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Analysis of Gut Microbiota from Aedes albopictus Mosquitoes Collected in Central Illinois

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Analysis of Gut Microbiota from Aedes *albopictus* mosquitoes collected in Central Illinois

by

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ABSTRACT

Aedes albopictus, Asian tiger mosquitoes, are vectors of a wide number of human viral diseases including, West Nile virus, Dengue virus, Chikungunya virus and (recently) Zika virus. A large body of evidence has suggested that microbiomes of mosquito midguts are closely associated with specific mosquito life processes such as nutrition, reproduction, aggregation and defense against toxins. In this study we characterize the bacterial flora of the midguts of adult female *Ae. albopictus* collected from woodlots and residential areas in Champaign and Coles Counties of Central Illinois (40 samples in each category). After extraction of DNA from dissected midguts, we used next generation sequencing (MiSeq V3) to obtain sequences spanning the V4 hypervariable region of the 16S rRNA gene. The bacterial sequences were analyzed with QIIME. After quality filtering and rarefying, we identified 551 operational taxonomic units (OTUs) from 114 samples. Of the top 30 most abundant OTUs, 31 genera were discovered in 22 families. According to an indicator species analysis, in Champaign County *Pseudomonas* (50%) and *Sediminibacterium* (63.5%) characterized the midguts of *Ae. albopictus* collected from residential areas and woodlots, respectively. For Coles County, the midguts of *Ae. albopictus* from residential area were well characterized by the OTU for *Bradyrhizobiaceae* (49.3%), and by *Janthinobacterium* (51.2%) for woodlots. In general, the composition of bacterial communities differed between both trapping locations and land use types, with some overlap occurring in the residential sectors. In contrast, alpha-diversity measures were largely similar across locality, but differed between land use types, with greater species richness (Chao1), heterogeneity (Shannon Index) and equitability in the midguts of mosquitoes collected from

wooded areas. In conclusion, the midgut bacterial community composition and diversity of *Ae. albopictus* varies by land use and location. Further studies on whether and how such differences in midgut biota influence variation in vectorial capacity traits are warranted.

ACKNOWLEDGMENTS

I would like to thank the Medical Entomology Lab at University of Illinois, Urbana-Champaign, for their devote efforts to this project, as well as Dr. Chang-Hyun Kim and Dr. Christopher Stone for their guidance, as well as Dr. Thomas Canam for his contributions. I'd also like to thank my parents and extended family, as well as my partner, for their support during the long journey I have taken through my education, as well as the Department of Biological Sciences and Eastern Illinois University for gifting me an opportunity I couldn't have imagined.

I would like to thank, above anyone, Dr. Antony Oluoch who has become far more than just a mentor for this project, but a confidant, friend, and a guide through the entirety of my undergraduate career. I will always cherish the help you have given me.

Lastly, I would like to thank every mosquito that made this project possible.

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INTRODUCTION

Mosquitoes of the species *Aedes albopictus* (Figure 1) are vectors of a wide number of human viral diseases including West Nile virus (1), Dengue virus (2), Chikungunya virus (3) and (recently) Zika virus (4, 5). Mosquito midguts play a central role in pathogen transmission. After a blood meal from an infected host, there occurs crucial intermediate development and replication within the midgut before eventual dissemination into secondary sites such as salivary glands (6). The midgut can modify and impede vector transmission via the influence of digestive enzymes and innate immunity (7, 8).

Also present in the mosquito midgut are bacteria of various species. The origin of midgut bacteria and the determinants of subsequent colonization are variable. Immature mosquitoes may acquire bacteria through their habitats (17), while blood feeding adults may acquire bacteria via their blood meal (from host skin, for example) (18). Finally, there is evidence for trans-stadial acquisition of these midgut microbiota (19).

Recently, a large body of evidence has suggested that the midgut bacterial composition can vary within and between mosquito species, sex, stage of development, and habitat (9-11, 14). These bacteria can exist in a symbiotic relationship with host mosquitoes. In such instances, they could function in the synthesis of essential nutrients absent in food sources (15). On the other hand, these bacteria can be directly pathogenic to their host mosquitoes (16). Finally, such bacteria can affect vector susceptibility to antigen through multiple mechanisms, and thereby influence transmission efficiency (20). For example, the bacterium *Wolbachia pipientis* induces resistance in *Ae. albopictus* and *Ae. aegypti to* Chikungunya and Dengue

viruses respectively (21, 22). Such observations have spurred interest in the possibility of using these microbiota in pathogen and mosquito control strategies.

Recent development of metagenomic PCR and next generation sequencing have fueled wider and more accurate exploration of these organisms as a supplement to culture-dependent techniques traditionally used previously (20). While many such studies have been carried out in other medically important mosquito species in the *Culex* and *Anopheles* genera (23), our understanding of the midgut bacterial flora specifically involving *Ae. albopictus* is limited. Yet recent outbreaks of Zika virus disease transmitted by this species of mosquitoes are an indication of its significance in human health and disease (24).

Using high throughput MiSeq® sequencing of 16s rRNA, the main objective of this study was to characterize the microbial diversity and composition of the midguts of *Ae. albopictus* collected from various locations and land use types in Champaign and Coles Counties, Illinois.

MATERIALS AND METHODS

Mosquito collection

Between May 20 and October 14, 2017, mosquitoes were collected from multiple geographical locations in both Champaign and Coles counties of central Illinois (Figures 2 and 3) using standard CDC light traps baited with dry ice (Figure 4). These locations were grouped into wooded and residential land use types. Specimens were transported to the lab and identified to species level using standard unique morphological characteristics on the dorsum of the head, abdomen and legs (13). After morphologic identification, all adult female *Ae. albopictus* were stored at -80°C until further processing

DNA extraction and 16S rRNA gene library preparation

One hundred and sixty (160) adult female *Ae. albopictus* were randomly selected from each of 2 land use types (residential and woodlot) in both Charleston and Champaign (Table 1). The mosquitoes were sterilized as previously described by Muturi *et al*. (12). Each was rinsed three times in sterile water and surface disinfected in 70 % ethanol for 5 min, followed by a 5-minute wash in sterile Dulbecco's phosphate buffered saline (DPBS) solution (ThermoFisher, Waltham, MA), then a 5-cycle rinse (each cycle being 3 minutes) in sterile DPBS. Each surface-cleaned *Ae. albopictus* was placed in a drop of sterile DPBS under a stereo dissecting microscope and the midgut was removed. Midguts were individually suspended in 1.5-mL microcentrifuge tubes containing 50 µL RNAlater solution (ThermoFisher, Waltham, MA) and stored at -80 °C until DNA isolation. Total DNA from each midgut was extracted using DNeasy Blood & Tissue kit

(Qiagen, Valencia, CA) following manufacturer's recommendation except that the volume of final elution buffer was 50 µL. DNA was quantified using an Epoch spectrophotometer (BioTek, Winooski, VT) and its quality assessed using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). For bacterial characterization, we targeted the V4 hypervariable region of the 16S rRNA gene (292 base pairs) using the following primer set: forward 5`-GTGYCAGCMGCCGCGGTAA-3` and reverse 5`-GGACTACNVGGGTWTCTAAT-3`.

Library preparation and sequencing were conducted at the W. M. Keck Center for Comparative and Functional Genomics at the University of Illinois at Urbana-Champaign. DNA of each mosquito sample was amplified using the above primer set on the Fluidigm® microfluidics quantitative PCR platform with appropriate linkers and sample barcodes. The final Fluidigm® libraries were pooled in equimolar ratio for sequencing. The final denatured library pool was spiked with 10% non-indexed PhiX control library (Illumina®) and sequenced by 2 × 300 nt paired-end sequencing on the Illumina® MiSeq® V3 Bulk system. The PhiX control library provides a balanced genome for calculation of matrix, phasing and pre-phasing, which are essential for accurate base-calling. The libraries were sequenced from both ends of the molecules to a total read length of 300 nt from each end. Cluster density was 964 k/mm^2 with 85.9 % of clusters passing filter. We present the results for the forward reads only because reverse reads, which are not independent from the forward reads, showed the same patterns among samples. This is expected because single-end reads are known to be sufficient to observe the same relationships among samples that are revealed with paired-end reads.

