Introducing an Environmental Microbiome to Axenic Insectary Reared Mosquitoes Alters Host and Microbe Blood Digestion Phenotypes

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Abstract

The microbiota of *Aedes aegypti* has been the subject of much research due to the potential role of the microbiome in mosquito physiology, development, and vectorial capacity. Axenic mosquitoes were colonized with environmental bacteria to compare microbiota acquired from the environment to insectary reared counterparts, particularly regarding blood meal digestion. Observationally, environmentally colonized mosquitoes showed faster blood digestion than insectary mosquitoes. 16S rRNA gene sequencing revealed that the diversity and community structure of the midgut microbiomes were distinct between the groups, with the environmental microbiomes having a greater diversity and larger temporal dynamics over the course of the blood meal. Metagenomic prediction from the 16S rRNA gene sequence data pointed to functional genes such as hemolysins differing between the two microbiomes. Additionally, only bacteria cultured from the environmental mosquitoes demonstrated hemolytic ability. Presence of these hemolytic bacteria may explain the observations of differing blood digestion rates in the mosquito. These data show that microbiomes of mosquitoes colonized from an environmental water source differ taxonomically and functionally from those from the insectary, with potential influences on host blood digestion. Thus, the axenic mosquito model can be employed to interrogate various microbiome compositions and link them to phenotypic outcomes of the host.

Introduction

Aedes aegypti mosquitoes are the primary vector for numerous globally important viral diseases such as Dengue fever, Zika, and Yellow fever¹. Members of the genus *Aedes* are anautogenous, meaning the females require a blood meal for egg production, which is also the point when they become infected with arboviruses²⁻⁴. Mosquitoes harbor a microbiome composed of bacteria, fungi, protists, and viruses, and several studies have linked the microbiome to host phenotypes such as development, digestion, reproduction, and pathogen susceptibility⁵⁻⁸. As with many metazoans the majority of the microbes associated with the mosquito reside in the gut, and as such potentially interact directly with viruses as they are ingested in the bloodmeal⁹⁻¹². Thus, the blood meal represents an important stage for possible vector control interventions¹³. However, there are still considerable knowledge gaps concerning what is occurring with the midgut microbiota during blood meal digestion.

The role bacteria play in blood meal digestion remains a significant knowledge gap. It is generally thought that the blood meal induces proliferation of gut bacteria^{7,14,15}, and antibiotic treatment of female mosquitoes slowed blood digestion and reduced egg laying, suggesting an important role for bacteria in blood meal digestion⁷. In contrast, axenic (microbe-free) mosquitoes could completely digest a blood meal and subsequently lay eggs, indicating bacteria are dispensable to mosquito blood meal digestion^{10,16}. However, this does not preclude specific microbial taxa from either enhancing or inhibiting blood meal digestion¹⁷. To date, studies investigating the relationship between the microbiome and blood meal digestion have employed culture-dependent techniques such as counting and characterizing cultivable bacteria¹⁸ or low-resolution culture-independent methods, such as sequencing 16S rRNA gene

clone libraries¹⁵. These methods resulted in a low-resolution picture of the microbiome, making it difficult to recognize taxonomic shifts within the community. A secondary limitation is that most studies were performed on insectary reared mosquitos which raises the question of how well insectary reared mosquito microbiomes reflect what happens in the environment. It is well documented that insectary reared mosquito microbiomes differ from those of field caught mosquitoes^{6,19,20}. Due to these differences, there is a critical research need to determine if patterns observed in the microbiome of insectary reared mosquitoes have any predictive value for mosquitoes in the wild.

This study employed our recently developed method to generate axenic mosquito larvae to imprint an environmentally acquired microbiome onto insectary reared *Ae. aegypti*^{16,21}. The bacterial populations were characterized by high-throughput sequencing of 16S rRNA genes to compare the composition of the insectary and environmental microbiomes and document temporal dynamics in the development of the midgut microbiome over blood meal digestion. Finally, through metagenomic prediction and culturing bacteria from the two respective microbiomes, we tested if there were potential phenotypic differences in the bacteria that made up the insectary and environmental microbiomes, specifically in their ability to breakdown and digest blood.

