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An alternative approach for standard disease vector control is the use of attractants. For the last several years, Dr. Wasserberg and his team have been studying oviposition attractants of *Phlebotomine* sand flies, vectors of the Leishmaniases agents. They have discovered that gravid females are attracted and stimulated to lay eggs in larval conditioned media and that this attraction is driven by bacterial kairomones. They also recently found that larval media conditioned with sand fly larvae was more attractive than medium aged for the same amount of time in the absence of larvae. Furthermore, both attraction and oviposition responses were shown to increase in response to both aged and larval conditioned media when compared to fresh larval media. Here, I tested the hypothesis that larval conditioning and medium aging affect the microbial community in a manner that affects the oviposition behavior of sand flies. The specific goals were to: (1) Compare the bacterial community secondary succession dynamics between aged and larval conditioned media; (2) Determine the effects of larval and post larval stages on microbial community structure and composition. To address these aims, fresh larval food was sampled as a baseline and aged and conditioned media were sampled at four time points (weeks 2, 4, 6, and 9). All samples were collected and analyzed using 16S amplicon sequencing. A total of 42 taxa were identified. The bacterial communities of week-2 larval-conditioned and aged media differed significantly from the baseline fresh medium with no significant change thereafter. However, the communities did not differ between the conditioned and aged media types at each time point. When grouped into larval and post-larval stages and compared to the corresponding aged media of same time points, the bacterial communities changed for both media types when compared to the baseline. Additionally, the bacterial communities of both the larval and post-larval media differed significantly from the aged media at both corresponding time points, suggesting larval induced taxonomical divergence of the bacterial community.

EFFECTS OF SAND FLY LARVAL REARING MEDIUM CONDITIONING AND AGING ON THE BACTERIAL COMMUNITY STRUCTURE AND DYNAMICS.

by

Nayma Romo Bechara

A Thesis

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Approved by

Dr. Kasie Raymann
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DEDICATION

I dedicate this thesis to my husband Ricardo for his unconditional love and support, to my parents, Alberto and Margot, my sister Liz, and Emily and Marcus for believing in me and helping me to follow my dreams and achieve my goals.

APPROVAL PAGE

This dissertation written by Nayma Romo Bechara has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

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Click or tap to enter a date.

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4/19/2021

Date of Final Oral Examination

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CHAPTER I: INTRODUCTION

Leishmaniasis Epidemiology:

Leishmaniases are caused by the *Leishmania* protozoan parasite and transmitted by sand flies. Leishmaniases are distributed around tropical, subtropical, and arid regions of the world and are considered endemic to more than 98 countries. It is estimated that 350 million people are at risk of being infected by *Leishmania,* and there are 12 million new cases every year (Alvar *et al.* 2012, Torres-Guerrero *et al.* 2017, Burza *et al.* 2018),). There are more than 20 species of *Leishmania* that can cause human diseases (Ashford 2000). The common clinical forms of leishmaniases are visceral (VL), cutaneous (CL), and mucocutaneous (MCL). The most threatening form of the disease is VL, which is caused by *Leishmania donovani* and *L. infantum/L. chagasi*. This disease affects the body's internal organs, including the spleen, liver, bone marrow, and it is usually fatal if left untreated. It has been stated that more than 90 percent of the world's reported VL cases occur in Bangladesh, India, Nepal, Sudan, and Brazil (Mondal *et al.* 2009, Bern *et al.* 2010, Burza *et al.* 2018). Cutaneous Leishmaniasis (CL) is the most recurrent form of the disease and can be caused by different *Leishmania* species. At the location of sand fly bite, a very particular skin ulcer is produced, which may persist for an extended period and can cause disfiguring scars. It is estimated that 90 percent of CL are found in Iran, Saudi Arabia, Algeria, Afghanistan, Syria, Sudan, Peru, and Brazil. Even without treatment, CL rarely causes severe illness but may produce scarring from the lesions (Desjeux 2004, Burza *et al.* 2018). Mucocutaneous leishmaniasis (MCL) is the least common form of the disease, mostly occurring in Central and South America. The MCL infection usually targets the mouth, nose, pharyngeal nodes and can produce massive destruction of the mucosal membranes leading to extreme disfigurement (Murray *et al.* 2005, Burza *et al.* 2018).

Leishmaniasis is considered a neglected tropical disease due to its prevalence in regions of the world with a high index of poverty (World Health Organization 2010) The lack of funding, reporting, good health care programs, and prevention systems are a current concern (Hotez *et al.* 2012). Additionally, key drivers of *Leishmania* are human migration, deforestation, urbanization, conflict, and climatic change - along with the increment of the density and distribution of sand flies (Wasserberg *et al.* 2003, Maroli *et al.* 2013, Showler and Boggild 2017) and the lack of early detection of the infection and vaccination raise the need for control of the disease.

LEISHMANIA PARASITES LIFE CYCLE:

Leishmania has a digenetic life cycle that alternates between the vertebrate host and the sand fly vector (Dostálová and Volf 2012). When a sand fly ingests blood from an infected vertebrate host containing phagocytic cells with the parasite's immotile stage - the amastigotes - the changes in environmental conditions (from host to vector) initiate a morphological transformation. Once in the midgut of the sand fly, the amastigote transforms into the extracellular motile stage -the promastigote- which attaches to the midgut. It then divides and travels to the proboscis of the sand fly. During the subsequent blood meal, infected sand flies inject the promastigote to the vertebrate host, infecting phagocytic cells and transforming into the amastigote. The amastigotes multiply and begin to infect other cells causing the spread of the infection in the vertebrate host (Claborn 2010, Dostálová and Volf 2012).

Sand Flies

Phlebotomine sand flies (Diptera: Phlebotominae) are part of the family *Psychodidae*, which contains over 800 species (Seccombe *et al.* 1993, Bates *et al*. 2015). From the six recognized genera, only two contain hematophagous species: *Lutzomyia* in the New World and *Phlebotomus* in the Old World (Maroli *et al.* 2013). Sand flies are small flies with a wide geographic distribution over arid, tropical, and subtropical regions(Lane

1993). They are a significant public health concern around the world because they transmit zoonotic diseases to humans, such as bartonellosis, arboviral infections, and leishmaniasis (Ready 2013).