Illumina OTU analysis and statistics

The QIIME version 1.9.0 pipeline was used to demultiplex and quality filter the forward and reverse reads using defaults. Barcodes and primer sequences were removed using Fastx toolkit operated in QIIME. The operational taxonomic units (OTUs) were chosen and identified at 97 % similarity using *de novo* OTU picking process. OTUs accounting for <0.005 % of the total sequences were discarded to reduce the problem of spurious OTUs that may result from random sequencing errors and are likely to overestimate the overall diversity. Due to variations in the number of sequences between samples (range 1 - 48,629 sequences), read depth was rarefied to 3022 reads per sample to retain adequate samples for statistical analysis and to standardize the sampling effort (Table 1). Alpha diversity metrics including Shannon diversity index, observed species, chao1, and evenness were generated in QIIME; analysis of variance (ANOVA) with Tukey adjustments was used to test the effect of site and mosquito species on these indices using the **R** statistical package. Bacterial communities were visualized using non-metric multi-dimensional scaling (NMDS) plots generated using vegan package in **R**. Non-parametric multivariate community analyses including indicator species analysis and Multi-Response Permutation Procedures (MRPP) were conducted using PC-ORD version 6.08. MRPP was used to test for differences in microbial communities between study sites while indicator species analysis was used to identify bacterial species that are strongly associated with land use types and locations. Indicator values range from **0** to **100%**, with a value of **100%** indicating that the species occurs in all samples of a treatment and are specific to those samples.

RESULTS AND DISCUSSION

Bacterial composition

Out of 160 samples dissected and processed, 114 samples remained after the quality filtering and rarefaction process (Table 1). These samples accounted for 551 OTUs. The top 30 most abundant OTUs, were definitively classified into 5 phyla (Figure 5) and 22 families (Figure 6) from which 31 genera were discovered (Figure 7). There were unclassified OTUs at each of these taxonomic levels. The phylum *Proteobacteria* was the most dominant in woodlots and residences in both Champaign and Charleston locations. The highest OTUs were derived from the family *Rickettsiaceae* in Champaign (38.5 %) and Charleston (44.2%) residences, as well as in Charleston woodlot (16.42%). However, in Champaign woodlots, the highest OTUs were derived from the family *Enterobacteriaceae* (15.9%). Indicator Species Analysis (ISA) was used to characterize the bacterial OTUs that were strongly associated with the midgut of *Ae. albopictus* from one location and land type over the other. One indicator species was identified for midgut of *Ae. albopictus* from each of land use type in Champaign: *Pseudomonas* (50%) from residential areas and *Sediminibacterium* (63.5%) from woodlots (Table 2). Three and 8 indicator bacterial species were identified for residential and woodlots in Charleston, respectively. The bacterial OTU of *Bradyrhizobiaceae* (49.3%) characterized residential area well, while *Janthinobacterium* (51.2%) did for woodlot in Charleston (Table 2). Only one indicator species identified for woodlots in Champaign had an indicator value greater than 60% (Table 2).

Bacterial diversity and richness

Bacterial species richness for Charleston was significantly greater than that of Champaign, particularly in the Woodlot areas (Figure 8; Table 3). The midgut of *Ae. ablopictus* from woodlots in Charleston was found to have the most diverse bacterial community amongst all 4 treatments. This observation was interesting because the Champaign-Urbana metropolitan area, with a population nearing 240,000, was expected to have a more diverse bacterial community, possibly due to humans and their activities providing a greater source of bacterial variety. However, Charleston, a much smaller town with approximately 22,000 people, showed a greater variety of fauna in this ecological zone than Champaign. Charleston has a woodier and more forested area than Champaign. In addition, the Embarras River may provide additional habitats and niches in which a greater variety of both flora and fauna may thrive. The midgut samples from woodlot in Champaign were more diverse than either residential treatment but did not show enough variation amongst species to be more diverse than the Charleston woodlots treatment. These findings suggest that bacterial diversity may be positively correlated with the diversity and abundance of vegetation surrounding mosquito habitats rather than anthropogenic factors. Furthermore, some human activities such as gardening and urbanizing may decrease diversity and abundance of plant species and consequently contribute to a reduction of bacterial diversity in mosquitoes from residential areas.

Composition of bacterial communities between Woodland and Residential areas

The composition of bacterial communities differed between trapping locations (Woodland or Residential), with some overlap occurring in the residential sectors (Table 4; Figure 9). According to the NMDS analysis based on occupancy data, bacterial communities in each category were different with the most dissimilar amongst Charleston woodlot and Charleston Residential communities (Figure 9 upper panel; Table 4). An additional NMDS analysis based on abundance of bacterial OTUs also agrees with the results from NMDS analysis of occupancy data (Figure 9 lower panel; Table 4).

It is interesting that Charleston woodlot and Charleston residence show the most separation in their bacterial communities when they are in the same geographical location indicating the role of vegetation rather than anthropogenic factors on bacterial diversity. Furthermore, Charleston residence is very similar to Champaign residence although they are miles apart. It is possible that the human population plays a large part in this, as humans share much of the same bacteria between each other, making residential areas similar in microbial diversity. A study by Grice et al. (25) of twenty skin sites on each of ten healthy humans found 205 identified genera in nineteen bacterial phyla, with most sequences assigned to four phyla: *Actinobacteria* (51.8%), *Firmicutes* (24.4%), *Proteobacteria* (16.5%), and *Bacteriodetes* (6.3%). These findings are in close agreement with our observations of the mosquito midguts where we found OTUs mapping to the same phyla (plus *Cyanobacteria*). Additionally, the woodiness and composition of Charleston forests must be a contributing factor in the diverse community

retained in the Charleston woodlots as the Champaign woodlot shows much more overlap and similarity with other bacterial communities of a different treatment than the Charleston woodlot (Figure 9).

Many of these genera could be commonly expected when their habitats are taken into account. *Sediminibacterium*, for example, is a genus that is associated with ground-water. We detected the OTU of the bacteria from all sampling locations and land use types with the highest proportion (9%) in Champaign woodlot (Figure 7). This would be expected in mosquitoes because they lay their eggs within water sources, especially those found in the woodlot. *Pseudomonas* is a common bacterial strand found on human skin, this makes sense considering it was prominent (13%) in Champaign residence (Figure 7). This genus has also been found to emit an unattractive odor that mosquitoes serving as malaria vectors dislike (26). The 16S rRNA sequence of *Bradyrhizobiaceae* has been analyzed and was found to have great affiliation with organisms from different environments such as soil, plant, or animal hosts (27). It is a key component in nitrogen fixation, being found in a wooded area seems very common because of its ecological role and host preference (27). It is interesting that this genus was found (5%) in Charleston residence (Figure 7), but this could be due to shrubs and various plants or trees throughout the city. Charleston is also a rural area with a large farming community, as *Bradyrhizobiaceae* is commonly found on soybean plants, these bacteria could be brought in from surrounding fields.

Enterobacteriaceae were one of the most abundant and common genera throughout the sample locations (Figure 7). *Enterobacteriaceae* are a normal part of the gut flora found in the

intestines of humans and other animals, while others are found in water or soil, or are parasites on a variety of different animals and plants. The abundance of *Enterobacteriaceae* in the mosquito midgut has been found to be correlated significantly with the *Plasmodium* infection status with regards to malaria transmission (28).