Materials And Methods

Insectary rearing of Ae. aegypti mosquitos

Mosquitos were obtained from a colony of *Ae. aegypti* (Orlando strain) maintained in the Connecticut Agricultural Experiment Station (New Haven, CT) insectary at 27 °C with a 14:10 hour light: dark cycle. Adult females received a blood feed with sterile defibrinated sheep's blood using a circulating membrane feeder with a parafilm membrane¹⁶. Mosquitos were allowed to feed on the membrane until a majority were observed to have fed. Mosquitoes with a visible blood meal were separated and reared in a sterile rearing chamber²¹. This group is hereafter referred to as the insectary group.

Ae. aegypti colonized with environmentally sourced bacteria

To generate an environmentally relevant source of bacteria, we created an environment mimicking *Ae. aegypti* breeding sites. In June 2018, distilled water with added leaf litter and gravel, was left outside to stagnate over a period of 2 weeks in New Haven, CT. Water was then vacuum-filtered with a 10.0 µm polypropylene prefilter (Merick Millipore) to remove organic debris and organisms that may have inhabited the stagnant water, including endemic mosquito eggs and larvae. This would act as the source of colonizing bacteria for axenic larvae.

Axenic larvae were generated by surface sterilizing colony *Ae. aegypti* eggs (Orlando strain) using the egg sterilization and hatching procedure described previously^{16,21}. Larvae were transferred to a sterilized Pyrex container containing 700 ml of environment-sourced water. The water was supplemented with sterile 2% liver yeast extract to support larval development. Pupae were transferred with larval rearing

water to a sterile hatching container. After adult emergence, mosquitos were maintained on 10% sterile sucrose and kept in a sterile rearing chamber¹⁶. After seven days, the mosquitos were blood fed identically to the insectary group as described above. This group is hereafter referred to as the environmental or environment colonized *Ae. aegypti* group.

Midgut extraction

Blood-fed females were immobilized over ice, submerged in 70% ethanol for 1 minute, and placed in 20 µl sterile phosphate-buffered saline on a sterile slide for midgut dissection. Dissected midguts were placed in 200 µl sterile saline on ice for storage. Either six of four midguts were dissected from the insectary and environmental groups, respectively, at 0-, 24-, 48- and 120-hours post blood feeding (PBF). Each midgut was homogenized using a pellet pestle and vortexed in 200 µl of sterile saline. The homogenized midgut was divided into two volumes; 100 µl of midgut homogenate was employed immediately for bacterial culturing while the remaining 100 µl of homogenized midgut was stored at -20 °C for DNA extraction and 16S rRNA gene amplification and sequencing.

DNA extraction, amplification, and sequencing of 16S rRNA genes

The midgut homogenate stored at -20 °C underwent a quick thaw at 55 °C for 1 minute prior to DNA extraction using the DNeasy PowerSoil Kit (Qiagen) with the following modification, samples were homogenized in a mixer mill (Qiagen TissueLyser Retch MM301) for 1 min at 30.0 1/s frequency. A nested PCR strategy was employed because of the low recovery of bacterial DNA from individual mosquito midguts. The initial PCR to amplify the 16S rRNA genes was performed with the primer pair 27 F (AGAGTTTGATCMTGGCTCAG) and 1492 R (TACGGYTACCTTGTTACGACTT)²² at 10 µM and the following components: PCR Buffer 2.5 µl, dNTPs 0.5 µl, MgSO₄ 1 µl, Platinum Taq Polymerase (Invitrogen) 0.1 µl, 0.75 µl forward primer (0.3 µM), 0.75 µl reverse primer (0.3 µM), 14.4 µl nuclease free diH₂O, and 5 µl of DNA sample per reaction. Thermo cycler program: 95 °C for 3 minutes, 10 cycles of 95 °C for 45 seconds, 55 °C for 45 seconds, 72 °C for 1 minute and 45 seconds followed by 72 °C for 10 minutes and 4 °C for infinite hold. Initial PCR products were then purified using the E.Z.N.A. Cycle Pure Kit (Omega). A secondary PCR of the purified initial amplicons was carried out using the V4 amplifying primers 515 F (5'-GTGCCAGCMGCCGCGGTAA) and 806 R (5'-GGACTACHVHHHTWTCTAAT) using dual barcoded Illumina primers²². The reactions were run as described for the initial PCR above. Both PCR reactions were run with negative controls consisting of sterile water replacing the template DNA.