Sand flies, like all true flies, are holometabolous, and their life cycle consists of four complete life stages: egg, larva (4 instar stages), pupa, and the adult. Although sand flies do not require standing water for their development, relatively moist and warm environments are essential for survival. Due to their susceptibility to dehydration, most species of sand flies are nocturnal, and they often find suitable settings for development in animal burrows, human habitations, and other protected habitats (Claborn 2010).

Vector Control:

Vector control using broadcast insecticides is the principal approach used to control vector-borne diseases. Implementing vector control strategies limits pathogen transmission by minimizing or eliminating contact between humans and the vector (Wilson *et al.* 2020). To prevent the spread of *Leishmania*, some conventional vector control strategies like the spraying of insecticides, indoor and outdoor residual applications, the use of nets as physical barriers, destruction of breeding sites, and the use of repellents and topical insecticides have been implemented around the world (Cetin and Ozbel 2017). However, sand flies have been developing resistance to chemicals used as insecticides; for example, in India, a study has confirmed that in endemic areas of Kala Azar, *P. argentipes* has become resistant to Dichlorodiphenyltrichloroethane -DDT- (Dhiman and Yadav 2016).

Consequently, other ecological control strategies have been studied. One of these strategies is based on a systemic controlled approach, where insecticides are given orally or topically applied to a host. In 2018, a controlled study was performed using cattle as the host (Poché *et al.* 2018); they investigated the efficacy of providing a single dose of fipronil against *P. argentipes*; They found that fipronil kills 100% of both adults

and larvae over 21 days (Poché *et al.* 2018). In a more recent study, using gerbilline reservoir species *Meriones tristrami* and *Meriones crassus*, the residuality of fipronil and its effect on adult sand flies was evaluated; they found that residues persist for >31 days and that the survival of sand flies was significantly reduced when fed on fipronil treated *M. tristrami* and *M. crassus* (Tsurim *et al.* 2020).

Another control strategy is the attract-and-kill method, where the main goal is to lure the vector to the insecticide using baits or attractants. A study from 2015 used attractive toxic sugar bait (ATSB) indoors to bait and kill sugar feeding mosquitoes in five villages from the Niger river in Mali; They found that after a 50-day trial, there was a significant reduction of 90% of female and 93% of male mosquito populations (Qualls *et al.* 2015). Another study demonstrated that ATSB, when applied to vegetation or in bait stations, significantly reduced the female and male population densities of sand flies (Qualls *et al.* 2015). In addition to ATSB, sex pheromones have also been studied. Using laboratory and field bioassays, Chelbi *et al.* (2011) demonstrated that *P. papatasi* females are attracted to the pheromones emitted by small groups of males or a mixed group of females and males. They also found that there is no female response if no male odor is present, and that larger groups of males become repellent to young female flies suggesting that the presence and attraction to a sex pheromone is both sex and concentration-dependent (Chelbi *et al.* 2011).

Over the past decade, a method considered for the control of pathogens' transmission is based on the insect's microbiome. The gut microbiome of hematophagous insect vectors has been studied in laboratory-reared and wild-caught insects such as mosquitoes, triatomines, and sand flies (Azambuja *et al.* 2005). In mosquitoes, it was demonstrated that exposure to environmental microorganisms has a significant influence on the composition of the microbiome (Hegde *et al.* 2018). A study that aimed to characterize the bacterial communities of three different species of mosquitoes *An. gambiae, Ae. aegypti* and *Ae. atropalpus*, when reared under identical conditions,

showed that the anautogenous species (*An. gambiae* and *Ae. aegypti*) shared more similar bacterial communities than with the autogenous species (*Ae. atropalpus*), suggesting that larvae acquire their microbiome from the environment (Coon *et al.* 2014). Studies also indicate that the insect's gut is a selective habitat for microbes, which creates a difference in the bacterial diversity of the gut microbiome (Guégan *et al.* 2018, Hegde *et al.* 2018). Additionally, there is strong evidence that supports that insect gut microbiome plays a vital role in the growth, development, environmental adaptation, tolerance to toxic diets, higher resistance to pathogens, performance (Coon *et al.* 2014, Guégan *et al*. 2018, Sontowski and van Dam 2020), and vectorial competence (Weiss and Aksoy 2011, Boissière *et al.* 2012), which intensifies the need to investigate and understand the relationship between the host and it's microbiome.

MICROBIOME AND ITS INFLUENCE ON INSECT DEVELOPMENT:

Several studies on the microbiome of insects and their effects on development have been performed in the past two decades. In mosquitoes and some other dipterans, variation in the bacterial communities depending on the developmental stage and their feeding status has been observed (Gonzalez-Ceron *et al.* 2003, Sontowski and van Dam 2020). Using axenic (i.e., free of microorganisms) eggs from different species of mosquitoes, Coon *et al*. (2014) determined that although hatching rates were not different from non-sterilized eggs, axenic larvae that were kept under sterile conditions (sterile food and water) could not develop and survive for more than five days unless exposed to bacteria. They also found that development is not related to certain bacterial species or communities, which indicates that although they require microbes for development the microbiome composition is not important (Coon *et al.* 2014). In contrast, studies of *An. gambiae* have shown that when mosquito larvae were infected with *Asaia sp.*, there was a significant acceleration of the developmental rate showing evident differences after 72 hours (Mitraka *et al.* 2013). Regarding sand flies, Peterkova-Koci *et al.* (2012) demonstrated that the mortality of *Lutzomyia longipalpis* larvae increases 25% when reared in a medium free of bacteria (sterile medium). They

demonstrated that sand fly larvae reared on sterile medium had longer developmental time when compared to larvae reared in unsterile rabbit feces. Additionally, when bacterial isolates from the rabbit feces were inoculated to the sterile medium either individually and in combination, they supported larval development (Peterkova-Koci *et al.* 2012).

MICROBIOME AND ITS INFLUENCE ON ATTRACTION AND OVIPOSITION OF SAND FLIES:

In mosquitoes and sand flies, there is evidence that bacteria are a source of larval food (Lindh 2007; Maleki-Ravasan *et al.* 2015). Additionally, it has been demonstrated that the volatiles released by some of these bacteria influence host preferences and locations for oviposition. For example, it has been shown that microorganisms obtained from leaf infusions of white oak and bamboo produced specific bacteria-associated carboxylic acids and methyl esters that served as potent oviposition stimulants for gravid *Ae. aegypti* (Ponnusamy *et al.* 2008).

