they compare with those of the midguts. These findings open the way for further studies on their role in mosquito biology and ecology and their potential to be exploited for mosquito vector management such as strategies to interfere with egg hatching in the environment.

**Supplementary information**

**Supplementary information** accompanies this paper at https://doi.org/10.1186/s13071-020-04408-4.

**Acknowledgments**

We thank Millon Blackshear for his technical assistance and all members of the Medical Entomology Laboratory (INHS), Yani Kaldis, Rosemary Ogbonna, and Eddie Lewis, for assisting with fieldwork and running the experiments, and members of Allan Laboratory for providing valuable reviews to the manuscript. The authors would like to thank Heather Walker of the National Center for Agricultural Utilization Research (U.S. Department of Agriculture) for helpful technical assistance.

**Disclaimer**

Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the view of the U.S. Department of Agriculture. The mention of firm names or trade products does not imply that they are endorsed or recommended by the USDA over other firms or similar products not mentioned. USDA is an equal opportunity provider and employer.

**Authors’ contributions**

EOJ, BFA and CS conceived the study. EOJ conducted the experiment and analyzed the data. CHK assisted with conducting the experiments. CD processed the sequencing data and generated the OTU table. CS and CD
References

1. Yang B, Wang Y, Qian P-Y. Sensitivity and correlation of hypervariable regions in 16S rDNA genes in phylogenetic analysis: BMC Bioinform. 2016;17:135.

2. Güeltén M, Zouache K, Démichel C, Minard G, Van Tran V, Potier P, et al. The mosquito holobiont: fresh insight into mosquito–microbiota interactions. Microbiome. 2018;6:49.

3. Coon KL, Brown MR, Strand MR. Mosquitoes host communities of bacteria that are essential for development but vary greatly between local habitats. Mol Ecol. 2016;25:5806–26.

4. Consensus for publication Not applicable.

5. Gusmão DS, Santos AV, Marini DC, Bacci M, Berbert-Molina MA, José T. Mosquitoes rely on their gut microbiota in the midgut: a method to stimulate optimal hatch. Mosq News. 1978;38:515–21.

6. Coon KL, Brown MR, Strand MR. Mosquitoes rely on their gut microbiota for larval development and to larval contact in a treehole mosquito. Ecol Entomol. 2019;44:386–95.

7. Edgerly JS, Mayer MA. To hatch or not to hatch? Egg hatch response to larval density and to larval contact in a treehole mosquito. Ecol Entomol. 1992;17:28–32.

8. Livdahl TP, Koenekoop RK, Futterweit SG. The complex hatching response of Aedes eggs to larval density. Ecol Entomol. 1984;9:437–42.

9. Novak RJ, Shroyer DA. Eggs of Aedes triseriatus and Ae hendersoni: a new method to stimulate optimal hatch. Mosq News. 1978;38:515–21.

10. Osorio A, Espinosa R, Puerto M, Trujillo G, Bracho-Rangel G, Zavala A. Comparative analysis of the composition of Aedes aegypti (Diptera: Culicidae) related to climatic factors in northwest Ohio. Environ Entomol. 2015;44:1022–8.

11. Sharma P, Sharma S, Maurya RK, De T Das, Thomas T, Lata S, et al. Salivary gland harbors more diverse microbial communities than gut in Anopheles gambiae. Parasit Vectors. 2014;7:235.

12. Dong Y, Manfredini F, Demoupolos G. Implication of the mosquito midgut microbiota in the defense against malaria parasites. PLoS Pathog. 2009;5:1000423.

13. Hussain M, Lu G, Torres S, Edmonds JH, Kay BH, Khromykh AA, et al. Effect of Wolbachia on replication of West Nile virus in a mosquito cell line and adult mosquitoes. J Virol. 2013;87:851–8.

14. Ramirez JL, Short SM, Bahia AC, Saraiva RG, Dong Y, Kang S, et al. Chromobacterium Csp_P reduces malaria and dengue infection in vector mosquitoes and has entomopathogenic and in vitro anti-pathogen activities. PLoS Pathog. 2014;10:e1004398.

