Identifying the effects of sugar meal composition and concentration on microbiota diversity in the *Aedes aegypti* midgut.

Research Thesis

Presented in partial fulfillment of the requirements for graduation *with research distinction* in the

undergraduate college of The Ohio State University

By

Zi Ping Wong

The Ohio State University

August 2021

Project advisor: Sarah M. Short, Department of Entomology

Abstract:

Aedes aegypti are a well-known source of vector borne diseases (World Health Organization, 2020). These female mosquitoes transmit disease via blood feeding. Their midguts contain microbiota that influence their physiology (i.e., immune function). and are the tissue through which human pathogens invade after a blood meal. Sugar is a vital source of the mosquito diet, which mosquitoes obtain from flower nectar. Sugar alters microbiota composition in the midgut because sugar feeding affects mosquito physiology. Nectars have been found to have variable sucrose and fructose compositions. Thus, we were interested in investigating varying ranges of sucrose and fructose to see their effects on the microbiota in the mosquito midgut. We used *Aedes aegypti* Liverpool strain mosquitoes and provided adult females with 30% sugar meals where the sugar was either 100% sucrose, 100% fructose, or 50% sucrose and 50% fructose. We dissected midguts from five females per treatment, serially diluted the homogenates, and cultured the samples on tryptic soy agar (TSA) media. We found that very few plates grew bacteria, and of the few that did grow, treatment 1 (50% sucrose, 50% fructose) had the most bacterial growth on TSA plates. Among the bacteria we cultured, we only found two types of bacteria. These findings suggest either that the midgut microbiota does not grow favorably on TSA plates, there were few bacteria in the midgut at the time of the experiment, or few bacteria grew favorably with the applied sugar treatments. The bacteria we did find might be more prevalent in the mosquito microbiota or might be the only bacteria that grow in the treatments we applied. Further research would be required to identify these bacteria to species and confirm the repeatability of the results.

Introduction:

Aedes aegypti, the yellow fever mosquito, vectors Dengue Virus (DENV), Zika Virus (ZIKV), yellow fever, etc. which represent a major global public health burden (World Health Organization, 2020). Collectively, vector-borne diseases make up more than 17% of all infectious diseases, resulting in more than 700,000 deaths every year. Dengue is the most widespread vector-borne viral infection and is transmitted by *Aedes* mosquitoes (World Health Organization, 2020). Today, over 3.9 billion people are at risk of contracting dengue (World Health Organization, 2020). It is estimated that 96 million people suffer from Dengue and 40,000 lives are claimed every year (World Health Organization, 2020). In some Asian and Latin American nations, severe dengue is a leading cause of illness and death. Unfortunately, there is not medication for severe dengue and many other vector-borne diseases. However, early screening for dengue and preventative medicine reduces fatality rates to less than 1% (World Health Organization, 2021). Inhibiting these pathogens in the mosquito prior to human transmission is an effective method to prevent mosquito related diseases and symptoms (Ferguson, 2018; Gao et al., 2020).

Mosquitoes are continuously exposed to various microorganisms from the environment. The bacteria found in the mosquito is acquired from the environment and is called the microbiota. Microbiota can be found in the mosquito midgut and other locations such as the crop. Studies of the mosquito microbiota suggest that bacteria associated with mosquitoes nutritionally benefit the insect (Dennison et al., 2014). Moreover, studies suggest the microbiota plays a significant role in initializing and maintaining the mosquitoes' basal immune activity, immune homeostasis, and host

metabolism (Dong et al., 2009). The microbiota can also influence the mosquito's immune response, physiology, and susceptibility to human pathogens (Douglas, 2014; Gao et al., 2020).

Many insects feed on sugar meals such as flower nectar. In 1958, Downes observed *Aedes* mosquitoes of both sexes frequently visiting flowers for nectar as a food source (Downes 1958; Olson et al., 2020). Sugar is the primary component of flower nectar and is an essential dietary component for mosquitoes due to its energy and nutrient rich characteristics. Sugar is an important energy source to support flight behavior, mating and egg production (A. N. Clements, 1955; Van Handel 1985; Foster 1995). Additionally, studies suggest that sugar deficient environments efficiently reduce the population or survivorship of adult mosquitoes (Foster 1995: Okech et al., 2003: Impoinvil et al., 2004: Gu et al., 2011: Olson et al., 2020). Even though sugar is a food source, studies suggest that *Aedes* have become tremendously anthropophilic with minimal reliance on sugar meals (Edman et al., 1992: Harrington et al., 2001: Olson et al., 2020). Although, *Aedes* are less dependent on sugar meals, it is still a worthy area of study to study the effects of sugar on their microbiota. Additionally, researchers have observed a reproductive advantage in *Ae. Aegypti* fed exclusively blood versus blood with sugar (Costero et al., 1998: Olson et al., 2020). These factors may contribute to the exceptional ability of *A. aegypti* mosquitoes to transmit human pathogens and may also explain the increasing role of *A. aegypti* in arthropod borne viral transmission (Olson et al., 2020).

