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Causes and consequences of microbiome formation in mosquito larvae

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Abstract

1. The assembly of host-associated microbial communities is influenced by multiple factors, but the effect of microbiomes on host phenotypes is often not well understood. To address questions of food-web effects on host microbiome assembly, we manipulated the resource environment (grass only [G] vs. grass + nutrients [GN]), competition type (intra- vs. inter-specific) and density (high vs. low) for *Culex restuans* mosquito larvae. We predicted the microbial communities in fourth-instar larvae would differ between these environmental treatments and that these treatments would translate into differences in the adult phenotype.
2. Resource environment and density influenced the larval microbiome. In addition, the larval microbiome exhibited notable differences compared to the free-living microbial communities.
3. Resource-driven differences in the larval samples can be attributed to Arcobacteraceae being more abundant in larvae reared in the GN treatments relative to those reared in the G treatments and Comamonadaceae being more abundant in the G treatment. Although significant, the difference in community structure between density treatments was difficult to discern. This appears to be driven by Weeksellaceae only being abundant in the high-density, interspecific, GN treatment.
4. Rearing larvae to adulthood under severe food limitation resulted in low survival (<25%) in both resource environments. Approximately 60% of survivors to adulthood were male. Larvae reared in the intraspecific, G treatment had the shortest development time to adulthood and emerged as the smallest adults.
5. These results demonstrate how environmental variation can significantly alter the alpha and beta diversity of free-living microbes, which in turn can significantly affect host phenotype and critical life history traits, such as development time, size at adulthood, and survival. These findings highlight the importance of considering

environmental influences on microbiome diversity to understand and predict host outcomes, offering valuable insights for diverse applications in fields such as ecology, public health, and agriculture.

KEYWORDS
community assembly, competition, microbiome

INTRODUCTION

Host-associated microbes offer fundamental support to their hosts in a variety of fitness-related processes ranging from aiding in digestion and metabolism to promoting immune system function (Coon et al., 2014; Janssens & Stoks, 2014; Shin et al., 2011). These microbe-driven differences in host phenotype may alter the outcome of other aspects of the host's ecology such as competition for food and susceptibility to pathogens (Dickson et al., 2017). The application of high-throughput sequencing technologies to microbial ecology has allowed for a more mechanistic understanding of potential links between the microbiome and important fitness-related traits of the host and how this variation may influence the interactions of the host with the rest of the food web (Horner-Devine et al., 2004; McFall-Ngai et al., 2013; Petersen & Osvatic, 2018). However, our understanding of the factors that drive the assembly and stability of many host-associated microbial assemblages, and how they influence interactions of the hosts, remains limited.

Given their role as vectors of human diseases, the microbiome of both larval and adult mosquitoes has received considerable attention (Coon et al., 2022; Dada et al., 2021). A common finding is that the larval environment plays a major role in shaping the patterns of microbial communities in mosquitoes (Alfano et al., 2019; Boissière et al., 2012; Kim et al., 2015; Minard et al., 2013; Minard et al., 2018; Saab et al., 2020). Studies focusing on the nutritional quality of resources have demonstrated the effects of the larval habitat on development time to adulthood and adult wing length (an indicator of adult body size), both of which are strongly correlated with mosquito fitness and vector competence (Ameneshewa & Service, 1996; Hardy et al., 1983). However, the free-living microbes serve both as food items and potential symbionts, making the direct effects of nutrition challenging to separate from the effects of different gut microbial communities.

Recent research has begun disentangling the roles of nutrition versus microbial function on multiple aspects of the adult phenotype through a variety of methods, including axenic cultures, antibiotic treatments, and controlled dietary experiments (see reviews by Caragata et al., 2019; Cansado-Utrilla et al., 2021; Dada et al., 2021). Multiple studies have demonstrated an over-representation of certain microbial taxa in larvae relative to those present in the water, suggesting that microbes are not simply transient members in the gut that are either digested or expelled (Bascuñán et al., 2018; Duguma et al., 2013; Wang et al., 2011). Moreover, axenic larvae fail to develop, even when fed, suggesting a non-nutritional role for microbes (Coon et al., 2014). Therefore, even though the exact function of putative symbionts is unknown, multiple lines of evidence suggest that access to certain microbes can influence important fitness-related traits.

Given the importance of the characteristics of the larval habitat for access to those microbes, it is important to understand what is driving those free-living microbial communities beyond the well-known effects of the dominant plant matter in the system (Gardner et al., 2018; Muturi et al., 2013). For example, grazers in aquatic systems (e.g., mosquitoes and other insects, cladocera), most of which consume aquatic microbes, may differ in their foraging strategies and microhabitats (Thorp & Rogers, 2015). As a result, the presence or absence of certain species of grazers may influence the free-living microbial community through differential consumption or nutrient recycling, which can influence competition directly or via interference. Further, the composition of the grazer community may alter the behaviour of the focal hosts so that their feeding behaviour or encounter rate of microbes is different (Minard et al., 2013). In short, although it is well established that the characteristics of the larval habitat are important, considerable work remains to understand how the community composition of grazers can influence the host microbiome assembly of certain host species.

To address this question of food-web effects on host microbiome assembly, we reared wild-caught *Culex restuans* mosquito larvae in treatments that varied in resource environment (grass only vs. grass + nutrients), competition type (intraspecific vs. interspecific with the filter-feeding cladoceran *Simocephalus vetulus* [order: Diplostraca; family: Daphniidae]), and competition intensity (low vs. high). This species of mosquito is common in the region and is known to vector several human diseases, such as West Nile virus (Kramer et al., 2008; Rochlin et al., 2019). Given the extensive work on interspecific competition among mosquito species (Costanzo et al., 2005; Grigaltchik et al., 2016; Murrell & Juliano, 2008), we chose *S. vetulus* as a potential competitor. This cladoceran is found in local storm water sites with *C. restuans* at densities as high as 300 L^{-1} (Holmes et al., 2016, Holmes unpublished data). Given that a single adult *Simocephalus* can filter between 2 and 9 $mL \cdot hr^{-1}$ (Brito et al., 2006), 10 and 20 adults per 350 mL beaker were expected to be strong competitors of larval mosquitoes, especially in the stormwater drainage ditches where the two are often found co-existing in very shallow (<10 cm) aquatic habitats. We predicted that variation in the larval environment would alter the microbial composition of the larval microbiome and the free-living microbes, as well as the measured survival, development time, adult wing length, and adult sex ratio.