Prior to sequencing, the final PCR reactions were normalized using the Invitrogen SequalPrep Normalization kit (Invitrogen), and 10 µl of each sample was pooled. A Qubit assay was used to confirm the concentration of pooled DNA, using standard protocols. Amplicons, including negative controls, were sequenced at the University of Connecticut Microbial Analysis, Resources, and Services facility on the Illumina MiSeq v2.2.0 platform.

Sequence processing and analysis

Sequence reads were assembled into contigs, and quality screened using mothur v 1.43.1²³. Sequences having at least 253 base pairs in length with no ambiguous bases, and no more than eight homopolymer base pairs were retained. Potentially, chimeric sequences were identified using the VSEARCH algorithm²⁴, as implemented in mothur, and subsequently removed from further analysis. The OptiClust algorithm in mothur was then utilized to assign sequences to Amplified Sequence Variants (ASVs) with a cutoff distance of 0.00 (100% sequence identity). The ASVs were classified against the SILVA database v.138²⁵. Subsequent analyses of microbiome data were performed with the R software phyloseq package²⁶. Diversity statistics were calculated on datasets randomly rarefied to the size of the smallest dataset (n=4397). Non-metric multidimensional scaling (NMDS) analysis was performed on the rarefied datasets employing the Bray-Curtis similarity metric, and significant differences were identified with the adonis: Multivariate ANOVA (MANOVA) function from the VEGAN R package²⁷. Statistically significant differences in the relative abundance of taxonomic bins were performed with the STAMP software package²⁸ with recommended statistical tests. Metagenomic prediction was performed with the PICRUSt2 pipeline²⁹, and statistical testing for differentially abundant predicted KEGG orthologs was performed using unnormalized counts in the ALDEx2 software package in R^{30,31}.

Quantification and identification of cultivable bacteria

Serial dilutions of 100 µl of each midgut homogenate were spread plated on Tryptic Soy Agar (TSA) in triplicate and incubated at 30 °C for 48 hours. To enumerate culturable members of the bacterial population; the dilution set with ten to 300 well-separated colonies per plate was counted and the number of colony forming units (CFUs) per mosquito was calculated. One of the replicate plates with well-separated colonies for each midgut sampled was used for isolate selection and sequencing to identify the most prevalent bacterial isolates in each mosquito. A randomized selection of isolated colonies was performed using a numbered plate grid (10 x 10) and a randomized number generator. Five colony isolates from each plate were selected for DNA extraction and sequencing, similar to the procedure described by Hyde *et al.*³². To increase the diversity of recovered colonies, if a colony differed from other colonies through morphological features a representative was additionally selected for isolation. In this manner, a low-resolution profile of the culturable microbial community was generated to identify the numerically abundant populations, while still recovering a wide selection of the total diversity of cultured bacteria. A pure culture was obtained for each selected isolate (n=225) by serially streaking the isolate into single colonies over three generations. Once a pure culture was confirmed, a single colony was inoculated into 1.5 ml Tryptic Soy Broth and incubated at 30 °C with shaking for 48 hours. Bacterial pellets for DNA extraction were obtained by centrifugation of 1 ml of liquid culture. The remaining 500 µl liquid cultures were combined with 500 µl sterile 80% glycerol to make permanent frozen stocks, and stored at -80 °C.