Wolbachia was another genus that was most abundant across all sampling locations (Figure 7). *Wolbachia* are a widely studied group of bacteria in relation to mosquitoes. They have been found not only to halt the transmission of Zika virus (29) but also to reduce the blood-feeding success in mosquitoes that serve as vectors for Dengue Fever (30).

These predominant bacterial genera within the midguts of *Ae. albopictus* from various habitats lend further knowledge to the public from both a health and ecological standpoint. Ultimately, understanding the relationship between midgut microbiota and their mosquito hosts would lead to developing disease control measures that are ecologically and environmentally sound.

CONCLUSION

This study is the first description and comparison of microbial communities associated with *Ae. albopictus* in wooded and residential habitats in two distinct communities of central Illinois. To our knowledge, this is also the first such study involving *Ae. albopictus.* We have observed that bacterial species richness for Charleston was greater than that of Champaign, particularly in the woodlot areas. The Charleston woodlots presented the highest amount of species richness, heterogeneity, and evenness amongst all trapping locations. We conclude that the composition and diversity of bacterial communities differed based on land use type and location. Further studies on whether such differences in midgut biota affect disease transmission by mosquito vectors are warranted.

TABLES AND FIGURES

Table 1. The size of samples that were utilized for midgut dissection and statistical analysis.

City	Land use types	Dissected	Analysis
Champaign	Residential	40	25
	Woodlot	40	28
Charleston	Residential	40	28
	Woodlot	40	33

Table 2. Bacterial OTUs that characterize locations and land use types.

City	Land use type	OTU ID	Phylum[1]	Class[2]	Species/Other[3]	IV[4]	*p*
Champaign	Residential	581211	P	G	*Pseudomonas*	50	0.001
	Woodlot	332283	B	S	*Sediminibacterium*	63.5	0.001
Charleston	Residential	220528	P	Al	*Bradyrhizobiaceae*	49.3	0.001
		679879	P	Al	*Wolbachia*	42.4	0.001
		1124258	Un	Un	*Unassigned*	41.3	0.002
	Woodlot	353404	P	Bt	*Janthinobacterium*	51.2	0.001
		156446	P	G	*Acinetobacter*	49.4	0.001
		456174	P	Bt	*Oxalobacteraceae*	49.4	0.001
		250282	Ac	Ac	*Corynebacterium*	47.9	0.001
		1080486	P	Bt	*Polaromonas*	45.5	0.001
		878622	P	G	*Acinetobacter lwoffii*	42.4	0.001
		452119	P	Bt	*Comamonadaceae*	41.8	0.001
		996306	F	Ba	*Bacillus*	41.1	0.001

[1] Phyla abbreviations: Ac = Actinobacteria, B = Bacteroidetes, F = Firmicutes, P = Proteobacteria, Un = Unassigned

[2] Class abbreviations: Al = Alphaproteobacteria, Ac = Actinobacteria, Ba = Bacilli, Bt = Betaproteobacteria, G = Gammaproteobacteria, S = Saprospirae, Un = Unassigned

[3] The lowest classification based on de novo OTUs

[4] **IV** = indicator value, computed by Indicator Species Analysis

Table 3. Statistics showing significant differences in diversity/richness measures between land use types and a significant interaction between land use and locality for equitability. Asterisks indicate significant differences at 95% confidence level and above.

	Coefficients	Estimate Std.	Error	z value	Pr(>|z|)
Chao1	(Intercept)	2.79870	0.08978	31.172	< 2e-16 ***
	City_Charleston	0.19970	0.12192	1.638	0.101
	Landuse_Woodlot	0.65778	0.11921	5.518	3.43e-08 ***
	City_Charleston : Landuse_Woodlot	0.11157	0.16117	0.692	0.489
Shannon	(Intercept)	0.52471	0.15385	3.411	0.000648***
	City_Charleston	-0.09917	0.21681	-0.457	0.647365
	Landuse_Woodlot	0.40657	0.19427	2.093	0.036371*
	City_Charleston : Landuse_Woodlot	0.48452	0.26306	1.842	0.065497
Equitability	(Intercept)	0.47283	0.03449	13.711	< 2e-16 ***
	City_Charleston	-0.03894	0.04745	-0.821	0.41352
	Landuse_Woodlot	0.10312	0.04745	2.173	0.03189*
	City_Charleston : Landuse_Woodlot	0.17969	0.06491	2.768	0.00662**

Table 4. Multi-Response Permutation Procedures (MRPP) analysis.

Comparison	Ad	N	T	A	*p*
Residential in Champaign vs. Woodlot in Champaign	0.42 vs. 0.49	25 vs. 28	-7.19	0.09	< 0.05
Residential in Champaign vs. Residential in Charleston	0.42 vs. 0.40	25 vs. 28	-5.19	0.05	< 0.05
Residential in Champaign vs. Woodlot in Charleston	0.42 vs. 0.36	25 vs. 33	-12.05	0.13	< 0.05
Woodlot in Champaign vs. Residential in Charleston	0.49 vs. 0.40	28 vs. 28	-10.36	0.12	< 0.05
Woodlot in Champaign vs. Woodlot in Charleston	0.49 vs. 0.36	28 vs. 33	-10.19	0.11	< 0.05
Residential in Charleston vs. Woodlot in Charleston	0.40 vs. 0.36	28 vs. 33	-13.46	0.15	< 0.05

Ad = average within group distances for the mosquito species

N = sample size.

T = test statistic describing separation between groups.

A = chance-corrected within group agreement as log10

Figure 1. Female adult mosquito of the species *Aedes albopictus*. Distinct morphologic characteristics of the head, thorax, abdomen and legs were used to identify these mosquitoes.