MATERIALS AND METHODS

Laboratory experiments

To examine the effects of the environment on free-living and host-associated microbial communities and on the resulting mosquito traits (survival, development time, size at adulthood, and adult sex ratio), we conducted laboratory experiments using *C. restuans* mosquitoes reared under eight conditions.

Mosquito larvae were reared in infusions made from two separate diluted stock mixtures ('G'—grass only and 'GN'—grass + nutrients). Stock mixtures were prepared in sterilized 15 L buckets by adding 23.94 g grass clippings·L^{-1} to autoclaved deionized (DI) water (Jackson, 2004). The GN stock mixture, used to make the GN infusion, was enriched with nitrogen (6.58 mg·L^{-1} $NaNO_3$) and phosphorus (0.94 mg·L^{-1} KH_2PO_4) to mimic nutrient-rich local field sites (Holmes, 2019). We expected this addition of nutrients would modify the free-living microbial assemblages that serve as food resources for *C. restuans* and *S. vetulus* (Horner-Devine et al., 2003). The G stock mixture contained only grass clippings (no nutrient addition). After 7 days, the stock buckets were filtered through a sterilized 105 μm sieve to remove the grass. In an effort to ensure that food resources were limited so that the effects of competition could be observed, stock mixtures were diluted to produce 25% infusions (262.5 mL of filtered lake water and 87.5 mL of G stock or GN stock), which served as the two resource environments for experimental trials (G and GN infusion, respectively). We used individual sterile 400 mL Fisherbrand™ Tri-Cornered Polypropylene beakers containing 350 mL of infusion for each trial.

Culex egg rafts were field-collected using grass infusion-baited oviposition traps. Egg rafts were hatched individually in petri dishes and *C. restuans* first instars were identified based on the presence of a clear scale anterior to the sclerotized egg-breaker and then pooled (Burkett-Cadena, 2013). We collected *S. vetulus* (Haney, 2018) from a local pond (40°06′31″ N 88°10′37″ W) and maintained them in laboratory culture by feeding them the green algae *Ankistrodesmus falcatus*.

We examined the effects of both intra- and interspecific competition by varying the densities (low: 20 or high: 40) and ratios of *C. restuans* to *S. vetulus* (20:0, 40:0, 10:10 or 20:20) in two different resource environments (G or GN infusion). Once beakers were filled with either G or GN infusion, five replicates of each treatment were stocked with recently hatched (<12 h) *C. restuans* larvae, along with 10 or 20 adult *S. vetulus* individuals. Beakers were held at 25°C for 4 days and occasionally stirred with sterilized stirring rods to disrupt the surface film. After 96 h (Day 4), we recorded the number of surviving *C. restuans* larvae and sacrificed them for microbiome analysis. The larvae were surface sterilized in 70% ethanol for 10 min and then rinsed five times with Dulbecco's Phosphate-Buffered Saline (DPBS) solution (Thermo Fisher Scientific, Waltham, MA; Muturi et al., 2016). Sterilized larvae were placed individually in sterile 1.5 mL microcentrifuge tubes with 100 μL of sterile DPBS solution and stored in a −20°C freezer until DNA extraction for 16S rRNA sequencing. To assess the initial free-living microbial composition, three 15 mL samples from both the initial G and GN infusions were collected and centrifuged for 60 min at 1643 rcf. Pellets were retained and stored in a −20°C freezer until subsequent DNA extraction for 16S rRNA sequencing. To determine the final free-living communities in experimental beakers, 15 mL water samples were prepared as above from each experimental beaker after the *C. restuans* larvae and *S. vetulus* individuals were removed.

A second experiment used the same conditions with larvae grown to adult emergence. Only the high level of competition was used for this experiment (either 40 *C. restuans* larvae or 20 *C. restuans* larvae and 20 *S. vetulus*). Pupae were transferred to individual 50 mL conical tubes containing water, where they were held until emergence. Though not all larvae survived to pupation, all pupae emerged as adults. Adult mosquitoes were collected and stored in a −20°C freezer until their sex was determined and their wing lengths (an indicator of body size and proxy for fitness) were measured using the Leica Application Suite (LAS v4.0).

Microbiome identification

From the initial and final infusion samples and the sacrificed *C. restuans* larvae, we extracted the microbial DNA using a QIAGEN DNeasy PowerSoil Pro Kit following the instructions of the manufacturer (QIAGEN, 2017). To increase the amount of template DNA available for sequencing, three pools of three larvae were used for each beaker whenever possible. A majority of the 38 beakers (68.4%) had three pools of three larvae, seven (18.4%) had two pools of three larvae, four (10.5%) had one pool of three larvae and one (2.6%) had a single larva. Two beakers (grass – inter – high and grass + nutrient – intra – high) were excluded due to contamination. Extracted DNA samples were stored in a −20°C freezer and later quantified using a Nanodrop 1000 (Thermo Scientific, Pittsburg, PA). Samples were sequenced at the National Center for Agricultural Utilization Research in Peoria, IL.

The V3-V4 region of the 16S rRNA gene was amplified with the polymerase chain reaction (PCR) primers 341f and 806r (Caporaso et al., 2011; Muyzer et al., 2012) under the following thermocycling conditions: 95°C, 10 min, 35 cycles of 95°C, 30 s; 58°C, 30 s and 72°C, 60 s. A SequalPrep™ normalization plate (Thermofisher Inc., Waltham, MA) was used to clean and normalize the PCR amplicons. Sample sequencing was done using an Illumina MiSeq system with a MiSeq V3 2 × 300 bp sequencing kit.