All isolates underwent initial DNA extraction by a boiling lysis procedure and resulting DNA underwent 16S rRNA gene PCR amplification (see below). Those isolates that could not be successfully amplified on the first pass were subsequently regrown and DNA was extracted using the EZNA Bacterial DNA Kit

(Omega) according to the manufacturer's protocol. To amplify the V4 region of the 16S rRNA the 515 F and 806 R primer pair were employed. Cycling parameters were as described above for the second step of the nested PCR protocol. Gel electrophoresis confirmed amplicons of ~250 base pairs in length. PCR products were purified using the Mag-Bind PCR RxnPure Plus (Omega Bio-tek) kit. Plates were sent to the Yale University Keck DNA Sequencing Facility for Sanger sequencing with the 515 F primer.

Raw sequences were processed using Geneious 8.1.9 (<https://www.geneious.com>). Sequences reads were determined to be of sufficient quality if they had a minimum of 80% high-quality bases and no less than 212 total base pairs. In total, 188 out of 225 bacterial isolates were retained after sequencing and quality filtering. Sequences were aligned with the mothur v 1.43.1 program²³. A 0.01 (99% sequence identity) cutoff was used to calculate pairwise distances between aligned DNA sequences for assignment into operational taxonomic units (OTUs). Representative sequences for each OTU were obtained through mothur and classified using a combination of the SILVA reference database and a BLAST sequence query²⁵. Representative isolates of each OTU were used to determine phenotypic characteristics of the bacterial populations. The ability to utilize blood as a sole growth source was determined by growth on M9 agar plates supplemented with sterile sheep's blood at a 1% concentration and growth for 24 hours at 30 °C.

DATA ACCESSIBILITY

All 16S rRNA gene sequences for the 19 representative OTUs are available in GenBank under the accession numbers OL811754- OL811772. The 16S rRNA amplicon gene libraries are available in the NCBI SRA under BioProject accession number: PRJNA787624.

Results

Host blood meal digestion

During midgut dissections, observational differences in the extent of blood meal digestion between the environmental and insectary groups were noted (Fig. 1). The environmental mosquitos exhibited a shorter blood digestion time than their insectary counterparts. This discrepancy in digestion was observed as an accelerated decrease in size of the food bolus of the midguts at each time point sampled and a complete absence of blood within the gut for environmental mosquitos sampled at 48-hour time point (Fig. 1D). This differed from what was observed during dissection of the insectary midguts, which had a more gradual decrease in the blood bolus size and maintained blood within the gut at 48 hours. Given that the genetics of the mosquitoes were presumably relatively similar, as they were derived from the same colony of mosquitoes, these initial observations suggest that the two microbiome states were responsible for the altered host blood digestion rates.

Diversity and composition of 16S rRNA gene sequence libraries

16S rRNA gene sequence libraries were employed to interrogate bacterial diversity in the mosquito microbiome. After quality filtering, 3 287 101 high-quality sequences were clustered into 141 958 ASVs. The diversity statistics for the different mosquito cohorts are displayed in Table 1. Good's coverage values ranged from 89 to 96%, suggesting that the majority of expected sequence diversity was recovered with this sequencing effort. The number of ASVs was ~61% higher ($P= 0.022$) in the environment colonized mosquitos than the insectary reared group. This was further supported by Shannon's diversity index, which was also significantly greater ($P= 0.003$) for the microbiomes of the environmental colonized mosquitos (Table 1). Thus, these data support that mosquitoes colonized by an environmental water source harbored a significantly higher alpha diversity than the colony mosquitoes reared in the insectary. In comparison, time point data revealed no significant variance in the number of ASVs recovered, or Shannon's diversity index, between individual time points in either of the groups, suggesting that bacterial diversity was not significantly altered over the course of blood meal digestion.

NMDS analysis showed a clear clustering of the datasets separating the insectary and environmental colonized mosquitos (Fig. 2). This clustering was highly significant ($P< 0.001$) (Fig. 2A), although the dispersion of the groups was not significant, suggesting some overlap in the populations (Permutation; $P= 0.337$). Investigating the datasets over the time course of the experiment showed differing patterns between the insectary and environment colonized mosquitos. Analysis of the beta diversity of the microbial composition for midgut microbiota for insectary *Ae. aegypti* indicated homogeneity of microbial communities found at the different time points, with no significant separation between datasets due to timepoint sampling (Fig. 2B; $P = 0.489$). In contrast, when clustered by time point, the environmental colonized microbiomes displayed distinct and significant clustering (Fig. 2C; $P= 0.038$). These data indicate that any temporal shifts in the microbiome's composition over the course of blood meal digestion were particularly pronounced for the environmentally colonized mosquitoes.