In sand flies, it was demonstrated that the new world sand fly *Lu. longipalpis* has an oviposition preference (85.8 %) to rabbit feces containing microbes compared to sterile rabbit feces (14.2 %), suggesting that the microbiome present in rabbit feces produces chemical cues that stimulate oviposition (Peterkova-Koci *et al.* 2012). It has also been hypothesized that the attraction to larvae rearing substrate in *P. papatasi* sand flies is related to the microbiome (Marayati *et al.* 2015). As larvae forage and defecate into the medium, the gut microbiome is thought to alter the composition of the rearing substrate, making it more attractive to sand flies. Using a multiple-choice oviposition assay and an attraction assay Marayati *et al.* (2015) tested six different media, water (control), a medium where second and third instar larvae have been reared (i.e., conditioned medium), fourth instar and pupae conditioned medium, medium left from the emergence of adult flies (i.e., expired medium), larval food (a mixture of rabbit feces and rabbit chow), and rabbit feces. As a result, they were able to demonstrate that sand flies are more attracted and oviposited more eggs in the second/ third instar conditioned medium

followed by the fourth/ pupae conditioned substrate, indicating that the conditioning process (the feeding and defecating of larvae) could possibly be attributed to the involvement of their gut microbiome.

In another study, the bacteria obtained from the most attractive conditioned medium (second/third instar larvae rearing medium) was cultured and isolated (Kakumanu *et al.* 2020). They identified 12 bacteria (*Leucobacter holotrichiae, Microbacterium sorbitolivorans, Sphingobacterium daejeonense, Cellulosimicrobium cellulans, Luteimonas padinae, Alcaligenes faecalis, Sphingobacterium* sp*. ,Brevibacterium sediminis, Stenotrophomonas indicatrix, Bacillus zhangzhouensis, Pseudomonas nitrititolerans, Brevundimonas olei*), and found that there are some dose related attraction effects, particularly with a significant negative dose-dependent attraction, where female sand flies are attracted to the mix of twelve bacterial isolates only in a low cell density, but were significantly repelled at high cell concentrations. Overall, these results suggested that microbial volatiles play an essential role in the attraction process of *P. papatasi* to a breeding site (Kakumanu *et al.* 2020).

To evaluate if the attraction of gravid sand flies was associated with the larval conditioning process or simply due to medium's aging, Matthew Miller, a former graduate student in the Wasserberg lab, performed a preliminary experiment where he used 9000 first instar larvae to condition substrate for five weeks. He set up one fresh larval food jar (conditioned) and left another larval food jar -with no larvae- to age (aged) for the same amount of time. He performed attraction bioassays for each larval stage and 16S amplicon sequencing of the substrates. For the sequencing analysis a baseline sample was collected from the initial two jars. Fourteen days later, two samples were collected from each substrate (aged and conditioned) to evaluate any differences between the bacterial community structures in the media. In the attraction bioassays, he found a significant attraction for conditioned medium when compared to the aged medium until it expired; at that point, the medium became slightly repellent. This was

consistent with the larval medium conditioning hypothesis. However, based on 16S amplicon sequencing the bacterial communities of conditioned and unconditioned media did not appear to differ (Figure 1). Yet, both conditioned and unconditioned media appeared to differ substantially when compared to their respective baseline samples (Figure 1) (Miller, 2017. Unpublished data).

We recently performed attraction and oviposition bioassays in order to determine if gravid sand flies are attracted to and stimulated to lay more eggs on rearing medium conditioned by sand fly larvae (reared for 3 weeks until reaching 2nd/3rd instar stage) compared with larval rearing medium aged to the same amount of time (and under the same conditions) (Faw *et al.* 2021). We also evaluated if attraction and oviposition responses increase with time in comparison to the starting fresh larval food baseline. We found a significant 'time effect' in the sense that when compared to the baseline, both attraction and oviposition responses have increased. Larval conditioning of the rearing medium enhanced gravid female's attraction in comparison to the aged medium but did not have a significant effect on the oviposition response (Faw *et al.* 2021).