16S and metagenomic data analyses were completed by the High-Performance Computing in Biology (HPCBio) group at the University of Illinois. Illumina data targeting the entire V3-V4 region of the 16S ribosomal subunit were quality assessed using FASTQC (Andrews, 2016) and then processed using the TADA Nextflow-based workflow (https://github.com/h3abionet/TADA) that implements a containerized version of the DADA2 (v1.22.0, R v4.1.1) workflow (Callahan et al., 2016; R Core Team, 2019) for dereplicating and denoising reads to generate amplicon sequence variants (ASVs). In brief, sequences underwent primer sequence removal using primers 341f ('CTACGGGNGGCWGCAG') and 806R ('GGACTACHVGGGT

WTCTAAT'). Minimal quality trimming was then performed; forward sequences were trimmed to a length of 275 nt and reverse sequences were trimmed to a length of 215 nt, a maximum expected error (EE) score of 2 was utilized, and no unclassified bases were retained. Default steps were used to denoise reads and dereplicate into ASVs, followed by taxonomic assignment using the DADA2 implementation of the RDP Classifier (Lan et al., 2012) and the Silva v138.1 database (Quast et al., 2013) custom formatted by DADA2 developers (McLaren & Callahan, 2021). Multiple sequence alignment of the resulting ASV sequences was performed by DECIPHER v.2.22.0 (Wright, 2015), followed by a midpoint-rooted phylogenetic analysis using Fasttree v2.1.10 (Price et al., 2010) to produce a maximum likelihood tree used in data analysis steps. Raw counts, taxonomic assignments, and the phylogenetic tree for the ASVs were imported into R v. 4.2.1 using the package phyloseq v. 1.42.0 (McMurdie & Holmes, 2013). Initially, minimal filtering was performed to remove mitochondria and chloroplast ASVs, ASVs that were unassigned at the Phylum level, and samples with less than 5000 sequenced reads, leaving 18,508 ASVs for statistical analysis of alpha diversity metrics that assume raw data as input. Prior to the statistical analysis of beta diversity, ASVs underwent prevalence filtering with a threshold of 4% (present in six samples), followed by taxa agglomeration to the species level when possible.

Statistical analyses

Alpha diversity analyses of the larvae and free-living microbial communities (Observed, Chao1, Shannon's, and Faith's PD estimates) were performed using the R packages phyloseq and vegan v. 2.6-4 (Oksanen et al., 2016). After confirming the normality of the residuals and homoscedasticity, we used ANOVAs for each using the model: resource environment (G vs. GN) * competition type (intraspecific vs. interspecific) * density (low vs. high).

Beta diversity analyses were performed in R with phyloseq and vegan using relative proportion normalization to maintain community structure. To assess differences in microbial community composition, we used principal coordinate analysis (PCoA) and permutational multivariate analysis of variance (PERMANOVA). We conducted these analyses on both Bray–Curtis and weighted UniFrac distance matrices, as we were uncertain about the influence of phylogenetic relatedness on patterns of community relatedness. PERMANOVA tests for the larval samples were performed using vegan function 'adonis2' to assess the significance of differences in Bray–Curtis distance and weighted UniFrac distance using the model: resource environment (G vs. GN) * competition type (intraspecific vs. interspecific) * density (low vs. high).

We visualized the relative abundance of microbial families using the 'microViz' package (v0.10.6) in R (Barnett et al., 2021). Differential abundance analysis was performed using normalization methods developed for DESeq2 (Love et al., 2014) on ASVs that were agglomerated to the family, genus, and species levels. R code used to conduct this analysis can be found at: https://github.com/HPCBio/caceres-16S-mosquito-2022Sept/tree/main/src.

In the first experiment, where all larvae were sacrificed on day 4, we used a logistic ANOVA in SAS (Version 9.4) to identify the effects of resources, competition, and starting density, and the effects of their interactions on individual mosquito survival to day 4 (1: survived to day 4 vs. 0: did not survive to day 4). In the second experiment, where all individuals were reared to adulthood, we used a two-way MANOVA and the subsequent univariate ANOVAs to investigate the role of resource environment and competition type, as well as their interaction on survival to adulthood, time to emergence, size at emergence, and sex ratio at emergence of *C. restuans*.

RESULTS

Microbiome

The MiSeq sequencing of the 16S amplicon generated 3,945,816 sequences between the 140 mosquito pools and free-living samples (after the removal of one free-living sample due to an abnormally low read count of <5000). Reads per pool ranged from 10,281 to 50,994, with a mean of 28,184.4 +/− 823.3 (SE) sequences. From 18,508 ASVs, we identified 133 microbial families, 206 genera, and 112 species. Prior to beta diversity analyses (Figure 1) and taxonomic composition plots, we performed a prevalence filter of 4%, reducing ASVs to 13,616, and then we performed taxa agglomeration to the species level whenever possible, resulting in a total of 159 taxa.

The PERMANOVA analysis, based on Bray–Curtis dissimilarity matrices, revealed that resource environment and density had a significant effect on the larval *C. restuans* microbiome (Figure 1a and Table 1). However, when phylogeny was considered (weighted UniFrac), density was no longer significant (Figure 1b and Table 1). The inclusion of phylogeny led to a reduced separation of microbial communities, suggesting that evolutionary relationships partially obscured the distinct patterns seen in Figure 1a. Because competition type was not significant in either model, it was omitted from visualization (Figure 1).

Microbial communities differed between stock water, final free-living community, and larval community samples (Figure 2). Despite comprising less than 25% of the water samples (both final free-living community and stock), Clostridiaceae was the most abundant family in all larval samples (Figure 2). Bacteroidaceae, which dominated the stock water samples and remained present in the final free-living community, were extremely rare in the larval samples. Larvae that had been raised in G infusion (without nutrients) had more Comamonadaceae than did those larvae that had been raised in GN infusion (with nutrients). Arcobacteraceae comprised a greater proportion of the community for GN treatments for both final free-living and larval communities compared to G treatments.

Differential abundance analysis revealed several bacterial families that were significantly differentially abundant between water samples and mosquito larvae (Figure 3). Bacterial families from the phyla Proteobacteria, Bacteroidota, and Campylobacterota were primarily associated with water/free-living samples, whereas bacterial families from the phylum Firmicutes were mostly associated with larval microbiomes.