The 16S rRNA sequence datasets were then analyzed for taxonomic composition, and 14 bacterial phyla were identified. Three phyla accounted for a total of 97% of all 16S rRNA gene sequences: Proteobacteria (90.7%), Firmicutes (4.5%), and Bacteroidetes (3.2%). The proportion of Proteobacteria-related sequences varied widely between individual mosquitoes, reaching a low of 38% in the 0-hour insectary midguts up to 100% in several of the 24- and 48-hour midguts (Fig. 3). Yet, statistical testing did not identify any significant differences in phylum level bins between insectary or environmental colonized mosquitoes, or the time points within the different colonization groups.

The sequences were classified to the family level to investigate the communities at a deeper taxonomic level (Fig. 3). Within the insectary communities, the majority of sequences belonged to the family *Enterobacteriaceae* (54%), followed by *Acetobacteraceae* (28%). In comparison, the bacterial communities in the environment colonized mosquitos were predominantly composed of the families *Acetobacteraceae* (66%), *Burkholderiaceae* (36%), and *Enterobacteriaceae* (29%). When comparing the relative abundance of family level bins between the insectary and environmental microbiomes, insectary mosquitos harbored an overall greater relative abundance of *Enterobacteriaceae* ($P= 0.013$) and *Acetobacteraceae* ($P= 3.16e-2$; Supplemental Fig. 1), while the environmental midguts showed

significantly higher proportions of *Burkholderiaceae* ($P = 4.33 \times 10^{-3}$) and *Xanthomonadaceae*-related sequences ($P = 0.041$). Thus, these data support the observations from NMDS clustering (Fig. 2A), indicating compositional differences between the microbiomes sourced from the insectary and environment.

Regarding temporal dynamics over blood meal digestion, only the *Enterobacteriaceae* family underwent significant changes in relative abundance in the insectary mosquitoes ($P \leq 0.02$; Supplemental Fig. 2). For instance, *Enterobacteriaceae* accounted for 93% of all sequences at 48-hours, increasing from 22% at 0-hours. The temporal patterns in community succession were more readily apparent in the environmental group. For instance, the *Enterobacteriaceae* also showed significant shifts in relative abundance over blood meal digestion. They were relatively rare in the initial samples (2.9% of sequences), increasing to 59% by 24-hours ($P = 0.001$; Supplemental Fig. 3A). Also, significantly more abundant within the 24-hour period was *Aeromonadaceae* representing 17% of all environmental sequences, up from 0% in the initial samples ($P = 0.01$ Supplemental Fig. 3D). By 48-hours, the *Burkholderiaceae* became dominant, accounting for an average of 93% of sequences ($P = 0.01$; Supplemental Fig. 3B). Finally, by 120-hours, a significant increase in unclassified sequences within the order *Bacillales* (phylum Firmicutes) was observed. Taken together, these data demonstrate that the insectary and environmental-colonized mosquitoes harbored different microbiome structures that were further differentiated by their susceptibility to temporal shifts, with the environmentally sourced microbiome demonstrating larger shifts in composition over the course of blood meal digestion.

