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Research Article

RESEARCH ARTICLE

Microbial communities of container aquatic habitats shift in response to *Culex restuans* larvae

Ephantus J. Muturi[1,*,†], Christopher Dunlap[1] and Carla E. Cáceres[2]

[1]USDA, Agricultural Research Service, NCAUR, Crop Bioprotection Research Unit, 1815 N. University St Peoria, IL. 61604. USA and [2]Department of Evolution, Ecology and Behavior, School of Integrative Biology, University of Illinois at Urbana–Champaign, 505 S. Goodwin Ave., Urbana, IL 61801, USA

*Corresponding author: 1815 N. University St. Peoria, IL. USA, 61604. E-mail: Ephantus.Muturi@ars.usda.gov; ephajumu@yahoo.com

One sentence summary: This study demonstrates the strong effect of the white-dotted mosquito on bacterial communities in container aquatic habitats.

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†Ephantus J. Muturi, http://orcid.org/0000-0001-5905-065X

ABSTRACT

We examined how larvae of *Culex restuans* mosquito influences the bacterial abundance, composition and diversity in simulated container aquatic habitats. The microbiota of *Cx. restuans* larvae were also characterized and compared to those of their larval habitats. The presence of *Cx. restuans* larvae altered the bacterial community composition and reduced the bacterial abundance, diversity and richness. *Azohydromonas* sp., *Delftia* sp., *Pseudomonas* sp., *Zooglea* sp., unclassified Enterobacteriaceae and unclassified Bacteroidales were suppressed while *Prosthecobacter* sp., *Hydrogenaphaga* sp., *Clostridium* sp., unclassified Clostridiaceae and *Chryseobacterium* sp. were enhanced in the presence of *Cx. restuans* larvae. *Cx. restuans* larvae harbored distinct and less diverse bacterial community compared to their larval habitats. These findings demonstrate that *Cx. restuans* larvae play a key role in structuring the microbial communities in container aquatic habitats and may lower the nutritional quality and alter the decomposition process and food web dynamics in these aquatic systems. The findings also demonstrate that mosquito larvae are highly selective of the bacterial taxa from the larval environment that colonize their bodies. These findings provide new opportunities for more focused studies to identify the specific bacterial taxa that serve as food for mosquito larvae and those that could be harnessed for disease control.

Keywords: *Culex restuans*; detritus; bacterial communities; container aquatic habitats

INTRODUCTION

Plant detritus, primarily leaf litter, is the most dominant energy source in many ecosystems and a key determinant of trophic structure, community dynamics and ecosystem functioning (Mann 1988; Moore *et al.* 2004). However, detritus feeders face enormous nutritional challenge since plant detritus is typically a low-quality diet. It has a relatively high content of indigestible carbon-rich compounds such as lignin and cellulose, and a low content of nitrogen (proteins) (Mann 1988). To overcome this nutritional challenge, detritivores rely on heterotrophic microbial communities that colonize the leaf litter. These microbes initiate the decomposition process and convert indigestible leaf components into more digestible and nutritious microbial biomass (Barlocher 1985; Mann 1988; Koski, Kiorboe and Takahashi 2005). The combined effects of microbial decomposition process and detritivores feeding activity further breaks down the detritus particles, increasing the surface area available for more microbial colonization. The detritus particles become more nutritious and desirable to detritivores as the ratio of microbial biomass to plant tissue increases (Suberkropp and Arsuffi 1984; Chung and Suberkropp 2009). Therefore, heterotrophic microbes serve the dual role of primary decomposers

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and basal food resources for numerous groups of detritus feeders.

In container aquatic habitats such as tree holes, leaf axils, bamboo stumps, stormwater catch basins, discarded automobile tires and rain barrels, the community structure is regulated from the bottom up based on the quality and quantity of basal resources (Yee, Kaufman and Juliano 2007; Murrell and Juliano 2008; Juliano 2009). The primary source of carbon for the inhabitant invertebrate communities in these systems is derived from allochthonous leaf litter and other plant materials. This plant detritus is occasionally supplemented with other types of detritus such as animal tissues (e.g. invertebrate carcasses), animal wastes (e.g. feces) and stemflow (Carpenter 1982; Daugherty *et al.* 2000; Kitching 2000). Larvae of different insect species particularly mosquitoes are the top-level consumers in these aquatic habitats (Kitching 1971; Butler *et al.* 2007; Yee *et al.* 2012; Kim, Lampman and Muturi 2015; Harbison *et al.* 2017) and may partition the detritus resource through their diverse foraging behavior (Merritt, Dadd and Walker 1992; Yee, Kesavaraju and Juliano 2004). Although mosquito larvae consume protozoans, fungi and other microeukaryotes, bacteria are the primary food resource for many mosquito species (Walker, Olds and Merritt 1988; Merritt, Dadd and Walker 1992; Paradise and Dunson 1998). Different bacterial species are known to vary in their nutritional content and susceptibility to invertebrate digestive processes, and differentially affect the survival and development of mosquito larvae (Phillips 1984; Coon *et al.* 2014). Thus, both microbial composition and biomass are likely key regulators of mosquito communities in container aquatic habitats.