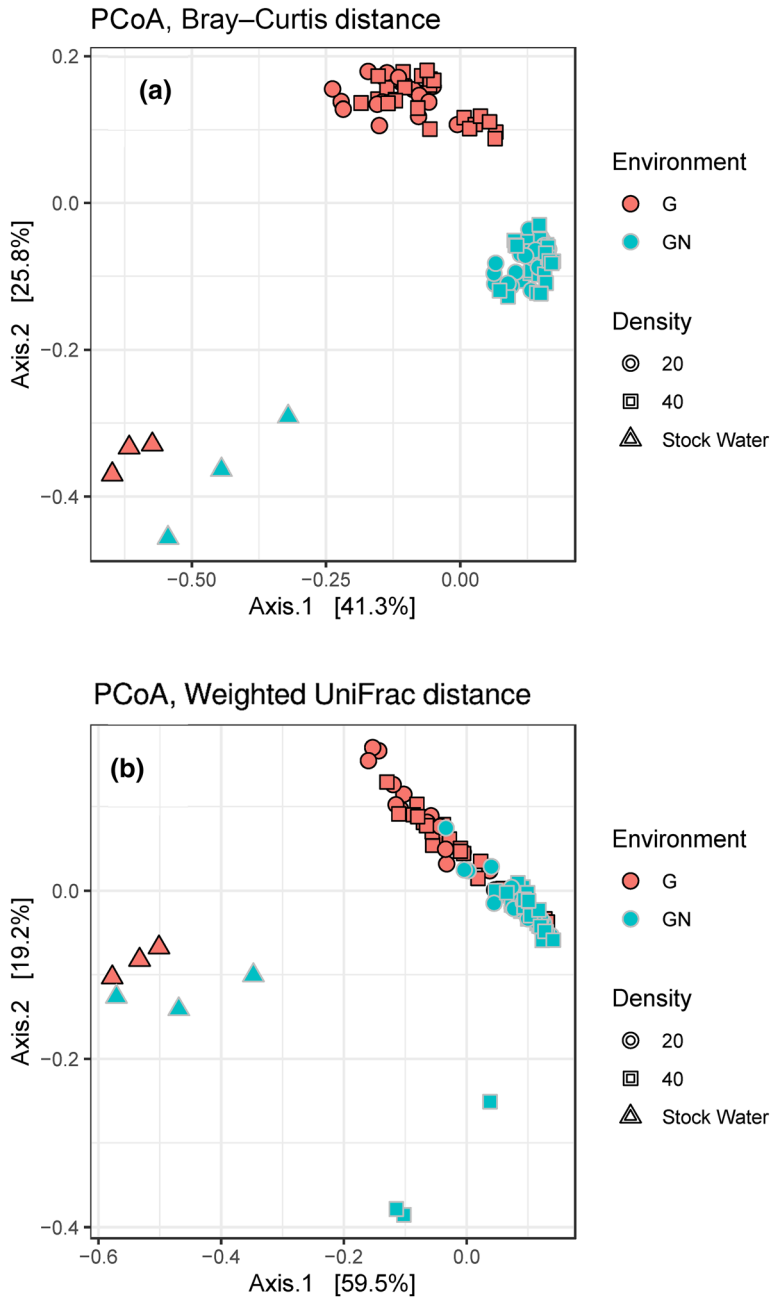

FIGURE 1 PCoA plots based on (a) Bray–Curtis and (b) weighted UniFrac dissimilarities of the microbial composition of mosquito larvae by experimental treatments (resource environment [G vs. GN infusion], competition [intra- vs. inter-specific], and density [low: 20 vs. high: 40]).

TABLE 1 Effects of experimental factors (resource environment: G vs. GN infusion, competition type: intra- vs. inter-specific, and density: low vs. high) on mosquito microbial communities.

PERMANOVA	Bray–Curtis		Weighted UniFrac	
Factor	R^2	p	R^2	p
Resource Environment	0.156	**0.001**	0.119	**0.001**
Competition	0.008	0.243	0.009	0.215
Density	0.019	**0.043**	0.02	0.053
Resource Environment × Competition	0.004	0.581	0.003	0.744
Resource Environment × Density	0.004	0.565	0.003	0.640
Competition × Density	0.001	0.952	0.002	0.875
Resource Environment × Competition × Density	0.001	0.954	0.001	0.904