In mosquitoes, the crop or ventral diverticulum is the main storage for sugar (nectar), before contents are transferred to the midgut for digestion and absorption

(Calkins, et. al, 2017). However, in blood feeding, blood is diverted to the midgut and bypasses the crop (Day, 1954: Calkins, et. al, 2017). Only adult females ingest blood because blood meals provide the necessary nutrients to complete egg maturation and complete their reproductive cycle (Olson, et. al., 2020), and blood feeding also facilitates energy for flight behavior (A. N. Clements, 1955). Thus, mosquitoes can become vectors of disease when they acquire blood from an infected host and transmit it to the next host (Olson et al., 2020). Importantly, when a female adult mosquito takes a blood meal from an infected vertebrate host, pathogens along with the blood are pulled into the midgut. The pathogens infect the gut cells, enter the hemolymph and salivary glands, and are transmitted when the infected mosquito bites the next host (Gao et al., 2020). Since the midgut is the first tissue a blood-borne pathogen encounters, it is relevant to our understanding of how pathogens infect mosquitoes.

Studies suggest that if mosquitoes feed on varying sugar concentrations they display different survivorship, behaviors, and activities (Sissoko et al., 2019). However, little research has been done to investigate how varying sugar compositions affect the midgut microbiota. Thus, it is our interest to investigate sugar compositions on female mosquitoes' midgut microbiota. In all hummingbird and bee pollinated flowers, nectar has been found to be sucrose-dominant with sucrose proportions ranging from 57.8% to 88.6% (Mathieu et al., 2001). Additionally, sucrose, fructose and glucose were found to be in nectar. On average, sucrose and fructose were found to be dominant compositions in nectar. Moreover, glucose was the only sugar that was not found exclusively alone in the nectar samples (Herrera et al., 2006). This suggests that

fructose and sucrose are the primary elements in nectar. Thus, we chose to utilize these sugars in our experiment.

In this work, we investigated how ingestion of different sugar types (sucrose and fructose) influences the midgut microbiota of *A. aegypti* mosquitoes. We hypothesized that the sugar meal, despite being stored in the crop, could potentially influence the midgut microbiota directly as a food source for bacteria in the midgut, or indirectly by affecting mosquito physiology and/or immune system signaling. It is also possible that sugar could influence bacterial growth in the crop, and the bacteria could then be carried downstream to the midgut.

Materials and Methods:

Mosquito Rearing:

Aedes Aegypti (Liverpool strain) was reared under 27C, 80% humidity, 14h:10h light: dark cycle. Approximately 400 eggs were cleaned with 3% bleach and rinsed twice with sterile reverse osmosis (RO) water. Eggs were submerged with sterile RO water and were hatched in a vacuum. Approximately 300 larvae were moved to plastic rearing pans (Figure 1) filled with 1L deionized water (DI) with 500µl of glycerol stock from a mosquito breeding site in Baltimore, MD and provided larval food (powdered fish food the first day and red pellet cat food after) ad libitum. On day 7, 3 cages were prepared with 45 pupae each (Figure 2). Males and females were housed together to create a more natural environment and to allow mating to occur. Sugar treatments were started once adults emerged. On day 11, sugar treatments and pupal cups were removed from

the cages. On day 13, midguts were dissected. Refer to Table 1 for a full schematic of the experiment.

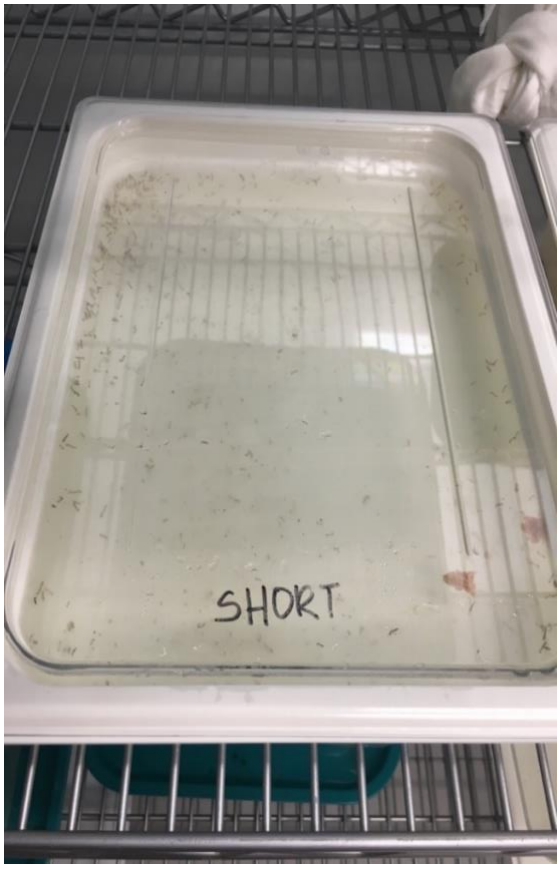

Figure 1: Larval rearing tray. Approximately 300 larvae were reared in a single pan that also contained 1L DI water. They were provided larval food ad libitum until pupation.