Given their close interaction with heterotrophic microbes, and the ease with which they can be manipulated, mosquito larvae within detritus-based container aquatic systems are suitable model organisms for addressing questions related to how aquatic microbial communities respond to grazing pressure from invertebrate consumers. Previous studies have attempted to address these questions by assessing how the microbial communities of container aquatic habitats fluctuate in the presence and absence of mosquito larvae. These studies have shown that mosquito larvae can have variable effects on diverse microbial communities. Water column protozoan densities were significantly reduced in response to grazing by *Ae. sierrensis* and *Ae. triseriatus* larvae (Washburn *et al.* 1988; Paradise and Dunson 1998; Eisenberg, Washburn and Schreiber 2000) while leaf-associated fungi were unaffected by the presence of *Ae. triseriatus* larvae (Kaufman *et al.* 2001). Bacterial abundance on leaf litter was significantly reduced in response to feeding by *Ae. triseriatus* larvae (Kaufman *et al.* 1999) while water column bacteria either increased (Kaufman *et al.* 1999), decreased (Walker *et al.* 1991) or remained unaffected (Paradise and Dunson 1998). *Aedes triseriatus* larvae caused a shift in bacterial and fungal communities in tree hole habitats (Kaufman *et al.* 1999; Kaufman, Chen and Walker 2008) while *Wyeomyia smithii* larvae had strong influence on abundance and composition of bacterial and protozoan communities in simulated habitats (Cochran-Stafira and von Ende 1998). Although mosquitoes from other genera such as *Anopheles* and *Culex* are of enormous medical significance, utilize container aquatic habitats for larval development, and exhibit different foraging behaviors from those of *Aedes* mosquitoes (Merritt, Dadd and Walker 1992; Yee, Kesavaraju and Juliano 2004), their interaction with microbial communities in their larval habitats remain poorly understood. A recent study by Duguma *et al.* (2017) revealed that both rotifer (*Habrotrocha rosa* Donner) and ciliate (*Paramecium* sp.) suppressed the growth of *Culex nigripalpus* larvae indirectly by disrupting other microbial groups (e.g. bacteria) that serve as food for mosquito larvae.

In this study, we used experimental microcosms simulating small container aquatic habitats to examine how the bacterial communities respond to larvae of the white-dotted mosquito, *Culex restuans*. We tested the hypothesis that the presence of *Cx. restuans* larvae would have strong effects on bacterial abundance, diversity and community composition. We also characterized the bacterial microbiota of *Cx. restuans* larvae from these microcosms. *Culex restuans* is known to thrive in container aquatic habitats of various nature and sizes including stormwater catch basins, tree holes, discarded tires and other natural and artificial water holding containers (Kling, Juliano and Yee 2007; Bara and Muturi 2015; Harbison *et al.* 2017). We focused on the water column bacteria since *Cx. restuans* larvae are known to filter-feed in the water column (Merritt, Dadd and Walker 1992). Results of this study improve current understanding of the relationship between bacterial communities and larval development in container aquatic habitats and may inform the design of more focused studies to decipher the role of specific bacterial taxa on mosquito larval nutrition.

MATERIALS AND METHODS

Experimental set up

Culex egg rafts were collected from nearby woodlots and residential areas in Peoria IL., using oviposition traps baited with grass infusion. The collections were done in May 2019, a time which is associated with large populations of *Cx. restuans*. In total, more than 200 *Culex* egg rafts were collected. Each egg raft was hatched individually in 12-well cell culture plates containing 3 mL of deionized water and a single first instar larva identified to species morphologically. All larvae were identified as *Culex restuans* based on the presence of a clear scale anterior to the sclerotized egg-breaker and were used for this study (Crabtree *et al.* 1995). Larvae from all egg rafts were pooled and rinsed 3 times with de-ionized water before adding them to the experimental microcosms.

The experimental setup included 30 tri-pour beakers (400 mL) filled with 350 mL of grass infusion that was prepared by fermenting 600 g of fresh grass in 50 L of tap water for 5 days. The fermentation was conducted outdoors in a shaded area. The infusion was filtered with a screen mesh to exclude large debris before it was dispensed into the experimental containers. The 30 containers were divided into three groups of 10 containers. Water samples (45 mL) from group 1 containers were immediately (Day 0) processed for analysis of initial microbiota (GFI samples). The 10 containers from group 2 were each stocked with 20 first instar larvae of *Cx. restuans* (GFM samples) and the 10 containers from group 3 were incubated without mosquito larvae (GFN samples). The containers were maintained at 26°C, 70% relative humidity (RH) and 10:14 h (light: dark cycle) and monitored daily for larval development. On day 4 when most larvae had matured to 4th instars, 45 mL of water samples from containers with and without mosquito larvae were collected after agitating the content. The larvae from each container were preserved in a −80°C freezer in pools of five larvae and three larval pools from each container were processed for analysis of their microbiota. Freshly collected water samples from all 30 containers were centrifuged at 5000 X *g* for 20 min. DNA was extracted from the resulting pellet using Power Soil DNA isolation kit according to the manufacturer's protocol (Mo Bio laboratories, Carlsbad, CA). Each mosquito larval pool was sur-

faced sterilized in 70% ethanol for 5 min and rinsed 3 times in sterile phosphate-buffered saline solution for 10 s before DNA extraction using the same kit used for water samples.