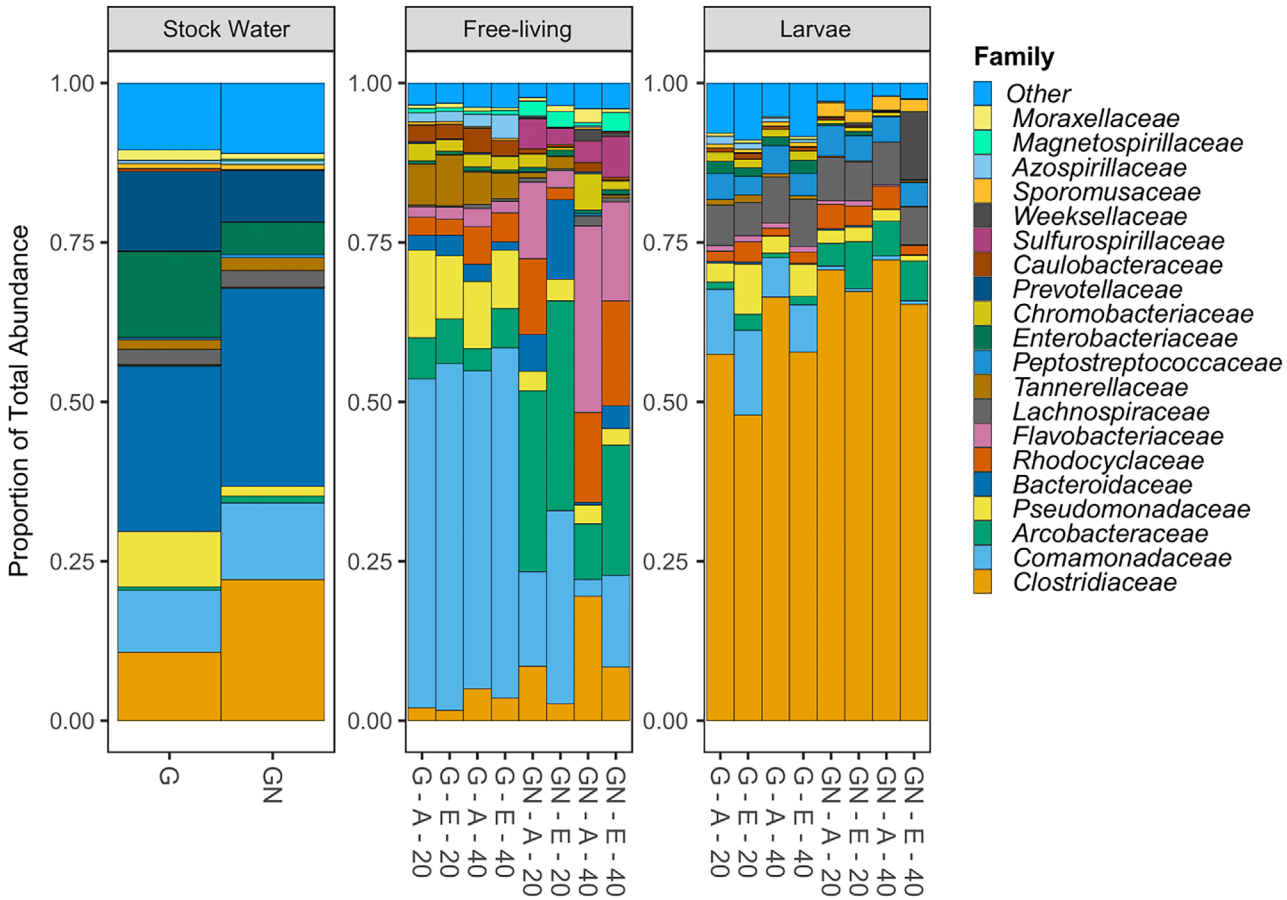

FIGURE 2 Proportion of total abundance of the top 20 most abundant microbial families in the stock water, post-experiment water (i.e., free-living communities), and mosquito larvae, aggregated by treatment. The remaining families were grouped as 'Other'.

The four measures of alpha diversity (Observed operational taxonomic units (OTUs), Chao1, Faith's phylogenetic diversity (PD), and Shannon diversity index) were higher in the larvae and stock water than in the free-living communities (Table 2). When considering phylogenetic relatedness, diversity (i.e., Faith's PD) was highest in both G and GN stock waters, followed by larvae in the G treatment. Larval microbial communities were significantly influenced by resource environment and larval density, but not by larval competition or their interactions (Table 3). Larvae raised in the G environment had more diverse communities than those raised in the GN environment (Table 2). The effect of density was also significant, with the low-density treatment being more diverse than the high-density treatment (Table 2). There was no discernible impact of competition or any interactive effects on any of the four alpha diversity measures.

Mosquito life history traits

Experiment 1: Effect of resource environment, competition type, and larval density on larval survivorship to day 4

Larval survival to day 4 was significantly affected by the resource environment. *Culex restuans* larval survivorship in GN resource was 76.4% and significantly higher than 50.1% in G resource ($\chi^2 = 48.5$, df = 1, $p < 0.0001$). Competition type also influenced survival, with larvae reared along with *S. vetulus* having an average survival of 70.0% compared to 56.6% survival for those reared without *S. vetulus* ($\chi^2 = 10.8$, df = 1, $p = 0.001$). There were no differences in larval survival between treatments differing in density ($\chi^2 = 0.16$, df = 1, $p = 0.69$) or due to any interactions (Resource*Density $\chi^2 = 0.15$, df = 1, $p = 0.69$; Resource*Competition $\chi^2 = 0.37$, df = 1, $p = 0.54$; Density*Competition $\chi^2 = 1.05$, df = 1, $p = 0.31$; Resource*Density*-Competition $\chi^2 = 3.74$, df = 1, $p = 0.053$).

Experiment 2: Effect of resource environment and competition type on survival to pupation, development time to adulthood, body size at adulthood, and adult sex ratio

The resource environment (Pillai's trace = 0.67, $F_{4,24} = 10.8$, $p < 0.0001$), competition type (Pillai's trace = 0.83, $F_{4,24} = 26.6$, $p < 0.0001$) and their interaction (Pillai's trace = 0.51, $F_{4,24} = 5.5$, $p < 0.0001$) influenced the four measured traits when analysed with MANOVA. Survival to adulthood was <25% in all treatments, which limits the ability to draw firm conclusions. The resource environment ($F_{1,37} = 19.23$, $p < 0.0001$), the competition type ($F_{1,37} = 107.82$,