Table 1: Experimental Timeline

Day:	1	2	3	4	5	6	7
	Eggs hatch	1st instars; fish flakes	2nd Instars; fish flakes	2nd instars; cat food (1 pellet)	3rd instars; cat food (2 pellets)	3rd instars; cat food (2 pellets)	4th instars; cat food (2 pellets); move pupae to cages; Night sugar treatment
Day:	8	9	10	11	12	13	
	Adults; Morning and night sugar treatment	Adults; Morning and night sugar treatment	Adults; Morning and night sugar treatment	Adults; Morning and night sugar treatment; Take pupal cups out	Adults; Morning and night sugar treatment	Adults; Morning sugar treatment; dissection	

Treatment Information:

There were 3 treatments (Table 2). Treatment 1 contained 50% sucrose and 50% fructose at a 30% sugar concentration. Treatment 2 contained 100% sucrose at a 30% sugar concentration. Treatment 3 contained 100% fructose at a 30% sugar concentration. Overnight, mosquitoes were provided with filter sterilized DI water. All liquid solutions were kept at 4°C and opened in a laminar flow hood to keep sterile. Cotton pads were used to absorb the sugar treatment poured in petri dishes and placed over mesh netting to allow mosquitoes to feed. A petri dish was placed on top of the mesh covering to prevent cotton pads from drying out.

Figure 2: Mosquito cages for housing adults. 45 pupae were placed in cages fitted with a partial mesh covering. Sugar-soaked cotton pads were placed on top of the mesh to allow mosquitoes to feed.

Table 2: Sugar treatment concentrations and compositions

Treatment	Sugar concentration	Sucrose Concentration	Fructose Concentration
1	30%	50%	50%
2	30%	100%	0%
3	30%	0%	100%

All sugar treatments were started at 5PM on Day 7, i.e., the day pupal cups were transferred to cages. Adults were provided sugar treatments twice a day at 9AM-10AM and 5PM-6PM (Table 3). Filter sterilized DI water was provided overnight.

Table 3: Treatment feedings timeline

9am-10am	10am-5pm	5pm-6pm	6pm-9am
Sugar treatment	Nothing	Sugar treatment	Sterile DI Water

Tryptic Soy Agar (TSA) Plates Preparation:

To culture bacteria growing in the mosquito midguts, roughly 50 Tryptic Soy Agar (TSA) plates were prepared. 500mL DI H_2O, 12.5g of Tryptic Soy medium powder, and 7.5g Agar were combined in an Erlenmeyer flask. The mixture was then mixed well with stirring plate. The media was autoclaved for 45 minutes on liquid cycle (121°C). The liquid medium was cooled at room temperature for 10 minutes before pouring into sterile petri dishes under a flame. Plates were then cooled at room temperature and stored upside down.

Digestive Tract Dissection:

A mechanical aspirator was used to extract mosquitoes out of cages. Females were cold-anesthetized and sterilized in 70% ethanol for 1 minute and rinsed twice with filter sterilized 1x PBS. Midguts were dissected on a sterile glass slide using forceps (Figure 3). Each sample was rinsed with filter sterilized 1x PBS and transferred to a

microcentrifuge tube containing 150µL of filter sterilized PBS. Two negative controls were collected by each person. These consisted of a sample collected as described above but absent mosquito tissue. Five midguts were dissected from each treatment and each midgut was stored in 150µL 1X PBS in an individual 1.5mL microcentrifuge tube. Samples were immediately homogenized and 100µL were spread on a TSA plate. Dissections were completed one treatment at a time to ensure samples were processed quickly. Negative controls were not diluted but were homogenized and plated.