Next-generation sequencing

DNA samples were quantified using qubit 4 fluorometer (ThermoFisher Scientific, Waltham, MA) and normalized to 5 ng/μL. The 16S rRNA library was prepared according to the 16S metagenomic sequencing library preparation protocol provided by Illumina. Briefly, the V3-V4 hypervariable region of the bacterial 16S rRNA was amplified using the following primer set: V3-V4 forward 5′ ⟨0:bold ⟩TCGTCGGCAGCGTCAGATGTG TATAAGAGACAG⟨/0:bold⟩CCTACGGGNGGCWGCAG and V3-V4 reverse 5′ ⟨0:bold ⟩GTCTCGTGGGCTCGGAGATGTGTATAAGAGA CAG⟨/0:bold⟩GACTACHV-GGGTATCTAATCC. The Illumina overhang adapter sequences (shown in bold) were added to the forward and reverse primers based on Nextera, Illumina 16S rRNA library preparation kit. Amplicon PCR to amplify the V3-V4 hypervariable region of the 16S rRNA was done in 25 μL reactions containing 12.5 μL of 2x KAPA HiFi HotStart ReadyMix, 5 μL of 1 μM each of the forward and reverse primers, and 2.5 μL of template genomic DNA. Thermocycling conditions were an initial incubation of 95°C for 3 min, followed by 25 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s and a final extension at 72°C for 5 min. The PCR amplicons were cleaned using the AMPure XP beads and index PCR was conducted in 45 μL reactions containing 25 μL of 2x KAPA HiFi HotStart ReadyMix, 5 μL each of index 1 and index 2 combinations and 10 μL of PCR grade water. Thermocycling conditions for index PCR were an initial incubation of 95°C for 3 min, followed by 8 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s and a final extension at 72°C for 5 min. The final libraries were cleaned using the AMPure XP beads, quantified using qubit 4 fluorometer, normalized to 4 nM and then pooled. The pooled library was denatured and mixed with Phix control spike-in of 10% as a sequencing control. Sequencing was performed on an Illumina MiSeq system using the MiSeq V3 2 × 300 paired-end sequencing kit. Demultiplexed reads were examined using CLC genomics workbench v8.5 (Qiagen inc., Valencia, CA) and low-quality reads and chimeras were removed. The reads were then paired, trimmed to fixed length and CLC Bio Microbial Genomics module was used for operational taxonomic unit (OTU) clustering. The Greengenes rRNA gene database was used to assign OTUs at 97% sequence similarity (DeSantis *et al.* 2006).

Statistical analyses

All statistical analyses were conducted using R version 3.3.2 (https://cran.r-project.org/bin/windows/base/old/3.2.3/) and PAST version 3.14 (Hammer, Harper and Ryan 2001). OTUs with less than 10 sequences were discarded as they may have resulted from sequencing errors (Bokulich *et al.* 2013). The remaining reads were rarefied to 4721 reads per sample to mitigate biases arising from different sampling depths across samples. Shannon diversity index, observed OTUs (richness) and chao1 were computed using *vegan* package in R (Oksanen *et al.* 2016) and non-parametric Kruskal–Wallis test was used for statistical comparisons among the four sample types. Non-metric multidimensional scaling (NMDS) with Bray–Curtis similarity matrix values was conducted using *vegan* package. *Phyloseq* package was used to generate NMDS plots to visualize within-group and between-group differences in bacterial communities among treatments. Stress values < 0.2 can be considered a good representation of the data and those > 0.3 are not considered to be valid (Quinn and Keough 2002). Permutational multivariate analysis of variance (PERMANOVA) with Bonferroni adjustment for multiple comparisons was conducted in PAST version 3.14 to determine the statistical significance. Venn diagrams were created using the *limma* package to visualize the OTUs that were shared among treatments. METAGENassist was used for automated taxonomic-to-phenotypic mapping in order to assess the putative functional profiles based on 16S community composition (Arndt *et al.* 2012). Taxonomic abundance was used as the input file and normalized over sample by sum and over taxa by Pareto scaling. The data was then analyzed for 'oxygen requirement by phenotype' to determine how the presence of mosquito larvae influences the functional profiles of microbial communities.

Bacterial DNA quantification

Femto Bacterial DNA Quantification Kit was used to detect and quantify the bacterial DNA in water samples incubated with and without mosquito larvae. Quantitative PCR (qPCR) was conducted in 20 μL volumes containing 18 μL of the Femto Bacterial qPCR premix, and 2 μL of one of the following templates: sample DNA, bacterial DNA standards, or no template control. Thermocycling conditions were an initial incubation of 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 1 min and a final extension at 72°C for 7 min.