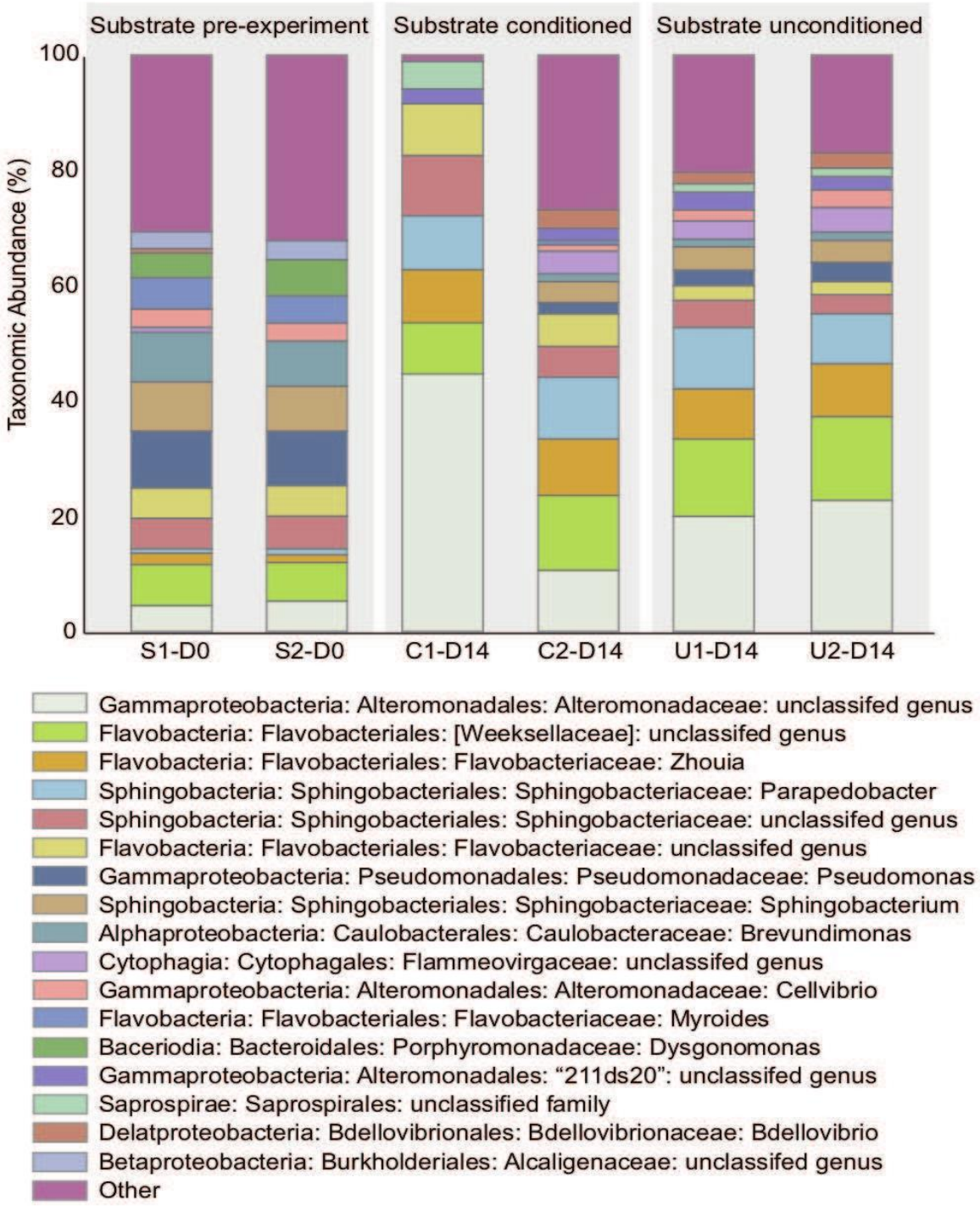


Figure 1: Description of the taxonomic abundance found in the preliminary study performed by Mathew Miller. S1 and S2 were collected as baseline samples, C1-D14 and C2-D14 were samples of the conditioned medium after fourteen days and samples, U1-D14 and U2-D14 were taken from unconditioned medium after fourteen days of aging.

Significance of the study:

The general goal of my thesis was to evaluate if the temporal and larval conditioning effects (Faw *et al.* 2021) on sand fly's oviposition behavior are driven by corresponding changes in the medium's bacterial community structure and composition. Behavioral studies suggest that the presence of microbes influences sand fly attraction and oviposition responses (Marayati *et al.* 2015; Kakumanu *et al.* 2020; Faw *et al.* 2021; Miller, unpublished data 2017). However, there remain many significant gaps in my understanding of the mechanisms responsible for this attraction. For example, it is still unknown what microbial communities are present in aged and conditioned rearing substrates, and if these microbes are important for larval growth and development. We also do not know the extent to which the aging and larval conditioning affects the microbial communities of the rearing substrate. Characterizing changes in the structure of the microbial communities in sand fly rearing substrates will provide insight into how conditioning and aging processes affect sand fly oviposition behavior and development. This knowledge could allow us to identify microorganisms or microbial communities that can act as sources for oviposition attractants or can impair sand fly's development and therefore be used as a biological control method.

Study Question

How do aging and larval conditioning affect bacterial community structure and dynamics of sand fly rearing substrates?

Study Goal

Since the impacts of aging and larval conditioning on the bacterial community has not been directly studied, the goal of this study was to characterize the successional dynamics of the bacterial community of aging and larval conditioned sand fly rearing substrates.

Specific Aims

The overall goal of this project was to determine how the conditioning and aging processes impact the bacterial community structure and abundance of sand fly rearing substrates. To answer this question, we addressed two specific aims:

Aim 1:

Compare bacterial community secondary succession dynamics between aged and larval conditioned media.

Hypothesis:

I hypothesized that larval conditioning of the rearing substrate (through digestion, breakdown, filtration of nutrients, and excretion) and aging (decomposition) processes would impact the bacterial community structure.

Prediction:

I predicted that there would be significant differences between the bacterial community structure of the aged and conditioned media. I also predicted that the bacterial communities of the larval rearing media would change over time.

Aim 2:

Determine the effects of larval and post larval stages on bacterial community structure and composition of the larval rearing media.

Hypothesis:

I hypothesized that the bacterial community structure will differ between larval and post-larval stages.

Prediction:

I predicted that there would be significant differences between the bacterial community structure of the larval and post-larval stages of the conditioned media and the corresponding timepoints of the aged media (weeks 2/4, and weeks 6/9). I also predicted that the bacterial community structure of the larval and post-larval stages of the conditioned media would differ from each other.

CHAPTER II: METHODS

Sand fly colony maintenance:

P. papatasi sand flies from Abkük, Turkey (2004) are maintained at the Ecology of Infectious Disease laboratory at the University of North Carolina at Greensboro. The sand flies are reared by following the mass-rearing methods described by Lawyer et al.(2017). They are maintained in incubators at 26°C, 80 % RH, at 14:10 light: dark photoperiod cycle. Female flies are blood-fed on live anesthetized mice (Harlan) (UNCG protocol #: 20-0011, June 2020). Adults are fed with 30% sucrose solution. Larvae are maintained in Nalgene jars with a 2.2 cm layer of Plaster of Paris on the bottom to ensure that moisture and drainage are kept and fed with fresh larval food, which is a mixture of fresh rabbit feces with rabbit chow (Purina) at a 1:1 ratio, and is fermented for three weeks in a chamber, air-dried, and ground into a powder.

Experimental design:

To characterize the bacterial communities of the conditioned and aging larval rearing media, all the materials including paintbrushes, Eppendorf tubes, wood sticks, and filter papers were autoclaved to avoid contamination of the samples. Fresh larval rearing medium was used as the starting point (baseline) of the experiment. This source was utilized to produce two types of experimental media: larval conditioned medium and non-larval conditioned but aged medium. Each medium had five replicates. Both media types were kept under similar conditions. Samples from the substrates were taken every 2 weeks for 6 weeks spanning the entire larval developmental period to adulthood. In the ninth week, one last group of samples were collected to obtain expired media samples. DNA extraction, library preparation and metagenomic sequencing were performed with each of the substrate samples (Figure 2).