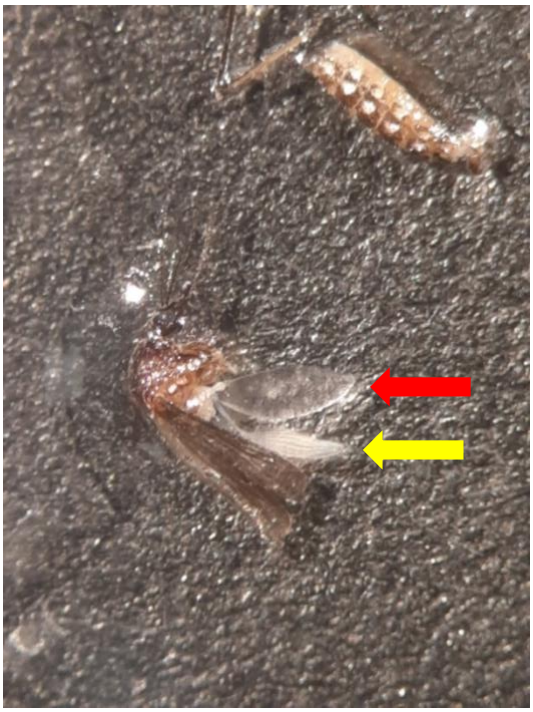

Figure 3: Dissected mosquito digestive tract. Adult female *A. aegypti* with abdomen separated from thorax and legs removed. The yellow arrow indicates the midgut, and the red arrow indicates the crop.

Homogenization:

Autoclaved blue pestles were cleaned with DNA away buffer and sterilized by autoclaving. The laminar flow hood was used to keep samples sterile. Pestles were used to manually break apart each midgut until torn, then a mechanical drill was used to homogenize thoroughly.

Plating:

Under sterile conditions, all samples were diluted 10^{-2} and 10^{-4} using filter sterilized 1X PBS. To dilute samples, 10 µL of homogenized sample was added to 990 µL of 1x PBS filter sterilized. The mixture was pipetted up and down to mix, then vortexed. 10 µL from the 10^{-2} dilution was then added to 990 µL filter sterilized 1X PBS pipetted up and down to mix and vortexed. 100 µL from each dilution (undiluted, 10^{-2}, 10^{-4}) was then pipetted onto a separate petri dish containing TSA media. Approximately 15 sterile glass beads were then added to each plate and swirled to spread homogenate then removed. Plates were placed upside down to grow bacteria colonies.

Incubation and Storage:

Plates were kept at room temperature (approximately 25°C) and allowed to grow for 7 days. One plate (1-2-M undilute; treatment-mosquito-tissue concentration) was moved to the fridge (4°C) to reduce overcrowding and pause growth. Another set of plates (1-1-M undilute, 2-1-M undilute, 3-1-M undilute, and 2-1-M dilute 10^{-2}) were incubated at 30°C to compare if a different temperature setting would change bacterial

growth. The undiluted plates had 10 µL and the dilute plate had 100µL homogenized sample. All remaining samples and dilutions were kept in the fridge at 4°C.

Results:

Majority of mosquito midguts contained no colony forming units.

For many samples, no cultivable bacteria were observed (Table 4). One sample from treatment one had approximately 800 colony forming units (Table 4). An exact count was not possible due to crowding on the plate (Figure 4). All dilutions from all other samples had fewer than 7 colony forming units. We used the number of colonies on each plate to calculate the number of CFUs found in each mosquito midgut and within each treatment group (Figure 5). Treatment 1 had 2 midguts with bacterial growth, treatment 2 had 1 midgut with bacterial growth and treatment 3 had 1 midgut with bacterial growth (Figure 5). In multiple cases, we observed one colony forming unit on a plate derived from a 10^{-4} dilution. In these cases, we suspect this is contamination, because if there were bacteria in the sample, the undiluted plate would have 10,000X the bacterial growth as the 10^{-4} dilution and we did not observe this (Table 4).

In cases where bacteria were successfully cultured, two types of colony forming units (CFU) were isolated.

Very few bacteria were collected overall but when bacteria were successfully isolated, two types of bacteria were observed. The first type (type A) was small and yellowish with an entire margin and a raised elevation. The second type (type H) was medium and yellowish with an entire margin and a flat elevation (Table 5, Figure 4).

Table 4: Quantification of colony Forming Units cultured from *A. aegypti* midguts on Tryptic Soy Agar media