RESULTS

The V3-V4 hypervariable region of 16S rRNA was used to characterize the bacterial diversity of mosquito larvae and water from their larval environment. The treatments were initial grass infusion (GFI), grass infusion without mosquito larvae (GFN), grass infusion with mosquito larvae (GFM) and mosquito larvae. After quality trimming, chimera checking step, removal of sequences with less than 10 reads and rarefaction at an even depth of 4721 reads, a data set consisting of 278 539 reads was recovered for downstream analysis. Microbial richness (Kruskal–Wallis chi-squared = 47.44, df = 3, $P < 0.0001$) and Shannon diversity index (Kruskal–Wallis chi-squared = 45.91, df = 3, $P < 0.0001$) were significantly lower in larval samples compared to the other treatments (Table 1). Bacterial richness and Shannon diversity index were also significantly lower in water that had contained mosquitoes (GFM samples) compared to water that had been incubated without mosquitoes (GFN samples).

Overall, a total of 351 OTUs were detected across sample types. To determine the shared bacterial richness among the four treatments, Venn diagrams displaying the overlaps between the treatments were developed. The total number of OTUs identified in GFI, GFN, GFM and larval samples were 272, 263, 267 and 231, respectively, with 130 OTUs occurring in the four sample types (Fig. 1a). There were also some OTUs that were shared between two or more sample types as well as those that were unique to each sample type. A total of 19 unique OTUs were found in GFI samples compared to 5 OTUs in GFN samples, 3 OTUs in GFM samples and 10 OTUs in larval samples. To assess how the presence of mosquito larvae affected the bacterial communities, we compared the bacterial richness among the GFI, GFN and GFM treatments (Fig. 1b). A total of 178 OTUs were shared among the three treatments and 32, 7 and 19 OTUs were unique to GFI, GFN and GFM treatments, respectively. A

Table 1. Alpha diversity indices (± SE) for *Culex restuans* larvae and water samples from aquatic microcosms with and without *Cx. restuans* larvae. GFI, initial grass infusion, GFN, grass infusion without mosquito larvae, GFM, grass infusion with mosquito larvae.

Treatment	N	Shannon	Observed	Chao1	Coverage
GFI	10	3.74 ± 0.07^{ab}	184.90 ± 3.27^{ab}	214.37 ± 5.13^{a}	86.47 ± 1.47
GFN	9	3.90 ± 0.02^{a}	182.89 ± 2.98^{a}	216.91 ± 5.92^{a}	84.55 ± 1.28
GFM	10	3.70 ± 0.03^{b}	164.60 ± 1.85^{b}	205.63 ± 8.27^{a}	80.98 ± 2.66
Larvae	30	2.40 ± 0.02^{c}	82.60 ± 1.53^{c}	111.02 ± 2.83^{b}	75.19 ± 1.51

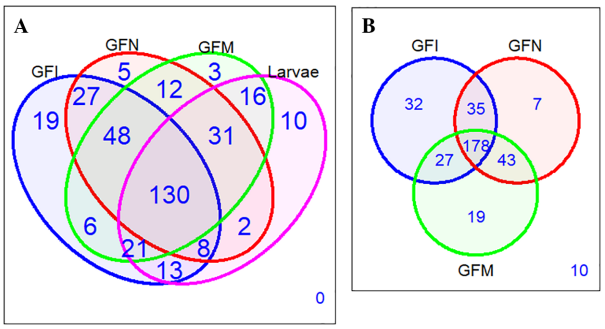

Figure 1. Venn diagram summarizing the overlap of bacterial OTUs among **(A)** all treatments, and **(B)** water samples. GFI, initial grass infusion, GFN, grass infusion without mosquito larvae, GFM, grass infusion with mosquito larvae.

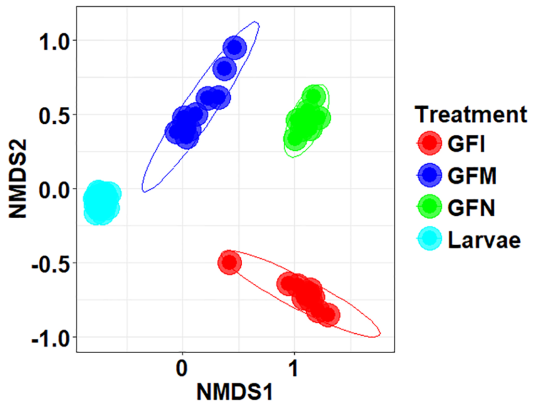

Figure 2. Non-metric multidimensional scaling (NMDS) ordination of Bray–Curtis distances between bacterial communities from different treatments. GFI, initial grass infusion, GFN, grass infusion without mosquito larvae, GFM, grass infusion with mosquito larvae.

total of 35 OTUs that were shared between GFI and GFN treatments were absent in GFM treatments and 43 OTUs that were shared between GFN and GFM treatments were absent in GFI treatment.

To determine the extent of similarity between microbial communities, beta diversity was calculated using NMDS ordination. The four sample types (GFI, GFN, GFM and larvae) had distinct bacterial communities based on NMDS ordination (Fig. 2, Table 2; NMDS stress = 0.10; PERMANOVA, $F = 269.2$, $P = 0.0001$). Mosquito larvae and GFN samples also had less individual variability in bacterial communities as indicated by smaller distances among these samples on the NMDS ordination compared to GFI and GFM samples.

The 351 OTUs detected in this study were assigned to nine phyla (Table 3). The majority of OTUs and sequences were from Proteobacteria and Firmicutes but some phyla that had

Table 2. PERMANOVA analysis results after Bonferroni correction for multiple comparisons. GFI, initial grass infusion, GFN, grass infusion without mosquito larvae, GFM, grass infusion with mosquito larvae.