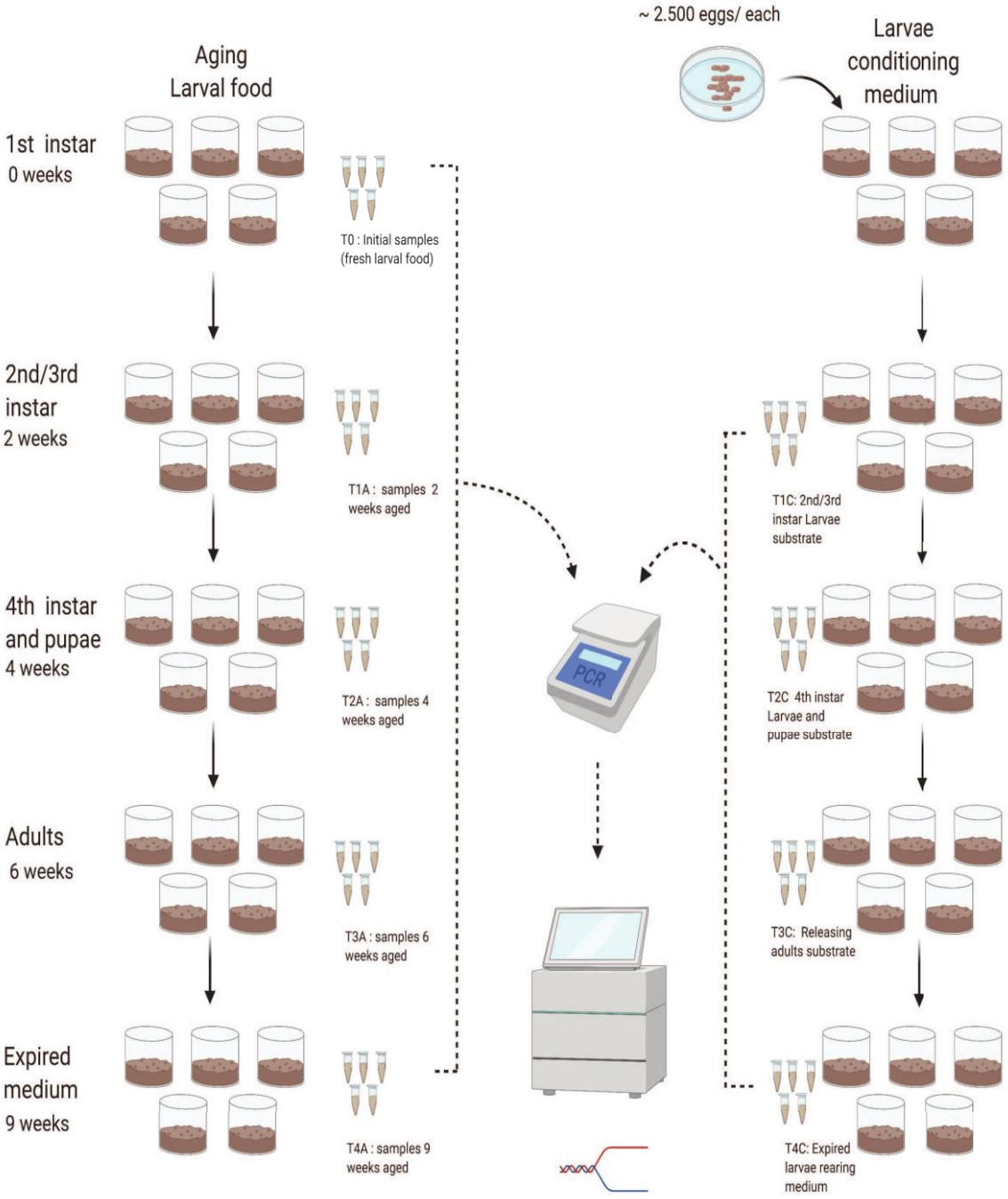


Figure 2: Description of the sample collection and sequencing processes. Sample collection began at week 0. After two weeks, samples from the substrates were collected again. The same procedure was repeated for the fourth week, sixth week and ninth week.

Conditioning and aging of larvae rearing medium:

To obtain a conditioned medium, approximately 2,500 eggs were placed in plastered 500 ml Nalgene jars. Once the larvae hatched, they were fed with the same fresh larval food daily for the first larval stage and then twice a week (Monday and Friday) from the second stage up to the fourth larval stage. For the aging medium, 500 ml Nalgene Jars with no eggs were used to put fresh larval food (at the same time and similar amount as the conditioned media) and left to decompose over time.

Sample Collection:

Sterilized 1.5 ml Eppendorf tubes, containing approximately 0.4 mg of each sample were collected using autoclaved wood sticks. All samples were labeled according to each group, replicate, and phase. When the larvae hatched, sample collection began at “Time point 0” with fresh larval food (baseline). After two weeks, which is “Time point 1,” samples from the substrates were collected again. The same procedure was repeated for “Time point 2” (fourth week), “Time point 3” (sixth week) and “Time point 4” (ninth week). A total of 45 samples were obtained and kept at -20ºC for further metagenomic analysis.

Metagenomic Microbial Characterization:

DNA EXTRACTION

DNA extraction of the samples collected from the experiment were obtained using the Pure link Microbiome DNA Purification Kit. To confirm that DNA was successfully extracted, DNA electrophoresis was performed. DNA was obtained from all 45 samples, which were used for further analysis.

LIBRARY PREPARATION AND SEQUENCING

The bacterial 16S rRNA gene V4 variable region was amplified using a polymerase chain reaction (PCR) with the primers 515F and 806R with illumina platform specific sequence adaptors attached: Hyb515F_rRNA: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGYCAGCMGCCGCGGTA -3' and Hyb806R_rRNA5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTAAT- Each reaction contained sterile distilled water, High-Fidelity (HF) buffer, the primers mentioned above, dNTP mix, and Phusion polymerase (New England Biolabs®) with the following reaction conditions: denaturation at 98° C for 30 s, followed by 20 cycles of 98° C for 10 s, 58° C for 30 s, and 72° C for 30 s, and a final extension at 72° C for 7 min. PCR products were electrophoresed in 2% agarose gel to confirm successful amplification. From the initial 45 samples, only 40 samples were successfully amplified (despite multiple attempts of amplification).

Amplified PCR products were cleaned by using a bead cleaning procedure with AxyPrep Mag beads. Once DNA was clean, Illumina index barcodes were added to the amplicons using the following reaction conditions: denaturation at 98° C for 2 minutes, followed by 15 cycles of 98° C for 10 s, 55° C for 30 s, and 72° C for 30 s, and a final extension at 72° C for 7 min. A final bead cleaning was performed, then DNA was quantified using a Qubit® 2.0 (Life Technologies) and all samples were pooled to equal concentrations for sequencing. The amplicons were sequenced using an Illumina iSeq100 (2x150 paired-end reads) in the Raymann Laboratory at the University of North Carolina at Greensboro. The samples were demultiplexed using the Illumina BaseSpace Sequence Hub (https://basespace.illumina.com/).