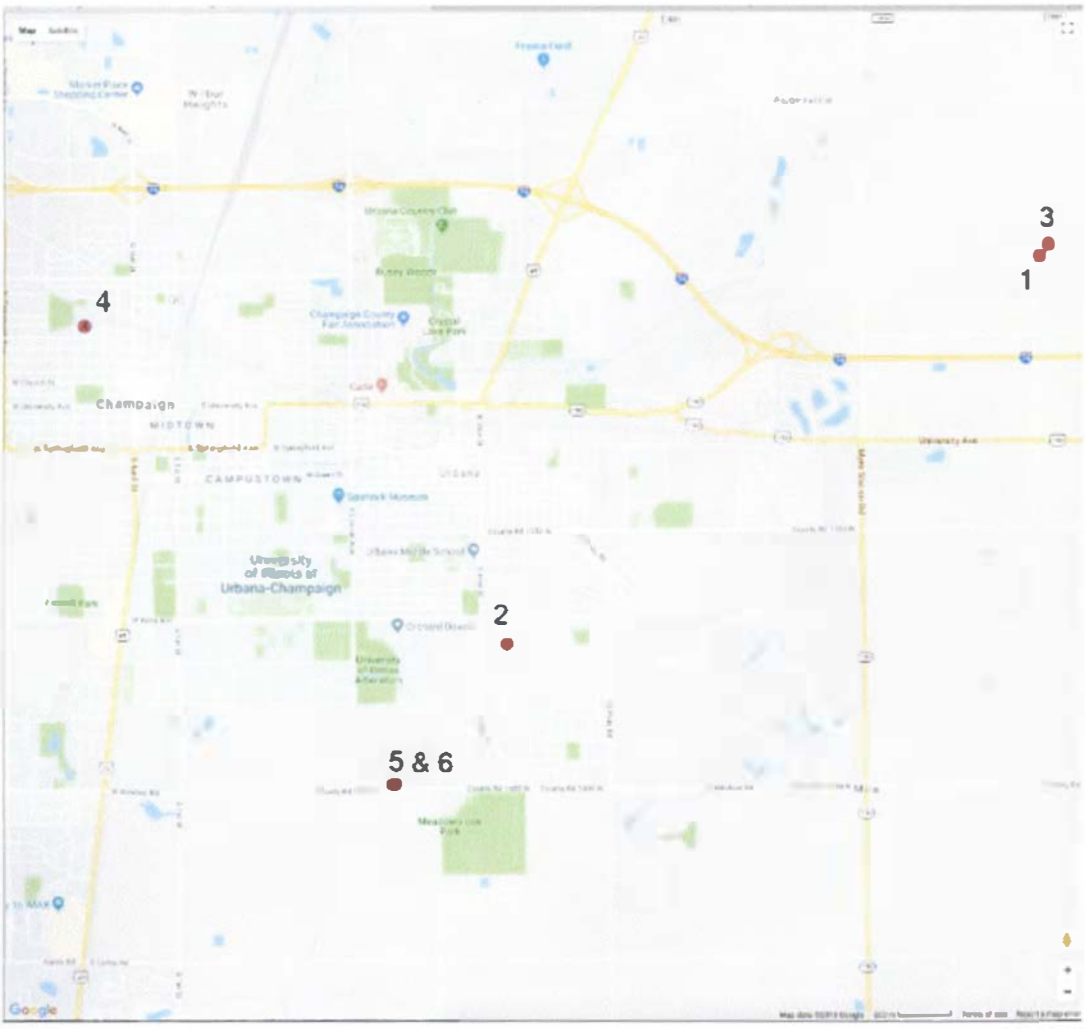

Figure 2. Geographical location of mosquito traps in Champaign County. These are derived from the following latitudes and longitudes:

1) 40.129	-88.143
2) 40.096	-88.202
3) 40.13	-88.142
4) 40.123	-88.249
5) 40.084	-88.214667
6) 40.084	-88.214262

Figure 3. Geographical location of mosquito traps in Coles County. These are derived from the following latitudes and longitudes:

1) 39.46	-88.163
2a) 39.49	-88.168
2b) 39.475	-88.175
3a) 39.486	-88.173
3b) 39.477	-88.175
4) 39.472	-88.172
5) 39.492	-88.167
6) 39.4761	-88.189

Figure 4. CDC light trap for collection of mosquitoes. The canister (left) was filled with dry ice which attracted mosquitoes into the motor driven trap (right). Traps were laid out between 15:00 and 09:00 hours.

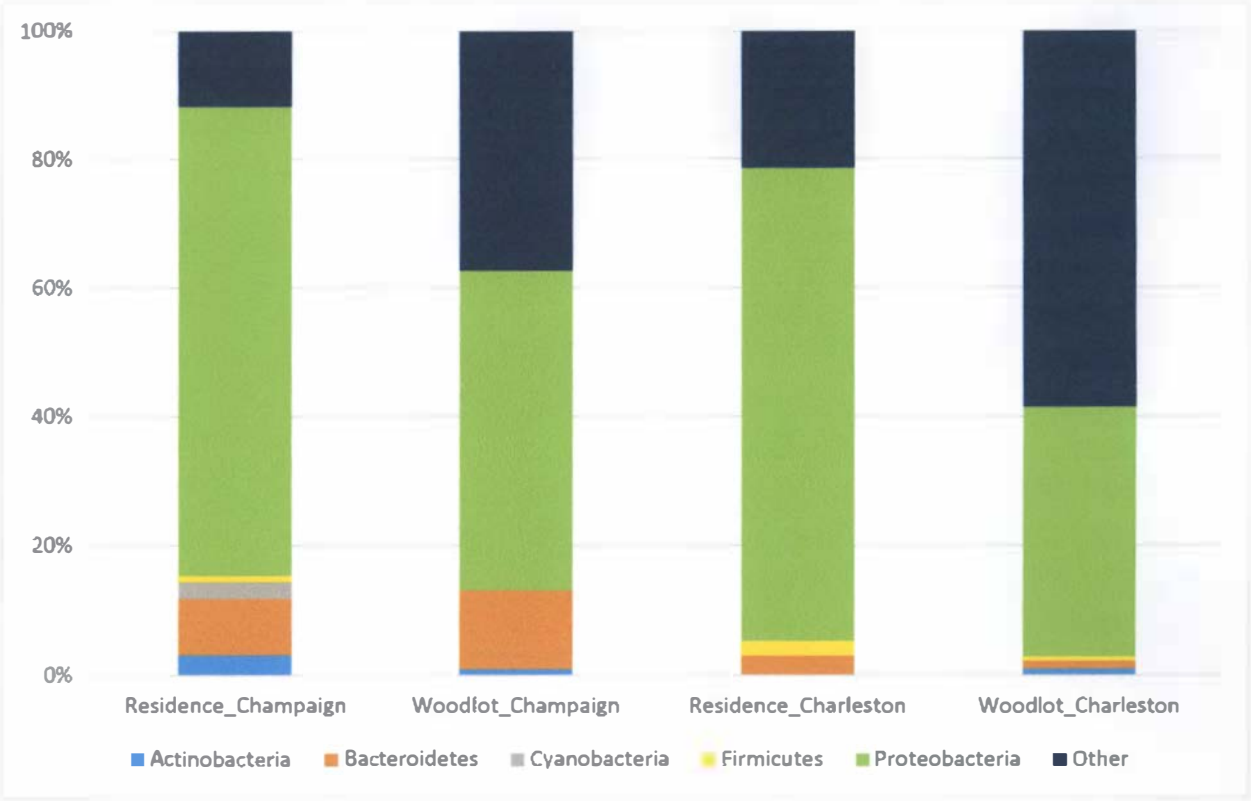

Figure 5. OTU abundance at phylum level for bacterial communities in *Aedes albopictus* amongst the 4 treatments (These were: Charleston woodlot and residential as well as Champaign woodlot and residential).