Comparison	F	P values
Larvae vs GFI	546.7	0.0006
Larvae vs GFM	266.2	0.0006
Larvae vs GFN	633.3	0.0006
GFI vs GFM	109.2	0.0006
GFI vs GFN	107.9	0.0006
GFM vs GFN	74.22	0.0006

fewer number of OTUs such as Bacteroidetes and Verrucomicrobia also accounted for substantial number of sequences in some treatments. The top 18 genera accounted for 85.8% of the total sequences and their relative abundance varied markedly across the four sample types (Fig. 3). The relative abundances of *Clostridium* sp. and unclassified Clostridiaceae were highest in larval samples, intermediate in GFM samples and lowest in GFI and GFN samples. *Magnetospirillum* sp. and unclassified Enterobacteriaceae were rare in larval and GFM samples and more abundant in GFI samples compared to GFN samples. *Pseudomonas* sp., *Rheinheimera* sp. and *Azospirillum* sp. were rare in larval and GFI samples and more abundant in GFN samples compared to GFM samples. *Hydrogenophaga* sp. and *Prosthecobacter* sp. were rare in larval and GFI samples and more abundant in GFM samples compared to GFN samples. *Azohydromonas* sp. was rare in larval samples and more abundant in GFI and GFN samples compared to GFM samples. *Delftia* sp. was rare in larval and GFI samples, and more abundant in GFN samples compared to GFM samples. *Chryseobacterium* sp. was less abundant in GFN samples compared to the other treatments. Unclassified Clostridiales was more abundant in larval and GFI samples compared to GFN and GFM samples. *Zoogloea* sp. was absent in larval samples and more abundant in GFN samples compared to GFM and GFI samples. Unclassified Bacteroidales was absent in larval samples, rare in GFM samples and more abundant in GFI samples compared to GFN samples.

The microbial communities from the four treatments differed in their putative oxygen consumption (Table 4). GFI and larval samples had a significantly higher proportion of anaerobic bacteria compared to aerobic bacteria (GFI: 44.3 vs 8.8%; Larvae: 96.1 vs 2.6%). In contrast, the GFN and GFM samples had a higher proportion of aerobic bacteria compared to anaerobic bacteria (GFN: 58.9 vs 20.7%; GFM: 50.3 vs 37.5%).

Bacterial loads differed significantly among GFI, GFN and GFM treatments. The bacterial loads in GFI and GFN treatments were 6.3 ± 0.6 and 6.1 ± 0.7, respectively and significantly higher than 0.5 ± 0.1 ng for the GFM treatment ($F = 44.12$, $df = 2, 18$, $P < 0.0001$).

Table 3. Phylum-level classification of bacterial communities from the four treatments. GFI, initial grass infusion, GFN, grass infusion without mosquito larvae, GFM, grass infusion with mosquito larvae, REL, relative abundance, OTUs, operational taxonomic units.

	GFI		GFN		GFM		Larvae	
Phylum	#OTUs	REL	#OTUs	REL	#OTUs	REL	#OTUs	REL
Acidobacteria	2	0.21	2	0.08	2	0.01	0	0.00
Actinobacteria	3	0.06	3	0.04	5	0.07	6	0.13
Bacteroidetes	28	22.80	28	13.97	33	11.62	21	1.05
Cyanobacteria	1	0.02	1	0.00	1	0.00	1	0.01
Firmicutes	117	27.85	75	15.30	80	36.60	102	96.22
Planctomycetes	0	0.00	1	0.00	1	0.00	0	0.00
Proteobacteria	116	48.28	147	69.09	138	39.85	96	2.14
TM7	1	0.20	1	0.04	1	0.33	1	0.01
Verrucomicrobia	4	0.42	5	1.33	6	11.37	4	0.37
Total	272	100.00	263	100.00	267	100.00	231	100.00