15. Gillett JD, Roman EA, Phillips V. Erratic hatching in Aedes eggs: a new interpretation. Proc R Soc London B Biol Sci. 1977;196:223–32.

16. Edgerly JS, Mayer MA. To hatch or not to hatch? Egg hatch response to larval density and to larval contact in a treehole mosquito. Ecol Entomol. 1992;17:28–32.

17. Livdahl TP, Koenekoop RK, Futterweit SG. The complex hatching response of Aedes eggs to larval density. Ecol Entomol. 1984;9:437–42.

18. Novak RJ, Shroyer DA. Eggs of Aedes triseriatus and Ae hendersoni: a new method to stimulate optimal hatch. Mosq News. 1978;38:515–21.

19. Fallis SP, Snow KR. The hatching stimulus for eggs of Aedes punctor (Diptera: Culicidae). Ecol Entomol. 1983;8:23–8.

20. Joyce AL, Melese E, Ha PT, Inman A. Population genetic structure of the Culex restuans Theobald and Culex pipiens L. (Diptera: Culicidae) in Michigan. J Med Entomol. 2008;45:20–7.

21. Ross HH. The colonization of temperate North America by mosquitoes and man. Mosq News. 1964;24:103–18.

22. Kunkel KE, Novak RJ, Lampman RL, Gu W. Modeling the impact of variable climatic factors on the crossover of Culex restuans and Culex pipiens (Diptera: Culicidae), vectors of West Nile virus in Illinois. Am J Trop Med Hyg. 2006;74:168–73.

23. Reiter P. A standardized procedure for the quantitative surveillance of certain Culex mosquitoes by egg raft collection. J Am Mosq Control Assoc. 1986;21:291–219.

24. Lampman RL, Novak RJ. Oviposition preferences of Culex pipiens and Culex restuans for infusion-baited traps. J Am Mosq Control Assoc. 1996;12:23–32.

25. Reiter P. A standardized procedure for the quantitative surveillance of certain Culex mosquitoes by egg raft collection. J Am Mosq Control Assoc. 1986;21:291–219.

26. Tuten HC. Habitat characteristics of larval mosquitoes in zoos of South Carolina, USA. J Am Mosq Control Assoc. 2011;27:111–9.

27. Jackson BT, Paulson SL, Youngman RR, Scheffel SL, Hawkins B. Oviposition preferences of Culex restuans and Culex pipiens (Diptera: Culicidae) related to climatic factors in northwest Ohio. Environ Entomol. 2015;44:1022–8.

28. Reiter P. A standardized procedure for the quantitative surveillance of certain Culex mosquitoes by egg raft collection. J Am Mosq Control Assoc. 1986;21:291–219.

29. Lampman RL, Novak RJ. Oviposition preferences of Culex pipiens and Culex restuans for infusion-baited traps. J Am Mosq Control Assoc. 1996;12:23–32.

30. Reiter P. A standardized procedure for the quantitative surveillance of certain Culex mosquitoes by egg raft collection. J Am Mosq Control Assoc. 1986;21:291–219.

31. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, et al. Global patterns of 16S rDNA diversity at a depth of millions of sequences per sample. Proc Natl Acad Sci USA. 2011;108. 4516–22.

32. Muyzer G, de Waal E, Uitterlinden AG. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol. 1993;59:695–700.

33. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, et al. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J. 2012;6:1621–4.

34. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, et al. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol. 2006;72:5069–72.
35. Birtel J, Walser J-C, Pichon S, Burgmann H, Matthews B. Estimating bacterial diversity for ecological studies: methods, metrics, and assumptions. PLoS ONE. 2015;10:e0123566.

36. Sanago YO, Kim CH, Lampman R, Novak RJ. A real-time TaqMan polymerase chain reaction for the identification of Culex vectors of West Nile and Saint Louis encephalitis viruses in North America. Am J Trop Med Hyg. 2007;77:58–66.