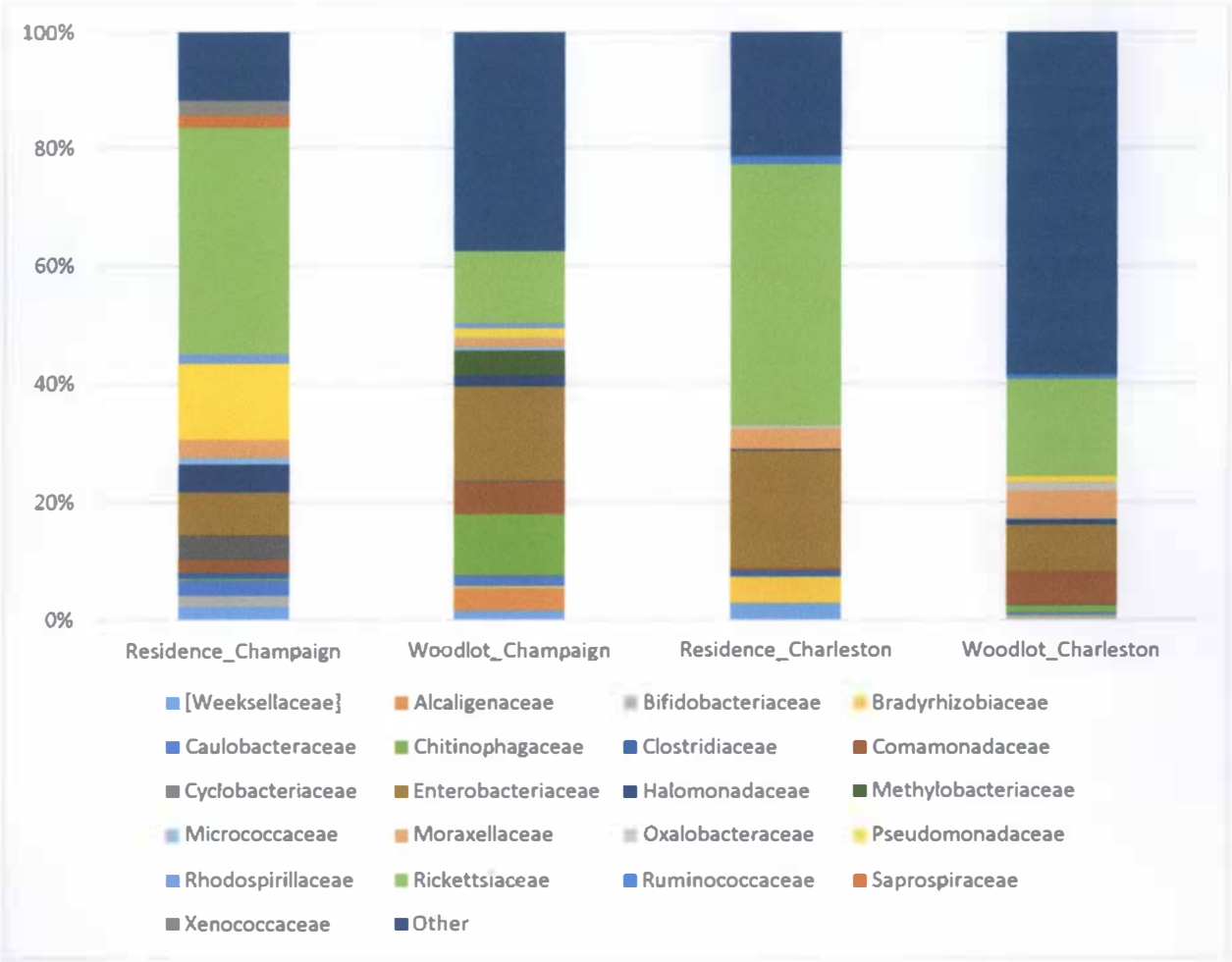

Figure 6. OTU abundance at family level for bacterial communities in *Aedes albopictus* amongst the 4 treatments (These were: Charleston woodlot and residential as well as Champaign woodlot and residential).

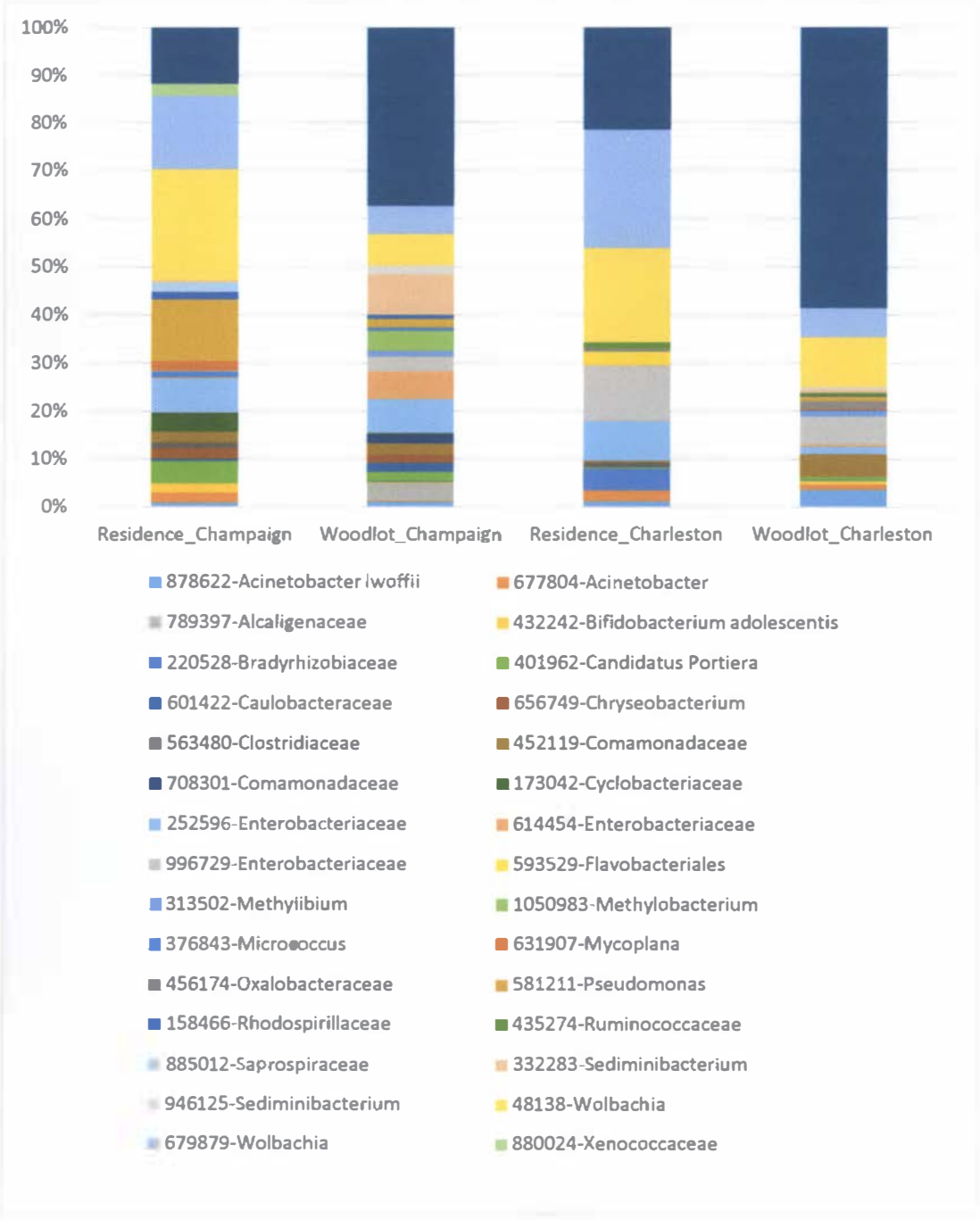

Figure 7. OTU abundance at genus level for bacterial communities in *Aedes albopictus* amongst the 4 treatments (These were: Charleston woodlot and residential as well as Champaign woodlot and residential).

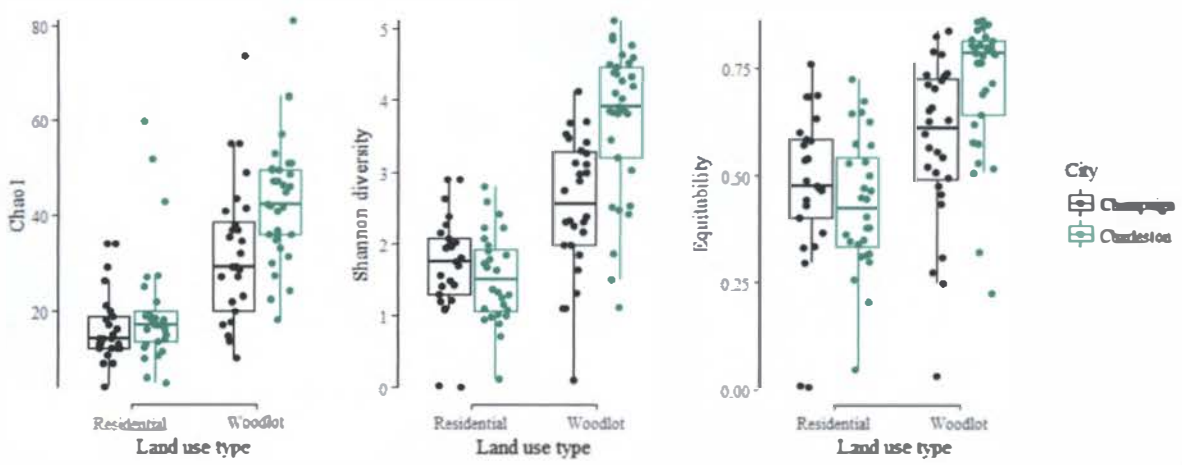

Figure 8. Estimates of species richness (Chao1), heterogeneity (Shannon Index), and evenness for *Aedes albopictus* microbiota populations from different localities and land use types.