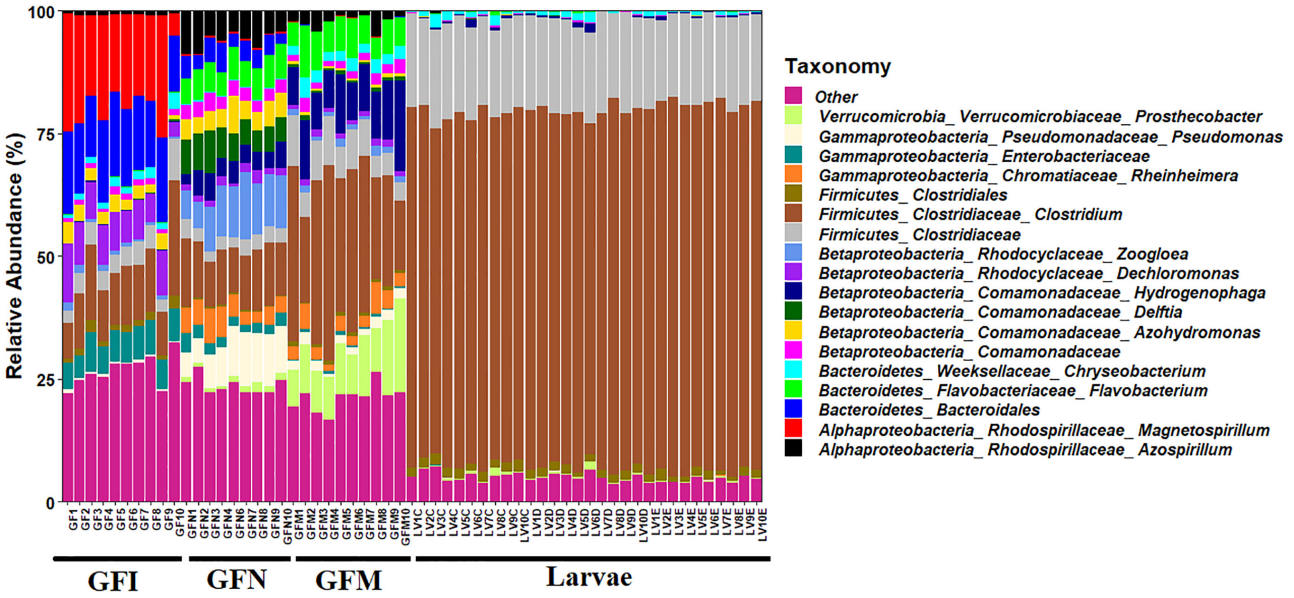

Figure 3. Relative abundances of bacterial taxa associated with different treatments. GFI, initial grass infusion, GFN, grass infusion without mosquito larvae, GFM, grass infusion with mosquito larvae.

Table 4. Oxygen requirements for bacterial communities detected in different treatments. GFI, initial grass infusion, GFN, grass infusion without mosquito larvae, GFM, grass infusion with mosquito larvae.

	Treatment			
	GFI	GFN	GFM	Larvae
Aerobic	8.8	58.9	50.3	2.6
Anaerobic	44.3	20.7	37.5	96.1
Facultative anaerobic	<0.01	1.7	1.1	<0.01
Unknown	46.7	18.7	11.2	1.3

DISCUSSION

In this study, we used experimental microcosms to investigate how the bacterial communities of container aquatic habitats respond to the presence and grazing by *Cx. restuans* larvae. Our results show that *Cx. restuans* larvae not only altered the water column bacterial composition, but also reduced the bacterial abundance, diversity and richness. These results suggest that *Cx. restuans* larvae play a major role in structuring their microbial food resources in container aquatic habitats. The findings enhance our understanding of the interactions between mosquito larvae and their microbial food resources in container aquatic habitats and present opportunities for further studies to identify the specific bacterial taxa that serve as food for mosquito larvae and to better understand the relative contribution of the larval environment as a source of mosquito microbiota.

Previous studies have shown that the presence of mosquito larvae can enhance, reduce or have little effect on the total bacterial abundance in the water column (Walker *et al.* 1991; Paradise and Dunson 1998; Kaufman *et al.* 1999). Our finding that *Cx. restuans* larvae reduced the total bacterial loads in the water column is consistent with previous findings that bacteria serve as a major food resource for mosquito larvae (Merritt, Dadd and Walker 1992).

The limited number of studies documenting the effect of mosquito larvae on microbial composition in container aquatic habitats have mostly focused on *Aedes triseriatus* (Kaufman *et al.* 1999; Kaufman *et al.* 2001; Kaufman, Chen and Walker

2008). In one of these studies, the presence of *Ae. triseriatus* larvae decreased the abundance of Pseudomonadaceae, consistent with our findings, and increased the abundance of Enterobacteriaceae, in contrast with our results (Kaufman *et al.* 1999). A separate study in tree holes revealed that *Ae. triseriatus* larvae suppressed members of Alphaproteobacteria and Bacteroidetes and enhanced the relative abundance of Betaproteobacteria (Kaufman, Chen and Walker 2008). In our study, *Cx. restuans* larvae had no significant effect on members of Alphaproteobacteria and suppressed the abundance of some members of Betaproteobacteria (e.g. *Azohydromonas*, *Zooglea* sp. and *Delftia* sp.) and Bacteroidetes (e.g. unclassified Bacteroidales) while enhancing the abundance of other members (e.g. *Hydrogenaphaga* sp. and *Chryseobacterium* sp.). Some members of Verrucomicrobia (*Prosthecobacter* sp.) and Firmicutes (*Clostridium* sp.) were also enhanced in the presence of *Cx. restuans* larvae, yet previous studies revealed they were not affected by the presence of *Ae. triseriatus* larvae. *Flavobacterium* sp. was not affected by the presence of *Cx. restuans* larvae although previous studies have demonstrated that this bacterial genus may be suppressed (Xu *et al.* 2008) or remain unaffected by *Ae. triseriatus* larvae (Kaufman, Chen and Walker 2008).