Treatment	Sample	Dilution	Type A	Type H	Total colonies
1	1	Undiluted	3[ab]	0	3[ab]
1	1	10^{-2}	0	0	0
1	1	10^{-4}	1[c]	0	1[c]
1	2	Undiluted	800	0	800
1	2	10^{-2}	7	0	7
1	2	10^{-4}	0	0	0
1	3	Undiluted	0	0	0
1	3	10^{-2}	0	0	0
1	3	10^{-4}	0	0	0
1	4	Undiluted	0	0	0
1	4	10^{-2}	0	0	0
1	4	10^{-4}	0	0	0
1	5	Undiluted	0	0	0
1	5	10^{-2}	0	0	0
1	5	10^{-4}	0	0	0
2	1	Undiluted	0	0	0
2	1	10^{-2}	0	0	0
2	1	10^{-4}	0	0	0
2	2	Undiluted	1	0	1
2	2	10^{-2}	0	0	0
2	2	10^{-4}	1[c]	0	1[c]
2	3	Undiluted	0	0	0
2	3	10^{-2}	0	0	0
2	3	10^{-4}	1[c]	0	0
2	4	Undiluted	0	0	0
2	4	10^{-2}	0	0	0
2	4	10^{-4}	1[c]	0	0
2	5	Undiluted	0	0	0
2	5	10^{-2}	0	0	0
2	5	10^{-4}	0	0	0
3	1	Undiluted	0	1	1
3	1	10^{-2}	0	0	0
3	1	10^{-4}	0	0	0
3	2	Undiluted	0	0	0
3	2	10^{-2}	0	0	0
3	2	10^{-4}	0	0	0
3	3	Undiluted	0	0	0
3	3	10^{-2}	0	0	0
3	3	10^{-4}	0	0	0
3	4	Undiluted	0	0	0
3	4	10^{-2}	0	0	0
3	4	10^{-4}	0	0	0
3	5	Undiluted	0	0	0
3	5	10^{-2}	0	0	0
3	5	10^{-4}	0	0	0
Negative control 1 (JR)			0	0	0
Negative control 2 (ZW)			0	0	0
Total colonies			811	1	812

[a]Approximation due to CFUs growing too close together

[b]On rim of plate
[c]Probable contamination, not included in total

Table 5: Characterization of colony Forming Units cultured from *A. aegypti* midguts on Tryptic Soy Agar media

Type	Size	Color	Opacity	Surface	Texture	Margin	Elevation
Type A	small	yellowish	opaque	dull	mucoid	entire	raised
Type H	medium	yellowish	opaque	dull	mucoid	entire	flat

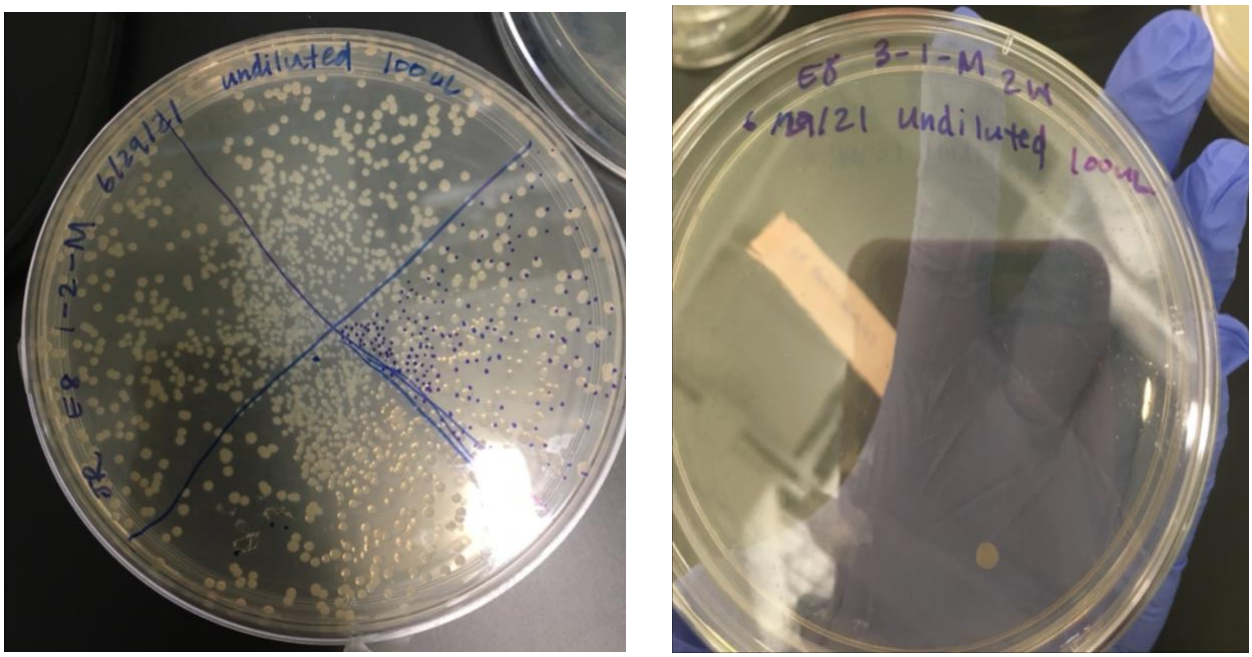

Figure 4: Cultured mosquito midguts bacteria types on Tryptic Soy Agar Media
A. aegypti midguts were isolated after sugar treatments. Bacteria type A is on the left and type H is on the right.