BIOINFORMATIC ANALYSIS

Forward and reverse paired-end reads were merged using FLASH (Magoc and Salzberg 2011) with minimum overlap of 5bp. Joined reads were quality filtered in QIIME2 (Bolyen *et al.* 2019) using the DADA2 pipeline (Callahan *et al.* 2016), which includes removal of PhiX and chimeric reads. Taxonomic assignment of the reads was performed using script “qiime feature-classifier classify-consensus-blast” using a classifier that was trained on the SILVA 16S reference database (SILVA Release 138.1, (Pruesse *et al.* 2007) and based on the specific primers we used for amplification and the length of our sequence reads. Taxonomic assignments were based on 97% or more sequence identity. Reads were filtered to remove all sequences corresponding to mitochondria, chloroplast, and unassigned taxa. Further filtering was performed to remove any taxa present in less than three samples or that were represented by fewer than 10 reads to account for sequencing errors.

The 16S sequence data was processed and analyzed using QIIME2 (Boylen *et al.* 2019). In order to perform a phylogenetic diversity analysis, a tree was created using the script “qiime phylogeny align-to-tree-mafft-fasttree” (Price *et al.* 2010, Katoh and Standley 2013). Alpha and beta diversity analyses were done using the script “qiime diversity core-metrics-phylogenetic” with a sampling depth of 1000. This depth was chosen to maximize the number of samples included in the analysis while maintaining enough reads per sample to be able to capture the richness of the dataset (Figure 3). Rarefying to 1000 reads per sampled resulted in a total of 29 samples for downstream analysis (5 samples of week 0, 3 samples of aged week 2, 2 samples of aged week 4, 5 samples of aged week 6, 1 sample of aged week 9, 2 samples of conditioned week 2, 2 samples of conditioned week 4, 5 samples of conditioned week 6, and 4 samples of conditioned week 9). Sequencing depths of 3000 and 5000 reads were also analyzed but showed little to no differences in the total number of taxa observed. The alpha and beta diversity group significance were tested using the scripts “qiime diversity alpha-group-significance” and “qiime diversity beta-group-significance” respectively.

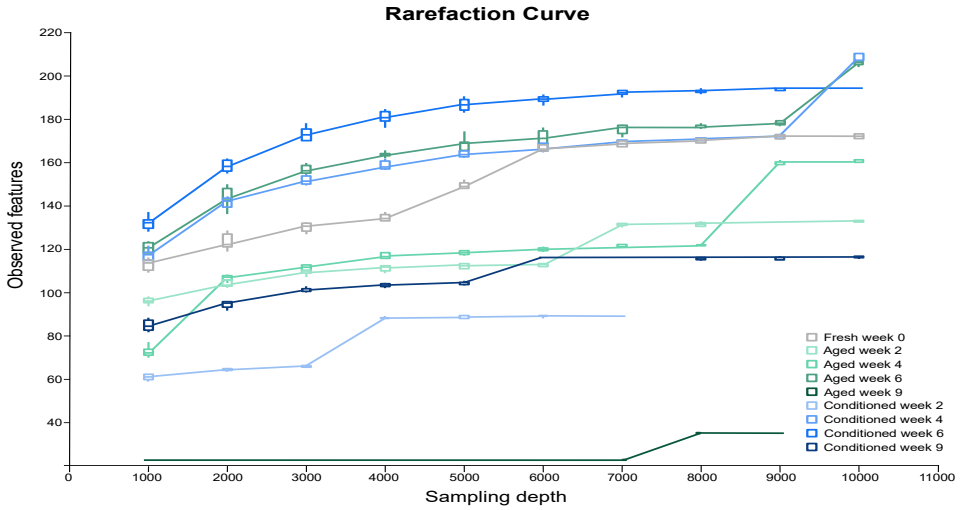


Figure 3: Rarefaction curve showing the sampling depths of the substrates (fresh larval food, and aged and conditioned media) per timepoint obtained after sequencing.

STATISTICAL ANALYSES:

The statistical analyses of the alpha and beta diversity were performed in Qiime2 (Boylen *et al.* 2019) using a Kruskal Wallis test and PERMANOVA test respectively. Data analysis including Faith's phylogenetic distances, Pielou's evenness index and number of Operational Taxonomic Units (OTU's) were used to describe alpha diversity. Boxplots and Wilcoxon rank sum tests were performed to study the relative abundance of individual taxa and their changes over time and across treatments. Bray-Curtis dissimilarity and weighted UniFrac were used to analyze beta diversity by comparing the differences between samples. To identify whether the changes were related to larval or post larval stages, alpha and beta diversity analyses were also performed on grouped samples, weeks 2 and 4 as larval stage and weeks 6 and 9 as post larval stage.

CHAPTER III: RESULTS

Aim 1. Evaluate the bacterial succession and community structure in aged and larval conditioned media.

To test the hypothesis that there is a difference between the bacterial community structure and successional dynamics of the aging and conditioned medium, 16S amplicon sequencing was performed on samples of baseline (fresh larval food -week 0-) and aging and conditioned media at different time points (week 2, week 4, week 6, and week 9). When analyzing the taxonomic diversity within the samples based on 97% sequencing clustering (Figure 4), a total 42 different taxa were identified (Figure 4). Because of the limitations of 16S amplicon sequencing as well as incomplete 16S reference databases, of the 42 taxa identified, I was able to classify 34 taxa to the genus, six to family (six taxa) and two taxa to order level.

Overall, I found that all three substrates shared two bacterial families (*Cyclobacteriaceae,* and *Pirellulaceae*) and 19 bacterial genera (*Glycomyces* sp*., Brachybacterium* sp*., Microbacterium* sp*., Cellulosimicrobium* sp*., Norcadiopsis* sp*., Patulibacter* sp*., Galbibacter* sp*., Sphingobacterium* sp*., Blastopirellula* sp*., Brevundimonas* sp*., Devosia* sp*., Ochrobactrum* sp*., Paracoccus* sp*., Cellvibrio* uncultured bacterium*, Cellvibrio* sp*., Pseudomonas endosymbiont of Onthophagus taurus, Pseudomonas* sp*., Luteimonas* uncultured bacterium*, Lysobacter* sp.). Fresh larval food and conditioned media shared two taxa absent in aged media (*Rhodococcus* sp., and *Microbacterium* ambiguous taxa), and fresh larval food and aged media shared two taxa not present in conditioned media (*Lactobacillus* uncultured bacterium*, and Parapusillimonas* sp*.*). With respect to aged and conditioned media, eight taxa (*Flavobacteriaceae, Parapedobacter* uncultured bacterium*, JG30 KF-CM45* uncultured bacterium*, Saccarimonadales* uncultured bacterium*, Planctomycetales* uncultured

bacterium, *LD29* uncultured bacterium, and *Luteolibacter* sp.) were found in both substrates that were absent in fresh larval food.