The differential effects of *Ae. triseriatus* and *Cx. restuans* larvae on microbial communities in their larval habitats may be due to differences in the feeding behavior of the two mosquito species which may expose them to different bacterial communities. *Culex restuans* larvae filter-feeds in the water column while *Ae. triseriatus* is known to split time between filter-feeding on the water column and feeding near or at the water surface (Merritt, Dadd and Walker 1992). The two mosquito species may also have different gut physiological conditions which may have variable effects on different bacterial taxa. The gut microbiota of mosquitoes is known to differ markedly between genera suggesting inter-genera differences in host gut physiologies (Mancini *et al.* 2018). Differences in methodologies between these studies may also have accounted for the observed differences. We focused on the water column bacterial communities while studies by Kaufman and colleagues focused on both water column- and leaf-associated bacteria. We also used grass infusion which is a high-quality food resource that enabled the mosquito larvae to mature quickly and thus shortening the duration of the experiment. In contrast, Kaufman and colleagues' studies used low-quality leaf litter that extended the duration of the study and may have contained different microbial taxa than were present in our grass infusion. Detritus from different plant species are known to host different microbial communities (Muturi, Orindi and Kim 2013; Gardner, Muturi and Allan 2018). Also, Kaufman and colleagues' studies used PCR-based cloning and sanger sequencing for bacterial detection, compared to the more powerful Miseq next generation sequencing that was used in our study. Thus, it is possible that our study may have detected some bacterial taxa that were not possible to detect using the methods employed in Kaufman and colleagues' studies.

Several factors may have accounted for the shifts in microbial composition and reduction in microbial diversity and richness in the presence of mosquito larvae. First, *Culex* larvae are known to filter-feed in the water column, and thus, the bacterial taxa thriving in the water column may have experienced more grazing pressure from the mosquito larvae compared to those thriving in other locations of the microcosms such as the bottom and air–water interface (Merritt, Dadd and Walker 1992). Thus, while containers without mosquitoes may have contained the entire bacterial community including those thriving in the water column, those with mosquitoes may have excluded the water-column bacteria through effects of larval feeding. However, we agitated the water samples in the microcosms prior to sample collection for microbial analysis and therefore, have no way of knowing the specific location of different microbial taxa before the containers were agitated. Second, some bacterial taxa are known to be more susceptible than others to digestion by mosquito larvae and other aquatic insects, and this may have substantial effect on bacterial richness, diversity and composition in container aquatic habitats (Sota and Kato 1994). For example, *Pseudomonas* sp. has been shown to be more easily digested by *Aedes* larvae and other aquatic invertebrates (Ladle and Hansford 1981; Sota and Kato 1994). *Aeromonas hydrophila* and *Citrobacter freundii* were also readily digested by larvae of freshwater mayfly, *Ephemera danica* while *Flavobacterium* sp. seemed to escape digestion via attachment to the hindgut wall (Austin and Baker 1988). Some bacterial taxa may also succumb to the redox potential and high pH in mosquito gut (Vallet-Gely, Lemaitre and Boccard 2008). Bacterial taxa that are readily susceptible to digestion and other conditions in the mosquito gut would be expected to decrease in response to mosquito feeding while those that are resistant to these conditions would either increase or remain unchanged in the presence of mosquito larvae. Thus, it is possible that the bacterial taxa that were suppressed in the presence of mosquito larvae such as *Azohydromonas* sp., *Delftia* sp., *Pseudomonas* sp. and *Zooglea* sp. were more susceptible to at least one of the above conditions in mosquito gut while those that were enhanced such as *Prosthecobacter* sp., *Hydrogenaphaga* sp., *Clostridium* sp. and *Chryseobacterium* sp. were more resistant to these conditions. The suppression of *Pseudomonas* sp. by *Cx. restuans* larvae is consistent with previous studies with *Aedes* mosquitoes (Sota and Kato 1994) while *Clostridium* sp. is known to be alkaline-tolerant (Engel and Moran 2013) and is therefore likely to survive ingestion by mosquito larvae and recolonize the aquatic habitats. Little is known about the fate of the other bacterial taxa following ingestion by mosquito larvae or other invertebrates and further studies are needed on this topic.

A third mechanism that may have potentially caused a shift in bacterial composition and diversity in our experimental microcosms is the selective effect of mosquito larvae on bacterial taxa indirectly through release of nitrogenous wastes that were utilized for growth or by consuming the protozoan communities that feed on bacteria and thus releasing some bacterial taxa from grazing pressure from protozoa. Bacteria are an important prey for protozoans (Fenchel 1987) and previous studies have demonstrated that larvae of some mosquito species feed on protozoans (Washburn *et al.* 1988; Paradise and Dunson 1998). We did not characterize the protozoan communities in our water samples and future studies focusing on the diverse microbial communities present in container aquatic habitats including bacteria, fungi and protozoa could be more revealing. Finally, our results revealed substantial differences in the proportion of aerobic and anaerobic bacteria in aquatic microcosms with and without mosquitoes. The stock grass infusion had a significantly higher proportion of anaerobic bacteria (44.3%) compared to aerobic bacteria (8.8%) which is not surprising because the infusion was fermented in an air-tight plastic container. In contrast, the proportion of aerobic bacteria were higher than those of anaerobic bacteria in GFN (without mosquitoes) and GFM (with mosquitoes) treatments. The ratio of aerobic bacteria to anaerobic bacteria was much higher in experimental microcosms without mosquito larvae (2.8) compared to those with mosquito larvae (1.3). These findings suggest that the presence

of mosquito larvae was associated with oxygen conditions that favored the growth of both aerobic and anaerobic bacteria while the absence of mosquito larvae resulted in oxygen levels that mostly favored proliferation of aerobic bacteria. Previous studies have reported an inverse relationship between dissolved oxygen and abundance of *Anopheles* and *Culex* larvae (Sunish and Reuben 2001; Muturi *et al*. 2007) suggesting that mosquito larvae likely contribute to oxygen reduction in their larval habitats. It is also possible that other chemical characteristics of the aquatic microcosms such as pH, conductivity and salinity shifted over time and in the presence of mosquito larvae leading to changes in bacterial communities.