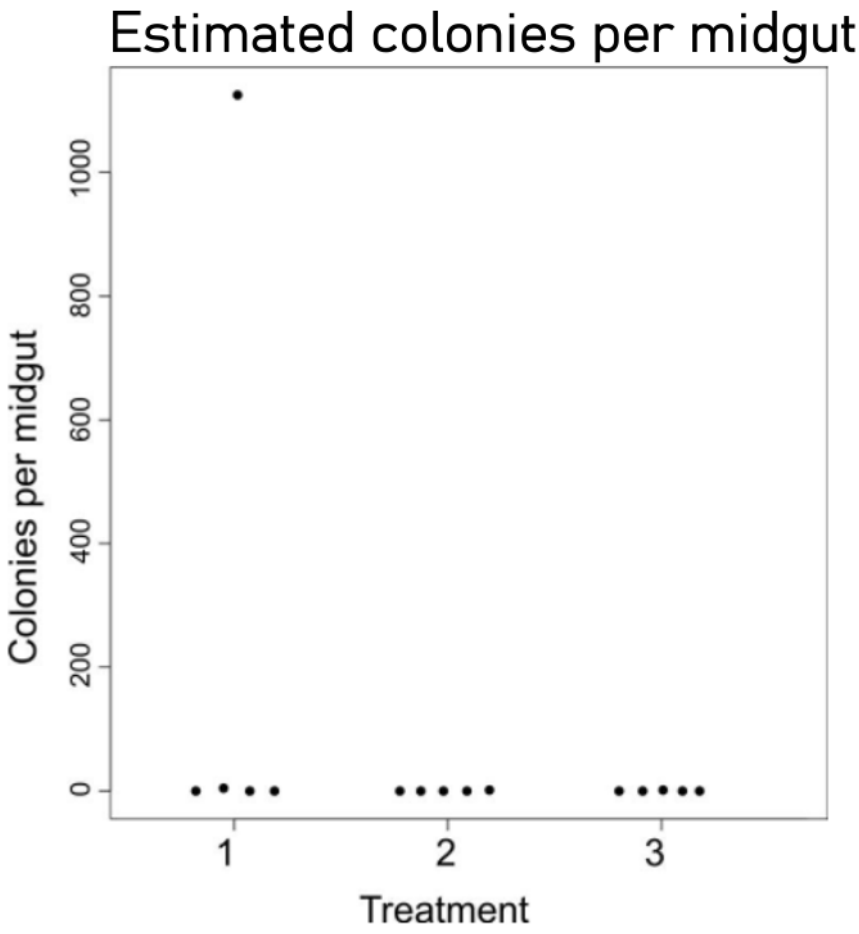

Figure 5: Estimated colonies per midgut cultured from *A. aegypti* midguts on Tryptic Soy Agar media. Very few midgut samples (n = 4) yielded bacterial growth and when bacteria were observed the numbers were generally very small, with the exception of one midgut in Treatment 1.

Discussion:

In this experiment, we attempted to investigate sugar composition and concentration effects on the midgut microbiota of *A. aegypti* adult female mosquitoes. We found 5 of 45 plates, and 4 individual midguts that grew bacteria. Treatment 1 (50% sucrose, 50% fructose) had the most bacterial growth on TSA plates with over 800 colony forming units (CFU) derived primarily from one sample. We found two types of

bacteria; the first type (type A) was small with a raised elevation. The second type (type H) was medium sized with a flat elevation. Size is dependent on the agar, environment, etc., thus, it is not a reliable factor to use to differentiate colonies. However, these two colony types did have different elevations suggesting they are not the same bacterial species.

Five midguts were homogenized from each treatment. Out of the 15 midguts sampled, colony forming units were only successfully cultured from four. Two midguts from treatment 1 (50% sucrose, 50% fructose), one midgut from treatment 2 (100% sucrose) and one midgut from treatment 3 (100% fructose) had bacterial growth. There was also a large range of bacterial growth found in the midguts with the maximum over 1000 colonies found. These results suggest that microbiota may not be present in the midgut, the microbiota does not favorably grow on TSA plates, or the treatments are not a favorable environment for microbiota growth. The bacteria found might be more common in the mosquitoes' microbiota or might be the only bacteria that grew with these treatments. Although, glycerol stock from a mosquito breeding site was added providing necessary microbiota for mosquito development, few microbiota was found on cultured TSA plates. More research is necessary to identify these bacterial species and confirm the repeatability of the results.

Treatment 1 (50% sucrose, 50% fructose) had over 800 CFU from two midguts, though most of the growth was from a single midgut. Treatment 2 (100% sucrose) had 1 CFU and treatment 3 (100% fructose) had 1 CFU. Treatment 1, therefore, had higher bacterial growth compared to all the other treatments. However, because so few samples yielded bacteria, we are unable to adequately assess the veracity or legitimacy

of this finding. It is possible that that treatment 1 might be more favorable to bacterial growth in the mosquito midgut because treatment 1 is most similar to a nectar's composition. However, we need to conduct more testing to confirm the reliability of these results.