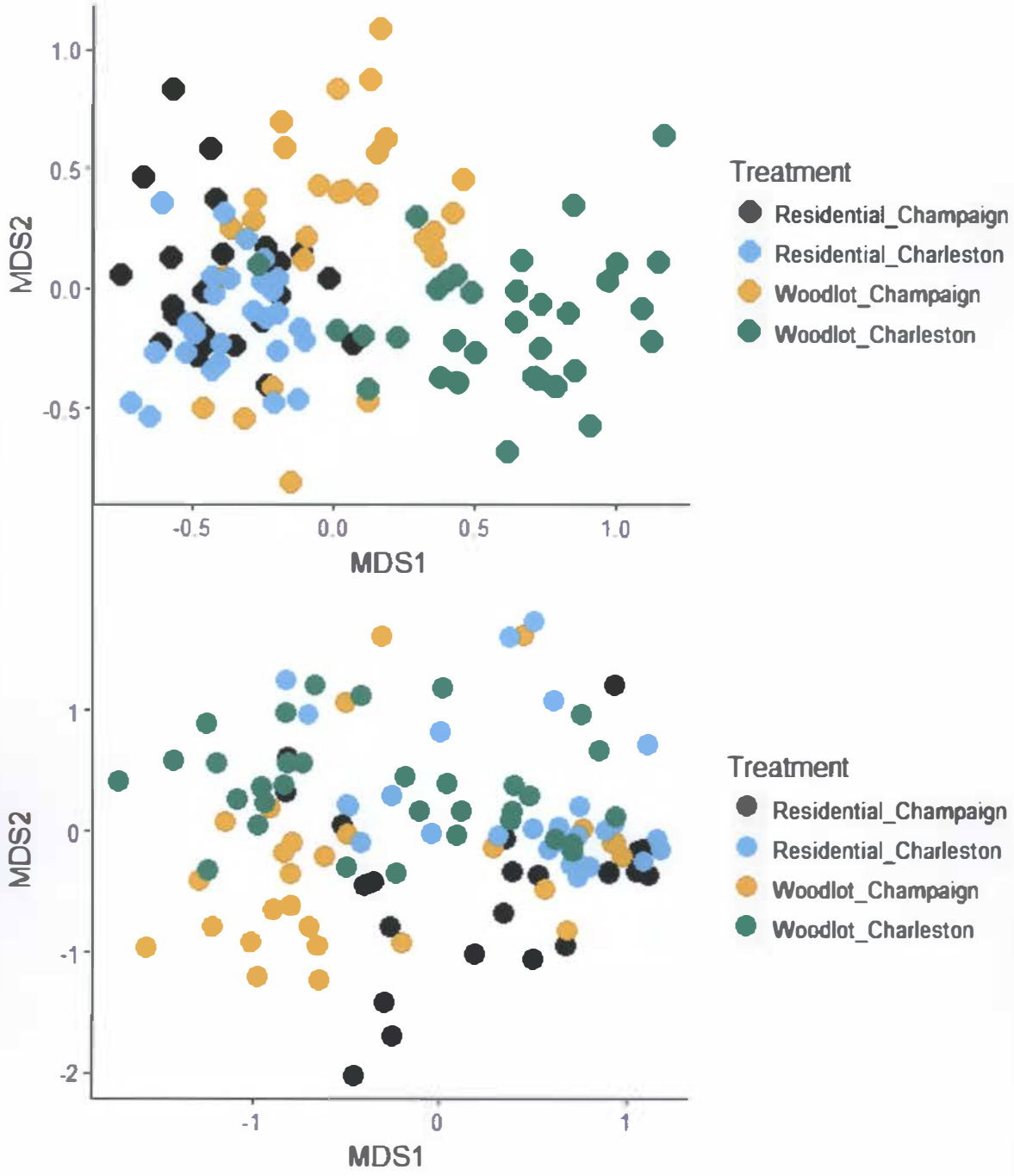

Figure 9. Non-metric multi-dimensional scaling (NMDS) analysis on occupancy (presence and absence) data (top) and on abundance (bottom).

APPENDIX

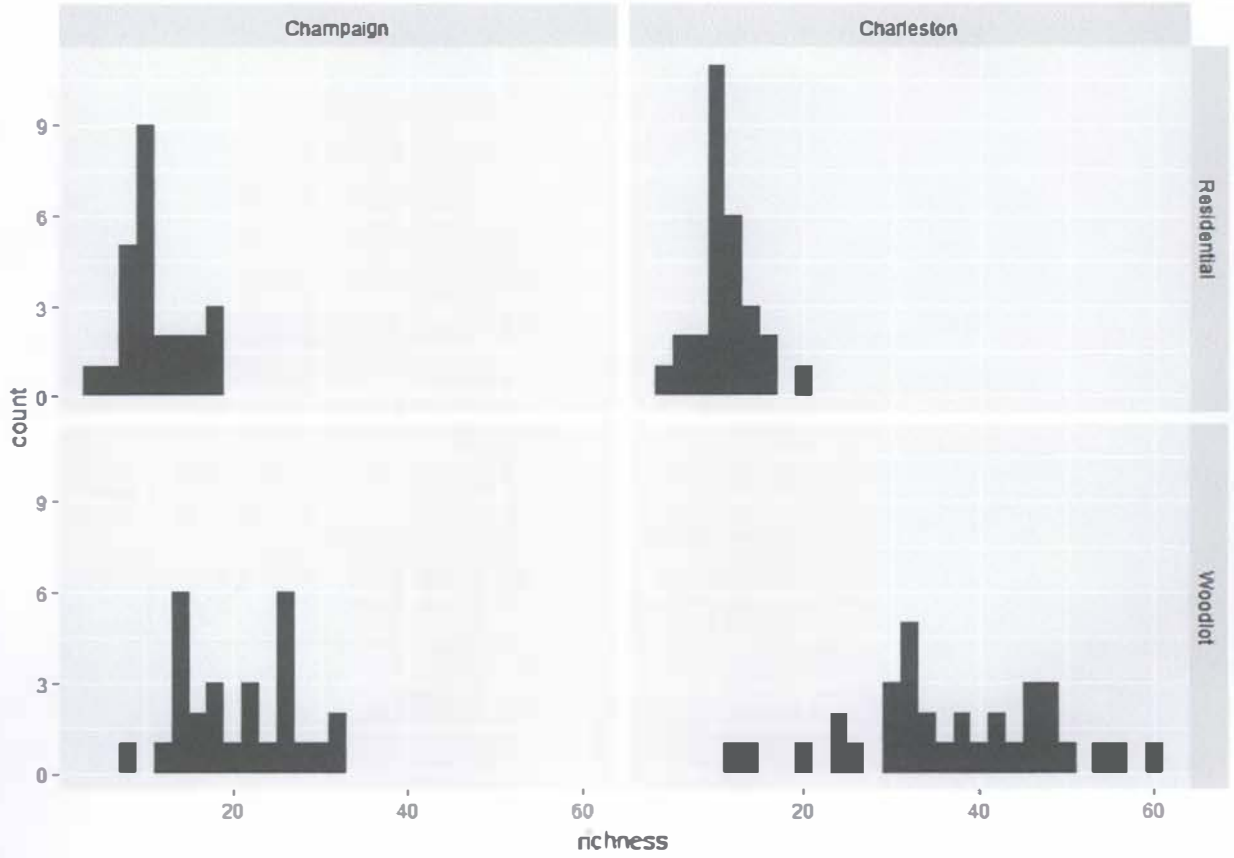

Figure A1. α-Diversity chart of species richness in bacterial communities in all treatment locations (Charleston woodlot and residential as well as Champaign woodlot and residential).