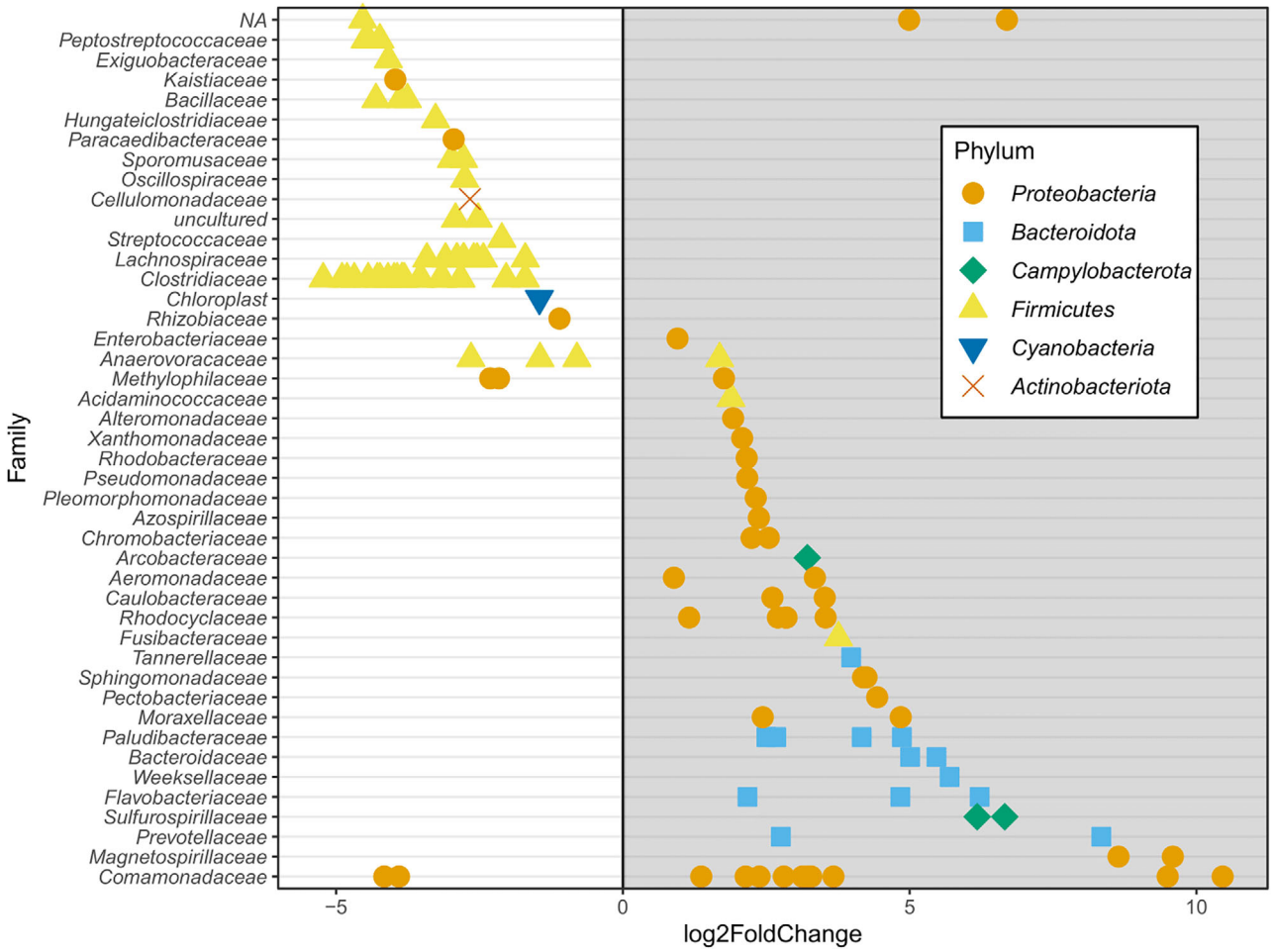

FIGURE 3 Log2-fold change in differential abundance of bacterial families between larval microbiome samples (left/white side) and free-living communities (right/shaded side). Only associations that were statistically significant at the 0.05 level are shown.

$p < 0.0001$) and the resource environment*competition type ($F_{1,37} = 15.34$, $p = 0.0004$) all influenced larval survival (Figure 4a). When larvae were reared in the presence of *S. vetulus*, adults emerged from all 10 beakers at an average of 4.3 adults per beaker when reared in G infusion and 1.8 adults per beaker when reared in GN infusion. Larvae reared in the absence of *S. vetulus* emerged as adults in only 7 of the 10 G infusion replicates, at an average of one adult per beaker, and in only one of the 10 GN infusion replicates, with a single adult emerging. This result is the opposite of what we found for survival to day 4 in experiment 1, where larval survival in GN infusion was higher.

For these survivors, the resource environment ($F_{1,24} = 24.44$, $p < 0.0001$), competition ($F_{1,24} = 14.91$, $p = 0.0007$) and the resource environment*competition interaction ($F_{1,24} = 13.25$, $p = 0.001$) influenced the time to emergence (Figure 4b). The significant interaction effect resulted from the fact that, with interspecific competition, time to adulthood was 22–24 days regardless of the resource environment. In contrast, surviving larvae that were reared under intraspecific competition eclosed after ~15 days in the G infusion compared to ~29 days in the GN infusion.

The results were similar for the size at emergence, with resource environment ($F_{1,27} = 4.94$, $p = 0.04$) and the resource environment*competition interaction ($F_{1,27} = 9.81$, $p = 0.0045$) influencing the wing size, while competition type did not ($F_{1,27} = 0.63$, $p = 0.43$; Figure 4c). The adult wings measured on average 2.89 mm when reared in G infusion in the presence of *S. vetulus*, 2.78 mm when reared in GN infusion in the presence of *S. vetulus* and 2.42 mm when reared with G infusion in the absence of *S. vetulus*. Only a single adult out of all 10 replicate beakers emerged from the treatment where larvae were reared in GN infusion without *S. vetulus* and its wings measured 3.06 mm. The majority (61%) of the surviving individuals were male, and there was no effect on the resource environment ($F_{1,24} = 2.90$, $p = 0.10$) or the competition type ($F_{1,24} = 1.91$, $p = 0.18$). Since so few individuals emerged from the intraspecific competition treatment, there was a significant interaction between the two competition types and resource environment ($F_{1,24} = 6.95$, $p = 0.015$; Figure 4d).

DISCUSSION

In this study, we examined how resource environment (grass [G] vs. grass + nutrients [GN]), competition type (intraspecific vs. interspecific) and initial density of competitors (20 vs. 40 individuals per 350 mL)

TABLE 2 Alpha diversity indices (Shannon, Chao1, Observed, and Faith's PD) for larval, free-living, and stock water microbial communities using ASVs defined by a 100% sequence similarity cutoff.

Type	Environment	Competition type	Density	Shannon	Chao1	Observed	Faith's PD
Larvae	G	Inter	20	7.86 ± 0.04	4957 ± 208	4398 ± 200	24.8 ± 0.7
		Inter	40	7.81 ± 0.06	4335 ± 321	3835 ± 299	23.6 ± 1
		Intra	20	7.87 ± 0.04	4925 ± 130	4370 ± 119	24.9 ± 0.6
		Intra	40	7.78 ± 0.04	4598 ± 126	4109 ± 108	23.8 ± 0.5
	GN	Inter	20	7.85 ± 0.04	4500 ± 214	4043 ± 208	23.2 ± 0.8
		Inter	40	7.62 ± 0.07	4244 ± 88	3851 ± 86	22.2 ± 0.4
		Intra	20	7.81 ± 0.02	4248 ± 159	3818 ± 169	22 ± 0.6
		Intra	40	7.67 ± 0.05	3829 ± 111	3444 ± 117	20.4 ± 0.6
Free-living	G	Inter	20	6.38 ± 0.17	3031 ± 180	2558 ± 134	20.6 ± 0.9
		Inter	40	6.56 ± 0.31	3570 ± 252	3030 ± 169	21.7 ± 1.4
		Intra	20	6.5 ± 0.19	3148 ± 228	2703 ± 180	20.4 ± 1.5
		Intra	40	6.54 ± 0.3	3493 ± 426	2940 ± 379	21.6 ± 1.4
	GN	Inter	20	6.49 ± 0.16	2961 ± 270	2550 ± 242	20.1 ± 1.4
		Inter	40	6.69 ± 0.27	3288 ± 450	2805 ± 377	22.2 ± 2
		Intra	20	6.59 ± 0.24	3421 ± 457	2898 ± 419	21.7 ± 1.6
		Intra	40	7.03 ± 0.32	3916 ± 387	3401 ± 385	23.4 ± 2
Stock water	G			7.53 ± 0.1	4854 ± 214	4155 ± 203	28 ± 1
	GN			7.46 ± 0.26	4881 ± 248	4284 ± 268	27.5 ± 0.7

TABLE 3 Statistical analyses for alpha diversity indices (Observed, Shannon, Faith's PD, and Chao1) of larval mosquito microbiome.