Most bacterial colonies found were type A. Type A made up of over 800 colonies whereas Type H only accounted for 1 colony. This suggests that type A might be more widespread in the *A. aegypti* midgut, or the treatments may have influenced the microbiota. Further research needs to be conducted to see if sugar composition plays an influential role in the microbiota composition.

In 4 cases, we observed only one colony forming unit (CFU) on a plate obtained from a 10^{-4} dilution. We suspect the CFUs on these plates are contamination since only the 10^{-4} has bacterial growth. If these samples did have bacteria, then we would have expected the undiluted plates to display 10,000X the bacterial growth, and the 10^{-2} diluted plates would display 100X the bacterial growth of the 10^{-4} dilution. Because these observations were not made, we assume the CFUs on these plates are the result of contamination and not bacteria derived from the midgut microbiota.

It is not clear where this contamination may have arisen from. Most plates (including the negative controls) had zero bacterial growth, which suggests that the TSA plates were sterile, and contaminants were introduced when plating. It is possible the plating technique may have introduced new contaminants as the homogenate may not have been spread evenly across the media. A couple plates had bacteria on the rims and lids suggesting poor technique when spreading the homogenate with beads or potentially contamination from the beads themselves.

Overall, our results indicate that we are unable to answer our original question of whether sugar treatments impact the midgut microbiota, as very few samples yielded cultivable bacteria. Many more samples will need to be collected, or the experimental procedures will need to be amended (e.g., a different bacterial media used) for a proper analysis to be performed. Also, continued practice of plating technique should be implemented to prevent contamination. Introduction of different mediums to see what medium is most appropriate for bacteria in the mosquito midgut may also increase our ability to collect useable data. Additionally, measuring bacterial load using qRT-PCR targeting the 16S bacterial RNA gene would potentially allow for higher sensitivity in measuring how sugar treatments are affecting the microbiota size. This method would also be capable of detecting changes in uncultivable microbes, which would give a more complete measurement of the microbiota. Another important adjustment to the protocol would be to alter the sugar concentration, as 30% may be too harsh for the microbiota to thrive in. It would also be beneficial to conduct sequencing on the bacterial colonies we did culture to confirm if they are different and to determine their identities. Unfortunately, we were unable to conduct these assays due to time constraints. Lastly, this experiment needs to be conducted multiple times to see if the individual mosquitoes we found to carry cultivable bacteria are anomalies or if sugar composition plays an influential role in their microbiota.

References:

1. A.N. CLEMENTS; The Sources of Energy for Flight in Mosquitoes. *J Exp Biol* 1 September 1955; 32 (3): 547–554. doi: https://doi.org/10.1242/jeb.32.3.547
2. Calkins TL, DeLaat A, Piermarini PM. Physiological characterization and regulation of the contractile properties of the mosquito ventral diverticulum (crop). J Insect Physiol. 2017 Nov;103:98-106. doi: 10.1016/j.jinsphys.2017.10.012. Epub 2017 Oct 28. PMID: 29107658; PMCID: PMC5708170.
3. Costero A, Edman JD, Clark GG, Scott TW. Life table study of Aedes aegypti (Diptera: Culicidae) in Puerto Rico fed only human blood versus blood plus sugar. J Med Entomol. 1998 Sep;35(5):809-13. doi: 10.1093/jmedent/35.5.809. PMID: 9775613.
4. DAY MF. The mechanism of food distribution to midgut or diverticula in the mosquito. Aust J Biol Sci. 1954 Nov;7(4):515-24. doi: 10.1071/bi9540515. PMID: 13229847.
5. Dennison NJ, Jupatanakul N, Dimopoulos G. The mosquito microbiota influences vector competence for human pathogens. Curr Opin Insect Sci. 2014 Sep 1;3:6-13. doi: 10.1016/j.cois.2014.07.004. PMID: 25584199; PMCID: PMC4288011.
6. Dong Y, Manfredini F, Dimopoulos G. Implication of the mosquito midgut microbiota in the defense against malaria parasites. PLoS Pathog. 2009 May;5(5):e1000423. doi: 10.1371/journal.ppat.1000423. Epub 2009 May 8. PMID: 19424427; PMCID: PMC2673032.
7. Douglas AE. The molecular basis of bacterial-insect symbiosis. J Mol Biol. 2014 Nov 25;426(23):3830-7. doi: 10.1016/j.jmb.2014.04.005. Epub 2014 Apr 13. PMID: 24735869; PMCID: PMC4385585.