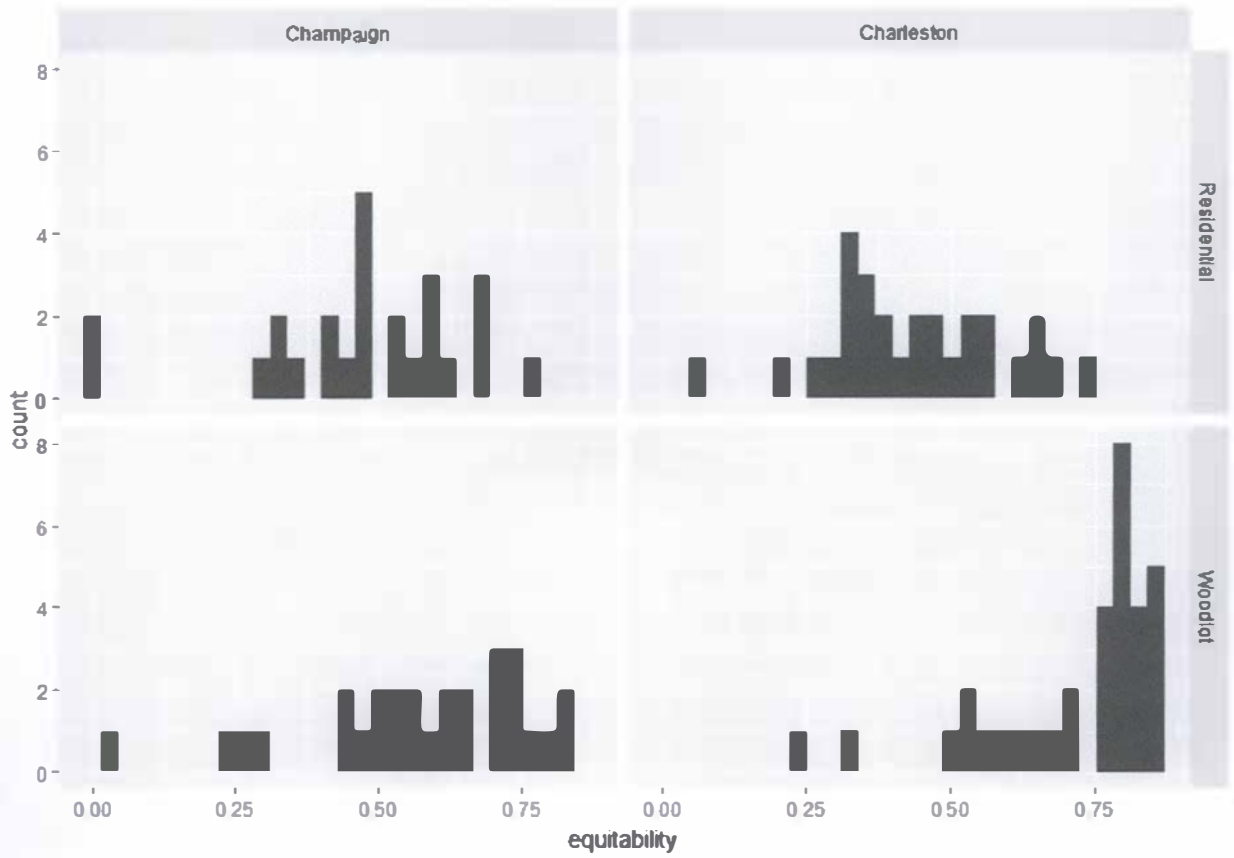

Figure A2. α-Diversity chart of the evenness of bacterial communities in all treatment locations (Charleston woodlot and residential as well as Champaign woodlot and residential).

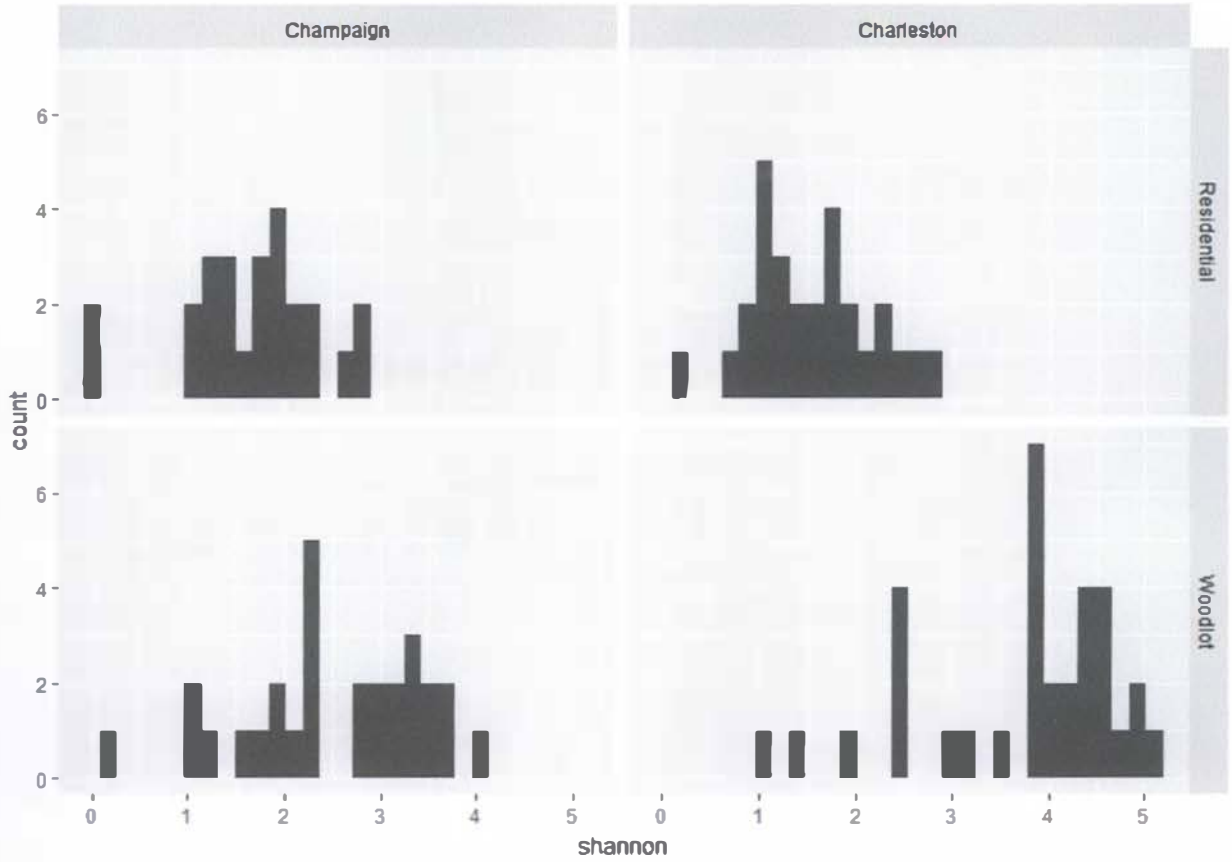

Figure A3. α-Diversity chart of the Shannon Index (a combination of equitability and species evenness amongst bacterial communities) of the 4 treatments (Charleston woodlot and residential as well as Champaign woodlot and residential).