Metagenomic prediction and functional gene profiles

Predictive functional profiling from the 16S rRNA gene data identified a total of 6788 KEGG orthologs in the mosquito microbiomes across the samples, with 3146 (46%) showing a significant difference in relative abundance between treatment groups (Fig. 4A). In general, clustering of the differentially abundant KEGG orthologs separated the insectary and environmental groups, except for the environmental 120-hours samples that predominantly clustered with the insectary group (Fig. 4A). In this regard, the functional predictions support taxonomic differences between the insectary and environmental microbiomes. We further interrogated genes with a known role in blood digestion, hemolysins responsible for lysing blood cells³³, to assess if a specific function tied to blood digestion may have differed in abundance over blood meal digestion (Fig. 4B-E). Several identified hemolysins demonstrated shifts in abundance between the insectary and environmental microbiomes. For instance, K11005 (alpha-hemolysin, *hlyA*) was only predicted in the insectary microbiomes, predominantly in the early time points (Fig. 4B). In contrast, K10948 (*hlyA*, Vibriotype) showed a peak solely in the environmental 24 PBF samples (Fig. 4C). K11039 (delta-hemolysin) (Fig. 4D) and K11068 (hemolysin III) (Fig. 4E) were predicted in both the insectary and environmental microbiomes, but with shifts in abundance over the course of blood meal digestion. These data suggest that specific microbial populations encoding different hemolytic genes shifted in abundance over the course of blood meal digestion, potentially related to the differences in observed blood digestion rates in the mosquito.

Isolation and characterization of viable bacteria over blood meal digestion

Bacterial abundance in *Ae. aegypti* midguts over the course of blood meal digestion were measured by viable cell counts (Fig. 5). Due to differences in the number of midguts sampled for each group and the failure to recover isolates at the 48-hour time point for the environment colonized mosquitoes, statistical comparisons between the two groups were not made. However, bacterial abundance tended to be higher in the insectary colonized mosquitoes than in the environment colonized mosquitoes. For example, at 24-hours for the environmental mosquitoes, viable cell counts were ~36% lower than for the same period in the insectary. Additionally, significant differences in viable cell counts between time points in each microbiome group were tested. Previous investigations into the dynamics of this community during the blood meal digestion indicated significant changes within microbial biomass as identified by recovered CFUs^{15,18}. We did not identify any significant differences between time points in either the insectary or environmental groups. However, for the environmental group, the data from the insectary mosquitoes were used to estimate the number of expected CFUs. As a result, the dilutions were too high, resulting in no bacteria being recovered at the 48-hour time point (Fig. 5B). As the plating was also performed with the same dilutions as the environmental 24-hour time point, we can surmise that there was likely a relatively large drop in viable cell counts by 48-hours in the environmental-colonized mosquitoes. These observations suggest that trends in bacterial abundance over the course of blood meal digestion are obscured by high inter-individual heterogeneity but generally support that environment-colonized mosquitoes show more pronounced differences in bacterial abundance over blood meal digestion.

Although isolates were collected over the time course of the experiment, for the purposes of taxonomic and phenotypic analyses the isolate data was compiled into a single dataset. A total of 151 bacterial isolates were obtained and characterized through sequencing of the 16S rRNA gene. The isolate sequences were clustered into OTUs (99% sequence identity), and a total of 19 OTUs were identified across the insectary and environmental mosquito midguts. Of these OTUs, ten belonged exclusively to the insectary microbiotas, while eight were only identified in the environmental group. Only one OTU was shared between the two groups, an isolate classified to the genus *Enterobacter* (Table 2). The classification of the cultured isolates revealed Proteobacteria to be predominant (90%). This result was comparable to the 16S rRNA gene sequence dataset in which ~90% of sequences also belonged to the phylum Proteobacteria (Fig. 3). Likewise, Bacteroidetes and Firmicutes were among the predominant phyla for both the sequence data and culturable isolates, with Firmicutes representing 4% of the sequence data and 2% of isolates. In comparison, Bacteroidetes represented 3% of the 16S rRNA gene sequences and 7% of the isolates, respectively. These comparisons indicate an approximate representation of the sequence-based community profiling within the culturable members isolated.

Representative isolates of each OTU were cultured on blood agar plates to score hemolytic capabilities. Additionally, the isolates were cultured on 1% Blood M9 minimal media to assess utilization of blood as a sole source of nutrients (Supplemental Fig. 4). All of the isolates from the insectary mosquitoes showed

gamma hemolysis (i.e. a lack of hemolytic ability), and none could grow on blood M9 media. In contrast, four of the representative OTUs from the environmental group showed gamma hemolysis, while two demonstrated alpha hemolysis (i.e. partial hemolysis and reduction of hemoglobin), and a further two isolates were capable of beta hemolysis, the complete lysis and clearing of blood cells (Table 2). All isolates that demonstrated hemolytic capabilities were also able to grow on M9 minimal media when supplemented with sheep's blood as the sole nutrient source.