37. R Development Core Team. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for statistical computing; 2017.

38. Rstudio Team. RStudio: Integrated development for R. Boston, MA: Rstudio Inc.; 2016.

39. Hammer Ø, Harper DAT, Ryan PD. PAST: Paleontological statistics software package for education and data analysis. Palaeontol Electron. 2001;4.9.

40. Bokulich NA, Subramanian S, Faith JJ, Gevers D, Gordon JJ, Knight R, et al. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. Nat Methods. 2013;10:57–9.

41. Hughes JB, Hellmann JJ, Ricketts TH, Bohannan BJ. Counting the uncountable: statistical approaches to estimating microbial diversity. Appl Environ Microbiol. 2001;67:4399–406.

42. Otzieszki T.D. A unified mathematical framework for the measurement of richness and evenness within and among multiple communities. Divers. Distributions. 2004;10:377–87.

43. McMurdie PJ, Holmes S. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS ONE. 2013;8:e61217.

44. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010;7:335–6.

45. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res. 2015;43:e47–e47.

46. Chao J, Wistreich GA, Moore J. Failure to isolate microorganisms from within mosquito eggs. Ann Entomol Soc Am. 1963;56:559–61.

47. Gardner AM, Muturi EJ, Allan BF. Discovery and exploitation of a natural ecological trap for a mosquito disease vector. Proc Biol Sci. 2018;285:20181962.

48. Muturi EJ, Kim CH, Bara J, Bach EM, Siddappaji MH, Wang Y, et al. Culex piperi and Culex restuans mosquitoes harbor distinct microbiota dominated by few bacterial taxa. Parasit Vectors. 2016;9:18.

49. Muturi EJ, Ramirez JL, Rooney AP, Kim CH. Comparative analysis of gut microbiota of mosquito communities in central Illinois. PLoS Negl Trop Dis. 2017;11:e0005377.

50. Kim CH, Lampman RL, Muturi EJ. Bacterial communities and midgut microbiota associated with mosquito populations from waste tires in east-central Illinois. J Med Entomol. 2015;52:63–75.

51. Wang Y, Gilbreath TM, Kukulta P, Yan G, Xu J. Dynamic gut microbiome across life history of the malaria mosquito Anopheles gambiense. PLoS ONE. 2011;6:e24767.

52. Zouache K, Raharimalala FN, Raquin V, Tran-Van V, Raveloson LHR, et al. First isolation of microorganisms from the gut diverticulum of Aedes aegypti (Diptera: Culicidae): new perspectives for an insect-bacteria association. Mem Inst Oswaldo Cruz. 2007;102:919–24.

53. Dada N, Sheth M, Liebman K, Pinto J, Lenhart A. Whole metagenome sequencing reveals links between mosquito microbiota and insecticide resistance in malaria vectors. Sci Rep. 2018;8:2084.

54. Juma EO, Allan BF, Kim CH, Stone C, Dunlap C, Muturi EJ. Effect of life stage and pesticide exposure on the gut microbiota of Aedes albopictus and Culex pipiens. J Vector Ecol. 2020;45:64–68.

55. Muturi EJ, Bara JJ, Rooney AP, Hansen AK. Midgut fungal and bacterial microbiota of Aedes triseriatus and Aedes japonicus shift in response to La Crosse virus infection. Mol Ecol. 2016;25:4075–90.

56. Apte-Deshpande A, Paingankar M, Gokhale MD, Deobagkar DN. Ser-rata adorifera a midgut inhabitant of Aedes aegypti mosquito enhances its susceptibility to dengue-2 virus. PLoS ONE. 2012;7:e40401.

57. Muturi EJ, Ramirez JL, Rooney AP, Dunlap C. Association between fertilizer-mediated changes in microbial communities and Aedes albopictus growth and survival. Acta Trop. 2016;164:54–63.