REFERENCES

1. Gubler DJ. The continuing spread of West Nile virus in the western hemisphere. *Clin Infect Dis.* 2007; 45(8):1039--46.

2. Gubler D, ed. Dengue and dengue hemorrhagic fever: its history and resurgence as a global public health problem. London (United Kingdom): *CAB International*; 1997.

3. Charrel RN, de Lamballerie X, Raoult D. Chikungunya outbreaks: the globalization of vectorborne diseases. *N Engl J Med.* 2007; 356(8):769–71. pmid:17314335.

4. Duffy MR, Chen TH, Hancock WT, Powers AM, Kool JL, Lanciotti RS, et al. Zika Virus Outbreak on Yap Island, Federated States of Micronesia. *New England J Med.* 2009; 360(24):2536--43.

5. Fauci AS, Morens DM. Zika Virus in the Americas—Yet Another Arbovirus Threat. *N Engl J Med.* 2016; 374(7):601--4.

6. Sim S, Jupatanakul N, Dimopoulos G. Mosquito immunity against arboviruses. *Viruses.* 2014; 6(11):4479–504.

7. Khoo CC, Piper J, Sanchez-Vargas I, Olson KE, Franz AW. The RNA interference pathway affects midgut infection- and escape barriers for Sindbis virus in *Aedes aegypti. BMC Microbiol*. 2010; 10:130. doi: 10.1186/1471-2180-10-130.

8. Paulson SL, Grimstad PR, Craig GB Jr. Midgut and salivary gland barriers to La Crosse virus dissemination in mosquitoes of the *Aedes triseriatus* group. *Med Vet Entomol*. 1989; 3(2):113–23.

9. Ramirez JL, Souza-Neto J, Torres Cosme R, Rovira J, Ortiz A, Pascale JM, et al. Reciprocal tripartite interactions between the *Aedes aegypti* midgut microbiota, innate immune system and dengue virus influences vector competence. *PLoS Negl Trop Dis.* 2012; 6(3):e1561.

10. Xi Z, Ramirez JL, Dimopoulos G. The *Aedes aegypti* toll pathway controls dengue virus infection. *PLoS Pathog.* 2008; 4(7):e1000098. pmid:18604274.

11. Wang S, Ghosh AK, Bongio N, Stebbings KA, Lampe DJ, Jacobs-Lorena M. Fighting malaria with engineered symbiotic bacteria from vector mosquitoes. *Proc Natl Acad Sci U S A.* 2012; 109(31):12734–9.

12. Muturi EJ, Kim CH, Bara J, Bach EM, Siddappaji MH. *Culex pipiens* and *Culex restuans* mosquitoes harbor distinct microbiota dominated by few bacterial taxa. *Parasit Vectors*. 2016; 9(1):18.

13. Darsie RFJ, Ward RA. Identification and geographical distribution of the mosquitoes of North America, north of Mexico. *Mosq Syst Suppl.* 1981; 1:1±313.

14. Werren JH (1997) Biology of Wolbachia. *Annu Rev Entomol* 42:587–609.

15. Dillon RJ, Dillon VM: The gut bacteria of insects: nonpathogenic interactions. *Annu Rev Entomol* 2004, 49:71–92.

16. Minard G, Mavingui P, Moro C: Diversity and function of bacterial microbiota in the mosquito holobiont. Parasit Vectors 2013; 6(1):146.

17. Pidiyar VJ, Jangid K, Patole MS, Shouche YS: Studies on cultured and uncultured microbiota of wild *Culex quinquefasciatus* mosquito midgut based on 16s ribosomal RNA gene analysis. *Am J Trop Med Hyg* 2004; 70(6):597–603.

18. Dharne M, Patole M, Shouche YS: Microbiology of the insect gut:tales from mosquitoes and bees. *J Biosci* 2006; 31(3):293–295.

19. Lindh JM, Borg-Karlson AK, Faye I: Transstadial and horizontal transfer of bacteria within a colony of Anopheles gambiae (Diptera: Culicidae) and oviposition response to bacteria-containing water. *Acta Trop* 2008; 107(3):242–250.

20. Muturi EJ, Ramirez JL, Rooney, Kim CH: Comparative analysis of gut microbiota of mosquito communities in central Illinois. *PLoS Negl Trop Dis.* 2017; 11(2):e0005377.

21. Blagrove, M. S. C., Arias-Goeta, C., Di Genua, C., Failloux, A.-B., & Sinkins, S. P. A. *Wolbachia w*Mel transinfection in *Aedes albopictus* is not detrimental to host fitness and inhibits Chikungunya virus. *PLoS Negl Trop Dis*, *7*, 2013: e2152.

22. Frentiu, F. D., Zakir, T., Walker, T., Popovici, J., Pyke, A. T., van den Hurk, A., O'Neill, S. Limited dengue virus replication in field-collected *Aedes aegypti* mosquitoes infected with *Wolbachia. PLoS Negl Trop Dis*, 2014; *8*, e2688.

23. Thongsripong, P., Chandler, J. A., Green, A. B., Kittayapong, K., Wilcox, B. A., Kapan, D. D., Bennett, S. N. Mosquito vector-associated microbiota: Metabarcoding bacteria and eukaryotic symbionts across habitat types in Thailand endemic for dengue and other arthropod-borne diseases. *Ecol Evol.* 2017; 8(2):1352-1368.

24. Oliveira M., Malinger A.S., G., Ximenes, R., Szejnfeld, P.O., Alves Sampaio, S., and Bispo de Filippis, A.M. (2016). Zika virus intrauterine infection causes fetal brain abnormality and microcephaly: tip of the iceberg? *Ultrasound Obstet. Gynecol.* 47, 6–7.

25. Grice, Elizabeth A.; Kong, HH; Conlan, S; Deming, CB; Davis, J; Young, AC; NISC

Comparative Sequencing Program; Bouffard, GG; et al. "Topographical and Temporal Diversity of the Human Skin Microbiome". *Science*. 2006; 324 (5931): 1190–2.

26. Verhulst N.O., Andriessen R., Groenhagen U., Kiss G.B., Schulz S., Takken W., van Loon J.J.A., Smallegange R.C. Differential attraction of malaria mosquitoes to volatile blends produced by human skin bacteria. *PLoS ONE*, 2010; Vol. 5, No. 12.

27. Jordan, D. C. Transfer of *Rhizobium japonicum Buchanan* 1980 to *Bradyrhizobium* gen. nov., a genus of slow-growing, root nodule bacteria from leguminous plants. *Int J Syst Bacteriol* 1982., 32:136–139

28. Joubert, D.A.,Walker, T., Carrington, L.B., De Bruyne, J.T., Kien, D.H., Hoang, Nle.T., Chau, N.V., Iturbe-Ormaetxe, I., Simmons, C.P., and O'Neill, S.L. Establishment of a Wolbachia Superinfection in *Aedes aegypti* Mosquitoes as a Potential Approach for Future Resistance Management. *PLoS Pathog.* 2016; 12, e1005434.29.

29. Ferguson, N.M., Kien, D.T., Clapham, H., Aguas, R., Trung, V.T., Chau, T.N.,. Popovici, J., Ryan, P.A., O'Neill, S.L., McGraw, E.A., et al. Modeling the impact on virus transmission of Wolbachia-mediated blocking of dengue virus infection of *Aedes aegypti*. 2015. *Sci. Transl Med.* 7, 279ra37.

30. Caragata, E.P., Dutra, H.L., and Moreira, L.A. (2016). Exploiting intimate relationships: Controlling mosquito-transmitted disease with Wolbachia. *Trends Parasitol.* 32, 207–218.31.