Interestingly, OTU 17 related to a species of *Bacillus* (phylum Firmicutes), was able to grow on the M9 blood media but did not demonstrate hemolytic activity on traditional blood agar (Table 2), suggesting it could grow on blood components without the attendant lysis of red blood cells. Thus, the majority (55%) of the isolates from the environmentally sourced microbiome showed some ability to lyse and/or digest blood, clearly differentiating them from the insectary isolates. Thus, these data support the metagenomic predictions from the 16S rRNA gene data (Fig. 5), showing a significant difference in the functional capabilities of the microbiota that make up the two different microbiomes.

Discussion

In this work, we show that our recently developed axenic mosquito model^{8,21}, can be employed to induce midgut microbiomes that differ in diversity and composition in colony *Ae. aegypti* mosquitoes. Axenic mosquitoes exposed to an environmental water source adopted a microbiome structure distinct from those reared in the insectary (Fig. 2A, Table 2). This result recapitulates a well described observation that the microbiomes of field-caught mosquitoes differ from those of mosquitoes reared in the laboratory³⁴. Importantly, bacteria such as *Stenotrophomonas maltophilia*, *Serratia marcescens*, and members of the *Chryseobacterium* sp., which were cultured from the environmental-colonized mosquitoes (Table 2) have previously been identified as members of the microbiome from field caught *Ae. aegypti*, suggesting the environmentally acquired bacteria from this study are broadly representative of bacteria commonly encountered in the *Ae. aegypti* microbiome^{35–39}. Of note, *Ae. aegypti* mosquitoes are not currently endemic to Connecticut, but with the ongoing geographic expansion of *Ae. aegypti* distribution caused by warming climates, *Ae. aegypti* populations could very well become endemic in this region in the coming years^{40,41}. In this respect, we do not propose that our data represent a “model” microbiome for *Ae. aegypti* mosquitoes. Instead, we present this data to highlight the effects that the source of colonizing bacteria and rearing conditions can have on the composition and function of the microbiome.

The compositional differences in the microbiome were associated with observational changes in the host's phenotype—namely, the rate of blood meal digestion. Mosquitoes colonized by an environmental microbiome showed nearly complete blood meal digestion by 48-hours, which was not the case for the insectary mosquitoes. The environmental-colonized mosquitoes displayed a microbiome that was more diverse (Table 1), temporally dynamic (Fig. 1,2), and differed in functional potential as assessed by both predicted metagenomes (Fig. 3) and growth phenotypes of isolated cultures (Table 2). The functional profiling points to a potential mechanism for differences in blood meal digestion rates due to the status of the microbiome. Specifically, hemolysin genes differed in composition and abundance between