Figure 4: Microbial relative abundance of fresh larval food (week 0) and aged media and conditioned media at different time points (week-2, week-4, week-6, week-9). Each bar represents an individual sample. Colors represent the relative abundance of each taxa.

COMMUNITY LEVEL EFFECTS

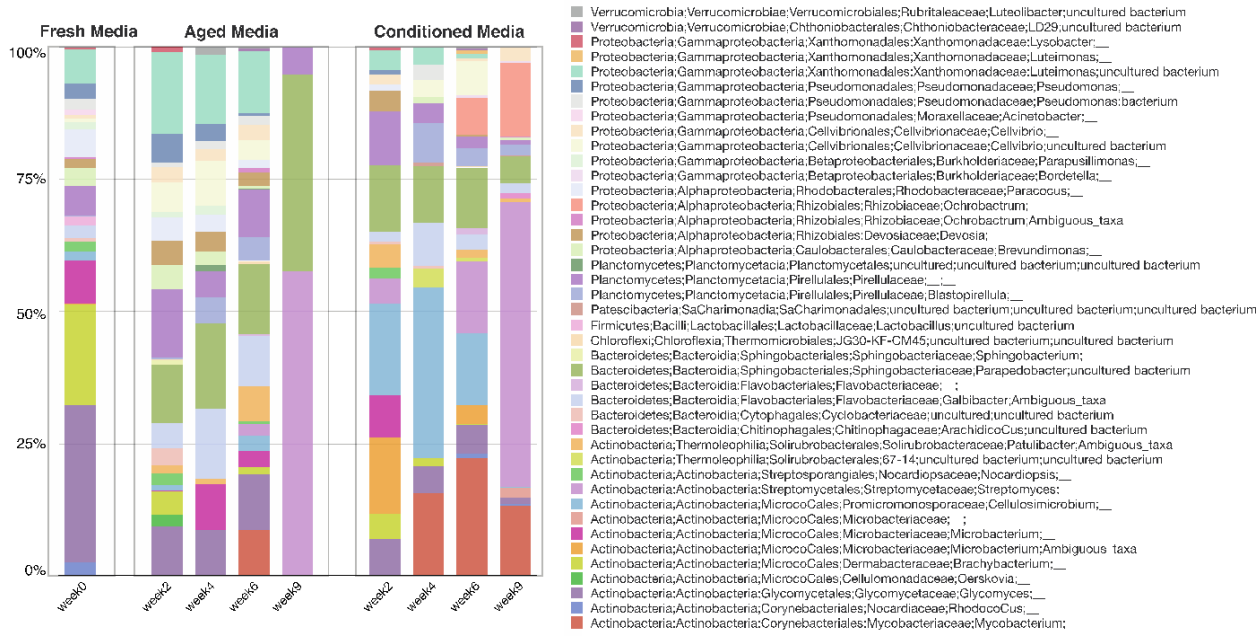


Figure 5: Relative abundance of the microbial communities of fresh larval food (week 0), aged media and conditioned media at weeks 2, 4, 6, and 9. Each bar represents the average relative abundance of taxa for all samples at the given timepoint. Colors represent the relative abundance of each taxon.

Based on the relative abundance bar plot (Figure 4), obvious differences were seen in the presence and abundance of the identified taxonomic groups between fresh larval food, conditioned media and aged media. The significance of taxon abundance across individual time points (weeks) was not tested due to low sample size, however, I found that some taxa were uniquely present in each substrate (see supplementary Appendix A). Only one taxon was found to be specific to fresh larval food (*Acinetobacter* sp.), and one was found to be specific to week-2 aged media (*Oerskovia* sp.). In terms of conditioned media, six unique taxa were found: *Luteimonas* sp. was only found in conditioned week-6, *Microbacteriaceae* was only found in conditioned week-9, *Ochrobactrum* sp. and *Bordetella* sp. were found in week-6 and week-9 conditioned media, *Arachidicoccus* uncultured bacterium was found in week-2 and week-9 conditioned media, and *Solirubrobacterales 67-14* uncultured bacterium was found in week-4 and week-6 conditioned media (see supplementary Appendix A).

ALPHA DIVERSITY

When analyzing alpha diversity to compare the diversity within the samples, I found that the bacterial communities of the conditioned, aged and fresh larval media showed some differences, but significant differences were only observed in phylogenetic diversity (a measure of biodiversity that incorporates the phylogenetic relatedness of the organisms). In terms of richness (Figure 6A) and evenness (Figure 6B), no significant differences were observed. However, for phylogenetic diversity (Figure 6C) I found that aged week-2 vs conditioned week-6 (*P*=0.025), aged week-6 vs fresh week-0 (*P*=0.047), and conditioned week-6 vs fresh week-0 (*P*=0.047) were significantly different.