Significantly low microbial diversity in mosquito larvae relative to water samples from their larval habitats has been reported previously (Wang *et al*. 2011; Duguma *et al*. 2013; Dada *et al*. 2014) and confirm previous findings that only a subset of bacteria from the aquatic habitat can colonize and persist in mosquito larvae. Firmicutes accounted for 96.2% of larval microbiota with family Clostridiaceae primarily *Clostridium* sp. forming the bulk of the sequences. Phylum Firmicutes is commonly reported in mosquito larvae, but not in as high relative abundance as reported in this study. Dada *et al*. 2014 reported Firmicutes to be the most abundant bacterial phyla in *Aedes aegypti* larvae accounting for 52% of the total sequences but accounted for only 2% of the total sequences in water samples from their larval habitats. In *Cx. tarsalis* larvae, one study found Firmicutes to comprise only 0.2% of the total sequences (Duguma *et al*. 2013) while another study by the same research group found Firmicutes to account for 6.0% of the total sequences (Duguma *et al*. 2015). The former study also showed that Firmicutes accounted for 0.01% of the total bacterial sequences in the water samples from their larval habitats while the latter study did not characterize the bacterial communities in the water samples. Firmicutes including members of class Clostridia were dominant in *Cx. nigripalpus* larvae collected throughout the study period covering summer, autumn and winter, but in *Cx. coronator*, they were only abundant in samples collected during winter (Duguma *et al*. 2017). However, this study did not characterize the bacterial communities of water samples from *Cx. nigripalpus* and *Cx. coronator* larval habitats. In the context of these findings, we speculate that *Cx. restuans* larvae are highly selective for members of family Clostridiaceae (phylum Firmicutes). The high abundance of Clostridiaceae in our water samples (39.2%) compared to 0.01–2.0% reported in water samples from previous studies may have facilitated their rapid colonization of mosquito tissues. It is also possible that some of the Clostridiaceae were acquired transstadially from the egg stage although we did not characterize the bacterial communities of 1st instar larvae before adding them into the microcosms.

Our results provide important insight into how larvae of *Cx. restuans* and other mosquito species with similar feeding behaviors may influence the bacterial communities in container aquatic habitats. However, we recognize that the simulated habitats used in our study may not be an accurate representation of the ecological interactions that occur under field conditions. In nature, larvae of multiple mosquito species co-occur in the same container aquatic habitats and rely on detritus from the surrounding vegetation as the main source of energy. Different detritus types affect the quantity and quality of food available for mosquito larvae by supporting different microbial composition and abundance (Gardner, Muturi and Allan 2018; Murrell and Juliano 2008; Muturi, Orindi and Kim 2013). Different container aquatic habitats may receive detritus from different plant species depending on their location and may therefore differ markedly in their nutritional quantity and quality. Bacterial communities associated with detritus form the bulk of mosquito larval food, although larvae of some mosquito species may also prey on protozoa and fungi (Walker, Olds and Merritt 1988; Merritt, Dadd and Walker 1992; Paradise and Danson 1998). These microorganisms may shift over time even in the absence of mosquito larvae and other macroinvertebrates, due to other factors that were not tested in this study such as intra- and inter-specific competition and changes in abiotic conditions of the container habitats. This fact coupled with the findings that some protozoan species prey on bacteria (Fenchel 1987; Duguma, Kaufman and Simas Domingos 2017) and that different mosquito species exhibit different feeding behaviors such as surface-feeding, suspension feeding and submerged feeding (Merritt, Dadd and Walker 1992), the interaction of mosquito larvae and microbial communities in natural container aquatic habitats is expected to be more complex than observed in the current study. Therefore, field studies incorporating both temporal and spatial dynamics and targeting larvae of multiple container-inhabiting mosquito species, their predators and diverse microbial communities including bacteria, fungi and protozoa are needed to facilitate comprehensive understanding of the trophic dynamics in container aquatic habitats.

In summary, this study demonstrates that mosquito larvae not only alter the microbial composition, abundance and diversity in container aquatic habitats, but also play an active role in selecting the microbial communities that colonize their bodies. Changes in aquatic microbial communities mediated by mosquito larvae may affect the quality and quantity of microbial food resources available for mosquito larvae and other container-dwelling invertebrate consumers and impact the decomposition process and food web dynamics in these systems. The bacterial taxa shown to respond to the presence of mosquito larvae should be investigated further to identify those taxa that are consumed by mosquito larvae. Further studies are also needed to identify which of the bacterial taxa identified in *Cx. restuans* larvae could be harnessed for control of *Culex*-borne diseases such as West Nile virus.

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