8. Downes J.A., The Feeding Habits of Biting Flies and Their Significance in Classification. 1958. Annual Review of Entomology. https://www.annualreviews.org/doi/abs/10.1146/annurev.en.03.010158.001341
9. Edman JD, Strickman D, Kittayapong P, Scott TW. Female Aedes aegypti (Diptera: Culicidae) in Thailand rarely feed on sugar. J Med Entomol. 1992 Nov;29(6):1035-8. doi: 10.1093/jmedent/29.6.1035. PMID: 1460619.
10. Ferguson, N.M. Challenges and opportunities in controlling mosquito-borne
11. infections. *Nature* **559,** 490–497 (2018). https://doi.org/10.1038/s41586-018-0318-5
12. Foster WA. Mosquito sugar feeding and reproductive energetics. Annu Rev Entomol. 1995;40:443-74. doi: 10.1146/annurev.en.40.010195.002303. PMID: 7810991.
13. Gao H, Cui C, Wang L, Jacobs-Lorena M, Wang S. Mosquito Microbiota and Implications for Disease Control. Trends Parasitol. 2020 Feb;36(2):98-111. doi: 10.1016/j.pt.2019.12.001. Epub 2019 Dec 19. PMID: 31866183.
14. Gu W, Müller G, Schlein Y, Novak RJ, Beier JC. Natural plant sugar sources of Anopheles mosquitoes strongly impact malaria transmission potential. PLoS One. 2011 Jan 20;6(1):e15996. doi: 10.1371/journal.pone.0015996. PMID: 21283732; PMCID: PMC3024498.
15. Harrington LC, Edman JD, Scott TW. Why do female Aedes aegypti (Diptera: Culicidae) feed preferentially and frequently on human blood? J Med Entomol. 2001 May;38(3):411-22. doi: 10.1603/0022-2585-38.3.411. PMID: 11372967.

16. Herrera CM, Pérez R, Alonso C. Extreme intraplant variation in nectar sugar composition in an insect-pollinated perennial herb. Am J Bot. 2006 Apr;93(4):575-81. doi: 10.3732/ajb.93.4.575. PMID: 21646218.
17. Impoinvil DE, Kongere JO, Foster WA, Njiru BN, Killeen GF, Githure JI, Beier JC, Hassanali A, Knols BG. Feeding and survival of the malaria vector Anopheles gambiae on plants growing in Kenya. Med Vet Entomol. 2004 Jun;18(2):108-15. doi: 10.1111/j.0269-283X.2004.00484.x. PMID: 15189235.
18. Mathieu Perret, Alain Chautems, Rodolphe Spichiger, Mauro Peixoto, Vincent Savolainen, Nectar Sugar Composition in Relation to Pollination Syndromes in Sinningieae (Gesneriaceae), *Annals of Botany*, Volume 87, Issue 2, February 2001, Pages 267–273, https://doi.org/10.1006/anbo.2000.1331
19. Okech BA, Gouagna LC, Killeen GF, Knols BG, Kabiru EW, Beier JC, Yan G, Githure JI. Influence of sugar availability and indoor microclimate on survival of Anopheles gambiae (Diptera: Culicidae) under semifield conditions in western Kenya. J Med Entomol. 2003 Sep;40(5):657-63. doi: 10.1603/0022-2585-40.5.657. PMID: 14596279.
20. Olson MF, Garcia-Luna S, Juarez JG, Martin E, Harrington LC, Eubanks MD, Badillo-Vargas IE, Hamer GL. Sugar Feeding Patterns for Aedes aegypti and Culex quinquefasciatus (Diptera: Culicidae) Mosquitoes in South Texas. J Med Entomol. 2020 Jul 4;57(4):1111-1119. doi: 10.1093/jme/tjaa005. PMID: 32043525; PMCID: PMC7334892.
21. Sissoko F, Junnila A, Traore MM, Traore SF, Doumbia S, Dembele SM, Schlein Y, Traore AS, Gergely P, Xue RD, Arheart KL, Revay EE, Kravchenko VD, Beier

JC, Müller GC. Frequent sugar feeding behavior by Aedes aegypti in Bamako, Mali makes them ideal candidates for control with attractive toxic sugar baits (ATSB). PLoS One. 2019 Jun 17;14(6):e0214170. doi: 10.1371/journal.pone.0214170. PMID: 31206547; PMCID: PMC6576782.

22. Van Handel E. Rapid determination of glycogen and sugars in mosquitoes. J Am Mosq Control Assoc. 1985 Sep;1(3):299-301. PMID: 2906671.

23. World Health Organization. (2020, March 2). *Vector-borne diseases*. World Health Organization. https://www.who.int/news-room/fact-sheets/detail/vector-borne-diseases.

24. World Health Organization. (2021, May 19). *Dengue and severe dengue*. World Health Organization. https://www.who.int/news-room/fact-sheets/detail/dengue-and-severe-dengue.