58. Juma EO, Allan BF, Kim CH, Stone C, Dunlap C, Muturi EJ. Effect of life stage and pesticide exposure on the gut microbiota of Aedes albopictus and Culex pipiens. J Sci Rep. 2020;10:9489.

59. Muturi EJ, Bara JJ, Rooney AP, Hansen AK. Midgut fungal and bacterial microbiota of Aedes triseriatus and Aedes japonicus shift in response to La Crosse virus infection. Mol Ecol. 2016;25:4075–90.

60. Apte-Deshpande A, Paingankar M, Gokhale MD, Deobagkar DN. Ser-rata adorifera a midgut inhabitant of Aedes aegypti mosquito enhances its susceptibility to dengue-2 virus. PLoS ONE. 2012;7:e40401.

61. Krajacic BJ, Huestis DA, Diao A, Yano AS, Diallo M, Krishna A, et al. Investigation of the seasonal microbiome of Anopheles coluzzii mosquitoes in Mali. PLoS ONE. 2018;13:e0194899.

62. Minard G, Tran FH, Van VT, Goubert C, Bellet C, Lambert G, et al. French invasive Asian tiger mosquito populations harbor reduced bacterial microbiota and genetic diversity compared to Vietnamese autochthonous relatives. Front Microbiol. 2015;6:970.

63. Gendrin M, Christophides GK. The Anopheles mosquito microbiota and their impact on pathogen transmission. In: Manguin S, editor. Anoph- eles mosquitoes - New insights into Malaria vectors. Rijeka: InTech; 2013. p. 525–48.

64. Thongtrispong P, Chandler JA, Green AB, Kittayapong P, Wilcox BA, Kapan DD, et al. Mosquito-vector-associated microbiota: metabarcoding bacteria and eukaryotic symbionts across habitat types in Thailand endemic for dengue and other arthropod-borne diseases. Ecol Evol. 2018;8:1352–68.

65. Manos J, Belas R. Proteobacteria: Gamma subclass. In: Dworkin M, Falkow S, Rosenberg E, Schleifer KH, Stackebrandt E, editors. The Prokaryotes: A Handbook on the Biology of Bacteria: Proteobacteria: Gamma subclass. 3rd ed. Singapore: Springer; 2006. p. 245–69.

66. Andrews ES, Xu G, Rich SM. Microbial communities within field-collected Culiseta melanura and Coquillettidia perturbans. Med Vet Entomol. 2014;28:125–32.

67. Audsley MD, Selezniov A, Joubert DA, Woolfitt M, O’Neill SL, McGraw EA. Wolbachia infection alters the relative abundance of resident bacteria in adult Aedes aegypti mosquitoes, but not larvae. Mol Ecol. 2018;27:297–309.

68. Checová L, Durnová E, Sikutová S, Halouzka J, Němec M. Characterization of spirochetal isolates from arthropods collected in South Moravia, Czech Republic, using fatty acid methyl esters analysis. J Chromatogr B. 2004;808:249–54.

69. Duguma D, Hall MW, Smartt CT, Debbonn M, Neufeld JD. Microbiota variations in Culex nigripalpus disease vector mosquito of west nile virus and saint louis encephalitis from different geographic origins. PeerJ. 2019;2019:6168.

70. Faissner I, Faissner W. Revision of the family Spironemidae Dolfini (Prostigmata, Hemimastigophora), with description of two new species, Spironema terricola n. sp. and Stereonema geiseri n. g., n. sp. J Eukaryot Microbiol. 1993;40:422–38.

71. Werren JH, Baldo L, Clark ME. Wolbachia: master manipulators of invertebrate biology. Nat Rev Microbiol. 2006;8:74–81.

72. Krajacic BJ, Huestis DA, Diao A, Yano AS, Diallo M, Krishna A, et al. Investigation of the seasonal microbiome of Anopheles coluzzii mosquitoes in Mali. PLoS ONE. 2018;13:e0194899.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.