microbiota hosted by the different microbiomes (Fig. 4B-C) and only isolates from the environmental microbiome carried out hemolysis in culture (Table 2). From these data we propose the following hypothesis. A “bloom” of a bacteria in the family *Aeromonadaceae*, occurring around 24-hours (Fig. 3, S3), may drive the accelerated rate of blood meal digestion in the environmental microbiome. This hypothesis is supported by the recovery of a bacterium related to *Aeromonas hydrophilia* (100% 16S rRNA gene sequence similarity) from the environmental microbiome capable of complete or beta-hemolysis (Table 2). Furthermore, the genome of *A. hydrophilia* encodes an ortholog of the *Vibrio*-type hemolysin (K10948⁴²), which also peaked in abundance at 24-hours in the predicted metagenomes (Fig. 4C). Thus, this organism seems to peak in abundance during the point at which blood digestion in the mosquito gut is maximal. There is precedence for the *Aeromonadaceae* playing a role in blood digestion in association with a host, as they are among the most prevalent bacteria that participate in blood digestion in the leech⁴³. However, a bloom of *Aeromonadaceae* in the mosquito's gut is unlikely to be a complete explanation for increasing rates of blood meal digestion, as many other microbial factors could also contribute to rates of blood digestion. For instance, when chitinases are added to the mosquito blood meal, rates of blood meal digestion increase, presumably due to a breakdown of the peritrophic matrix produced by the mosquito to sequester blood in the gut⁴⁴. In this study, bacteria in the genera *Serratia*, *Stenotrophomonas*, and *Chryseobacterium*, all known to produce chitinases⁴⁵, were also isolated from mosquito guts (Table 2). Therefore, blood meal digestion in the mosquito is likely a complex phenotype arising from bacteria/host and bacteria/bacteria interactions. The data presented here highlights the importance and utility of colonizing axenic mosquitoes with microbiomes of different composition and function, and linking those differences to mosquito phenotypes. This also opens the path for generating gnotobiotic mosquitoes, a state in which the composition of the microbiome is fully characterized, to specifically interrogate the influence of particular microbial taxa on mosquito phenotypes⁸. A recent call for standardization of mosquito microbiota research practices calls for the establishment of reproducible metrics to be employed in mosquito microbiome studies⁴⁶. We propose that axenic and gnotobiotic mosquitoes colonized by controlled microbiomes consisting of environmentally relevant microbes will be a central pillar of this effort.

Declarations

Competing Interests: The authors declare no competing financial interests

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Tables

Tables 1 and 2 are available in the Supplemental Files section

Figures

Figure 1

Observed blood digestion. A) Insectary *Ae. aegypti* female 24 hours after blood ingestion presenting a visible blood bolus (for illustrative purposes only). B) Pre-dissected environmental microbiome colonized female displaying lack of blood bolus at 48 hours after blood feed (C) Dissected midgut of blood fed female after 120 hours displaying lack of blood within midgut (D) Formed eggs of female lacking blood in the midgut after 48 hours post blood feed in an environmental colonized mosquito.

Figure 2

Non-metric multidimensional scaling (NMDS) ordination. For each ordination, inter-sample distances were calculated with the Bray-Curtis metric. Ellipses denote the 95% confidence level for the distribution of each group. The stress value for each ordination is indicated along with the P-value from a MANOVA test in the bottom inset panels. (A) All samples compared were grouped by microbiome status (insectary of environmental). (B) Time points within insectary midguts. (C) Time points within environmental midguts.

Figure 3

Taxonomic composition of 16S rRNA gene sequences at the family level. Families representing greater than 5% of total sequences are shown for each midgut. Phylum level assignments of the predominant families are also indicated: Proteobacteria shown in blue hues, Firmicutes shown in red hues, and Bacteroidetes shown in yellow. Families representing less than 5% of sequences are labeled as “other” in gray. Results of statistical comparisons of inter-group relative abundances are displayed in Supplemental Figures 1-3.

Figure 4

Functional profiling of predicted metagenomes. (A) Heatmap of significantly different KEGG orthologs inferred from PICRUSt2 metagenomic prediction. The dendrogram represents clustering based on Euclidean distances, using the differentially abundant KEGG orthologs as an input. (B-E) Estimated relative abundance of various hemolysins in the predicted metagenomes. Bars represent the average value among individually sampled mosquitoes along with the standard deviation of the mean (n=6 for insectary and n=4 for environmental). Note the different y-axis scales between panels.

Figure 5

Viable cell counts from blood fed mosquitoes. For each mosquito CFUs were determined by culturing on TSA media. (A) Insectary mosquitoes. (B) Environment colonized mosquitoes. Each point represents an individual mosquito. The mean is indicated by the black bar. The asterisks indicate mosquitoes from which no viable bacteria were recovered presumably due to over dilution during plating, particularly for the early (0 hours) and late (120 hours) sampling. The mean is indicated for each group. No viable bacteria were recovered from the environmental colonized mosquitoes at 48-hours. Plating was performed with 10^{-2} to 10^{-5} dilutions, based on our results from the insectary mosquitoes. Thus, this represents an upper limit for the possible viable cell counts in these mosquitoes. Note the different y-axis scales between panels.

Supplementary Files

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