ANOVA		Alpha diversity metric			
		Significance is bolded at $p < 0.05$			
Factor	df	Observed	Shannon	Faith's PD	Chao1
Resource Environment	1	**0.001**	**0.006**	**< 0.001**	**0.001**
Competition	1	0.356	0.738	0.096	0.323
Density	1	**0.009**	**< 0.001**	**0.011**	**0.003**
Resource Environment × Competition	1	0.057	0.794	0.055	0.060
Resource Environment × Density	1	0.627	0.083	0.875	0.613
Competition × Density	1	0.873	0.578	0.789	0.853
Resource Environment × Competition × Density	1	0.308	0.325	0.640	0.351
Residuals	89				

influenced the *C. restuans* larval microbiome. In a separate experiment, we also examined how resource environment and competition type influenced survival to adulthood, development time, sex ratio at emergence and size at adulthood. In the first experiment, the community structure of the free-living microbes in the initial infusions was similar despite the nutrient differences, but the two starting infusions were distinct from both the larvae and the final free-living community. The larval microbiomes diverged from the free-living water community, with larvae predominantly inhabited by Clostridiaceae, and water samples having relatively more Comamonadaceae. By the end of the trial, larval microbial communities differed for individuals reared in our resource environment and density treatments. However, there was no effect of density when phylogenetic relatedness of microbes was included. In the second experiment, larvae reared in G infusion with *S. vetulus* (interspecific competition) exhibited significantly higher survival rates compared to those reared in GN infusion alongside other larvae only (intraspecific competition). Moreover, larvae raised in GN infusion required a longer development period but emerged as larger adults compared to those reared in G infusion. Our findings suggest that the environmental differences we introduced played a pivotal role in shaping the mosquito microbiome. Larval microbiomes were dominated by Clostridiaceae, and taxa in this family also declined from the initial to final infusion, suggesting that they may be easily encountered and ingested. However, Bacteroidaceae also declined from the stock water to the final water and

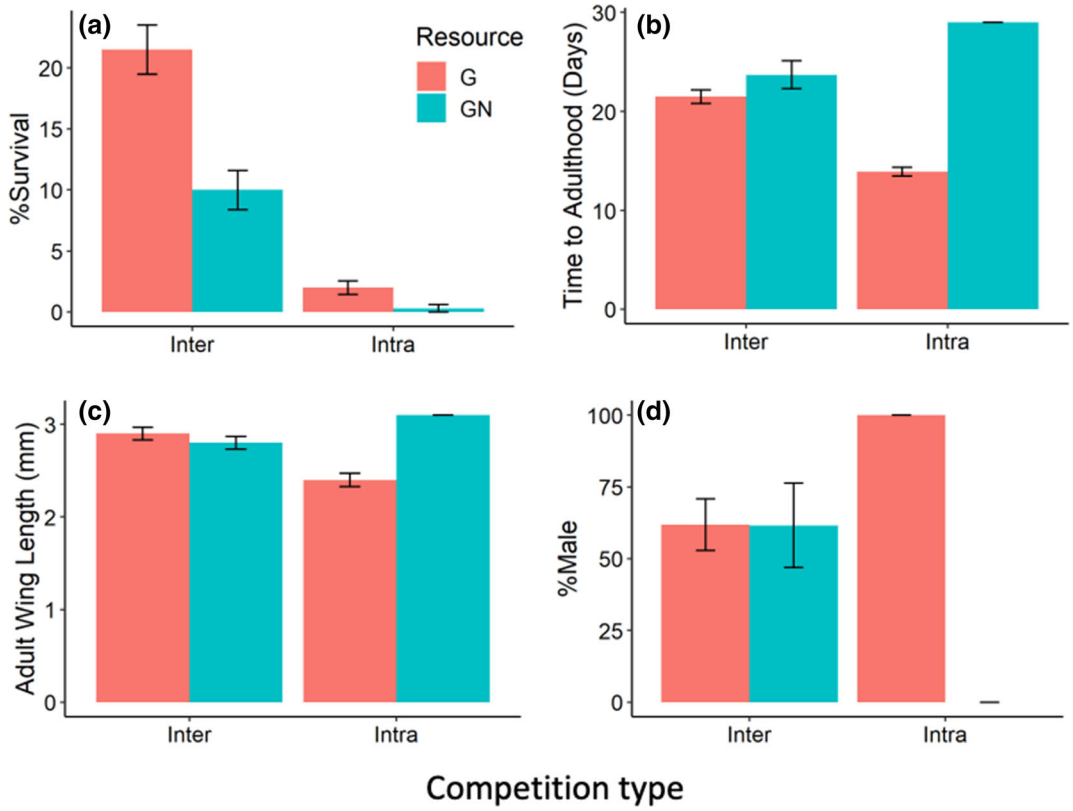

FIGURE 4 Results from Experiment 2. Graphed are: (a) the percent survival from hatching to adulthood, (b) the average number of days from hatching to adulthood, (c) the average wing length (in mm) of emerged adults, and (d) the percent of emerged adults that were identified as male for *Culex* mosquitoes reared with one of two resource environments (G vs. GN infusion) and one of two competition types (inter- vs. intraspecific).

was absent from the larval microbiome, suggesting that differential consumption cannot fully explain larval community structure. The observed difference in microbial communities between the mosquito larvae and their larval environment has been previously reported (Bascuñán et al., 2018; Duguma et al., 2013; Guégan et al., 2018; Wang et al., 2011). There are multiple potential mechanisms driving these differences, including: the rate at which the mosquito encounters and ingests microbes; differential colonization of, digestion in, and excretion from the gut; and population growth rates in the water column from the start to the end of the experiment. Species that are repeatedly found in the environment but rarely in the gut are either not encountered by the grazer or, if so, are likely quickly digested or are capable of accelerated growth in the environment as other species are consumed and their nutrients recycled (Muturi et al., 2020).

One issue with sequencing the microbiomes of aquatic filter feeders is that they include both bacteria that have colonized the gut and those that are passing through, either to be digested or excreted intact. Kaufman and colleagues (Kaufman et al., 1999; Kaufman et al., 2008) have attempted to disentangle resident symbionts from transient microbes that are providing sources of nutrition. One clue to identifying the symbionts lies in the species that are maintained through metamorphosis. Unfortunately, few studies to date have resolved those taxa to the species or even genus level, making it difficult to link bacterial species to potential symbiotic function (Guégan et al., 2018). Hence, the differences we detected here between the treatments can offer predictions for the appropriate manipulative experiments to help us sort the 'bacteria as food vs. bacteria as symbionts' problem in animals that have diets entirely comprised of microbes (Hammer et al., 2019).

The addition of nutrients to the water decreased alpha diversity in their associated larvae. Being raised at a lower density resulted in a more diverse microbiome compared to being raised at a higher density. Although we do not have a mechanism to explain this pattern, both differences in colonization dynamics of the host and the competitive ability of those microbes once established in the host are possible explanations. All four alpha diversity estimates are higher compared to several previous studies examining the microbiome of larval *Culex* (Duguma et al., 2015; Juma et al., 2021). We suspect that this discrepancy results from comparing our ASVs to OTUs. Since ASVs are based on exact sequence variants, we may have captured additional diversity relative to those that use OTUs. In fact, when we compared our current results with the initial analysis of the same data (Schwing, 2021), we found similar qualitative results of the effects of nutrient addition and density, but higher absolute values. With increased resolution, higher estimates of alpha diversity are possible.

We did not establish mosquito-free beakers, so we are unable to assess how much of the change from the initial to the final free-living

community was driven by the presence of the mosquitoes. In a similar study that included mosquito-free controls, Muturi et al. (2020) found that the community structure in mosquito-free controls changed over the course of 4 days, suggesting that some of the differences we observed may result in part from competition among bacterial species. Muturi et al. (2020) also found that the larval mosquitoes influenced the final community structure, which was likely the case in this study as well. In addition, we followed the protocol for the Powersoil kit with no modifications, which may have influenced our diversity metrics due to potential inhibitors in the water.

There is no doubt that conditions experienced in the larval habitat influence adult traits (Coon et al., 2014, 2016; Dickson et al., 2017; Juma et al., 2021; Romoli et al., 2021). The density of intraspecific or interspecific competitors is well known to influence survival, development time, and adult size (Alto et al., 2012; Blaustein & Chase, 2007; Juliano, 2009; Rowbottom et al., 2015), but how these interactions influence the larval microbiome, and how that microbiome assembly influences larval and juvenile traits is still being explored. Coon et al. (2014) demonstrated that axenic *Aedes* larvae failed to develop beyond the first instar, and Dickson et al. (2017) demonstrated the effects of the microbiome on time to pupation and adult traits, including adult body size, longevity, and vectorial capacity, by using gnotobotic *Aedes aegypti* mosquitoes. Given that experiment 1 collected larvae on day 4 of the experiment and experiment 2 collected adults more than 2 weeks later, any potential link between the larval microbiome and adult phenotype must be interpreted with caution.

Previous studies have also addressed how the microbial community may influence vector competence (Apte-Deshpande et al., 2012; Caragata et al., 2019). For example, Bennett et al. (2019) suggested that the high degree of variation found within populations in vector competence may be mediated by microbial communities. In particular, they pointed to the presence or absence of *Serratia* in *A. aegypti* influencing the susceptibility to dengue virus type 2 (DENV-2). Although we did not measure susceptibility to pathogens, we did find the effects of competition on survival in the intraspecific treatment, compared to when *Culex* larvae were reared with *Simocephalus*. Egizi et al. (2014), in examining competition between *Culex quinquifasciatus* and *Aedes japonicus*, found that these two species influence the free-living microbial communities in different ways, which may then influence the dynamics of the other competitor.

Similarly, our data revealed differences between intraspecific and interspecific competition, which may have been mediated through the presence or absence of certain free-living microbes. Our survival data also suggest the potential for microbe-specific effects. Although survival was higher in the grass + nutrient treatments during the first 4 days compared to that in the grass-only treatments (experiment 1), when we ran the experiment until emergence as adults, we recorded higher survival in the grass-only treatments compared to the grass + nutrient treatments (experiment 2). As the nutrients were expected to increase mosquito survival (Victor & Reuben, 2000), our results suggest that certain microbes that were less abundant in the grass + nutrient treatments may be beneficial to larval survival and development after the first 4 days. Moreover, in an attempt to ensure competition, we significantly limited the food, which resulted in an overall low survivorship, particularly in the interspecific treatment.

Mosquitoes influence human health and the economy as vectors of many deadly diseases. Future studies need to clarify to what degree each microbial taxa acts as a source of nutrition versus as a potential endosymbiont providing functional support to the mosquito host's physiology and fitness. As the interest in determining more precisely the drivers of microbiome assembly and the corresponding effects of those assemblages on mosquito host fitness expands, we can hope to see improvement in our ability to treat, reduce, predict, and prevent outbreaks of mosquito-borne disease (Dada et al., 2021).

AUTHOR CONTRIBUTIONS

Cameron D. Schwing: Writing – review and editing; conceptualization; investigation; writing – original draft; methodology; visualization; formal analysis; data curation. **Christopher J. Holmes:** Writing – review and editing; conceptualization; investigation; writing – original draft; methodology; visualization; formal analysis; supervision; data curation. **Ephantus J. Muturi:** Writing – review and editing; writing – original draft; conceptualization; methodology; visualization; resources; supervision; data curation; project administration; investigation; funding acquisition. **Christopher Dunlap:** Writing – review and editing; resources; data curation; investigation; methodology. **Jessica R. Holmes:** Writing – review and editing; investigation; validation; formal analysis; resources; data curation; software. **Carla E. Cáceres:** Writing – review and editing; conceptualization; funding acquisition; writing – original draft; methodology; visualization; formal analysis; project administration; supervision; resources.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data are available at https://github.com/HPCBio/caceres-16S-mosquito-2022Sept/tree/main/src.

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