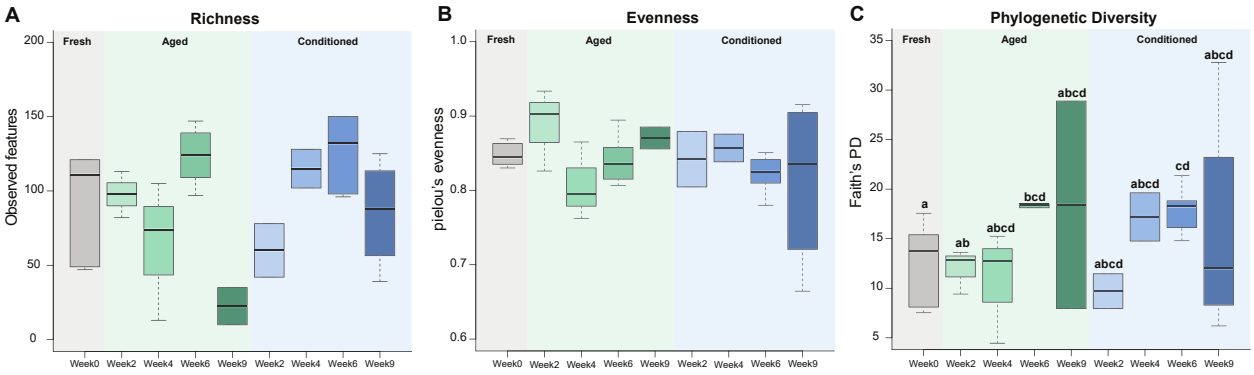
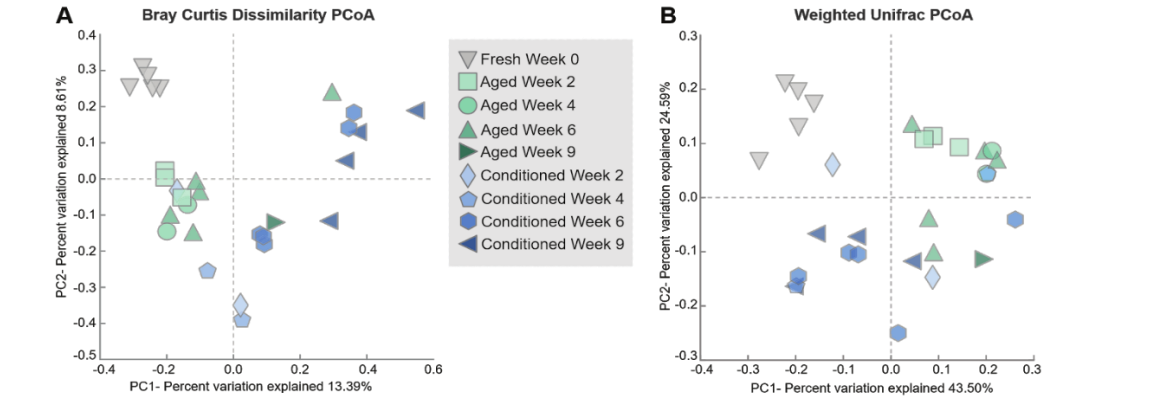


Figure 6: Alpha diversity measures of fresh larval food, aged media and conditioned media at each sampling time point based on A) richness with reference to the observed number of features(i.e. OTUs), B) evenness based on Pielou's evenness index and, C) and phylogenetic diversity measured by Faith's PD index. Letters signify p-value <0.05, Kruskal Wallis test. If no letters are present on a graph no significant differences were found.

BETA DIVERSITY

When assessing beta diversity to compare the bacterial community divergence between substrate types over time, significant differences were observed. Based on the quantitative non-phylogenetic beta diversity metric Bray Curtis (Figure 7A), significant differences were found. Specifically, significant temporal effects between fresh larval food week-0 and week-2 aged (*P*= 0.018), week-6 aged and week 0 fresh larval food (*P*

= 0.008), week-2 conditioned and week 0 fresh larval food (*P*= 0.047), week-6 conditioned and week-0 fresh larval food (*P*= 0.004), and week-9 conditioned and week 0 fresh larval food (*P*= 0.007) were found. Similarly based on weighted UniFrac, which incorporates the phylogenetic distances of the observed organisms (Figure 7B), significant differences were also observed between week-2 aged and week-0 fresh larval food (*P*= 0.019), week-6 aged and week-0 fresh larval food (*P*= 0.008), week-4 conditioned and week-0 fresh larval food (*P*= 0.047), week-6 conditioned and week-0 fresh larval food (*P*= 0.006), and week-9 conditioned and week-0 fresh larval food (*P*= 0.007). These results show evidence of temporal divergence in the bacterial community structure within each of the substrates. However, comparison of the bacterial communities of the aged and conditioned media did not reveal any significant differences between the substrates at the same respective time points. However, week-2 (*P*= 0.032) and week-6 (*P*=0.014) of the aged media are significantly different from week 9 conditioned, suggesting that after adult sand fly eclosure the community is altered significantly.



Statistically significant groups (PERMANOVA)

Group 1	Group 2	Permutations	pseudo-F	p-value	q-value
Aged_week2	Conditioned_week6	999	1.8205	0.015	0.1260
Aged_week2	Conditioned_week9	999	2.7258	0.035	0.1800
Aged_week2	Fresh_week0	999	2.0124	0.018	0.1260
Aged_week6	Conditioned_week9	999	2.0407	0.021	0.1260
Aged_week6	Fresh_week0	999	1.9280	0.008	0.0960
Conditioned_week2	Fresh_week0	999	2.0886	0.047	0.1855
Conditioned_week6	Fresh_week0	999	2.8073	0.004	0.0960
Conditioned_week9	Fresh_week0	999	4.3703	0.007	0.0960

Statistically significant groups (PERMANOVA)

Group 1	Group 2	Permutations	pseudo-F	p-value	q-value
Aged_week2	Conditioned_week9	999	9.0612	0.032	0.1920
Aged_week2	Fresh_week0	999	17.0956	0.019	0.1368
Aged_week6	Conditioned_week9	999	7.4504	0.014	0.1260
Aged_week6	Fresh_week0	999	16.3201	0.008	0.0960
Conditioned_week4	Fresh_week0	999	4.4739	0.047	0.2127
Conditioned_week6	Fresh_week0	999	9.1516	0.006	0.0960
Conditioned_week9	Fresh_week0	999	12.2096	0.007	0.0960

Figure 7: Beta diversity comparisons of fresh, aged, and conditioned media at each sampling time point. Principal coordinate analysis (PCoA) based on A) Bray Curtis dissimilarity B) Weighted UniFrac. Significance was tested using PERMANOVA with 999 permutations.

SPECIES SIGNIFICANCE

When comparing fresh larval food, aged media and conditioned media combined at all time points, 15 taxa (*Glycomyces* sp., *Cellulosimicrobium* sp., *Pirellulaceae* uncultured bacterium, *Brachybacterium* sp., *Streptomyces* sp., *Brevundimonas* sp., *Microbacterium* sp., *Parapedobacter* sp., *Devosia* sp., *Pseudomonas* sp., *Parapusillimonas* sp., *Lactobacillus* sp., *Ochrobactrum* sp., *Luteimonas* sp., and *Paracoccus* sp.) were found to be significantly differentially abundant between groups (Figure 8). Although we lacked power to statistically test differences at each time point, we were able to visualize differences in the relative abundance of each individual taxa (Appendix B).

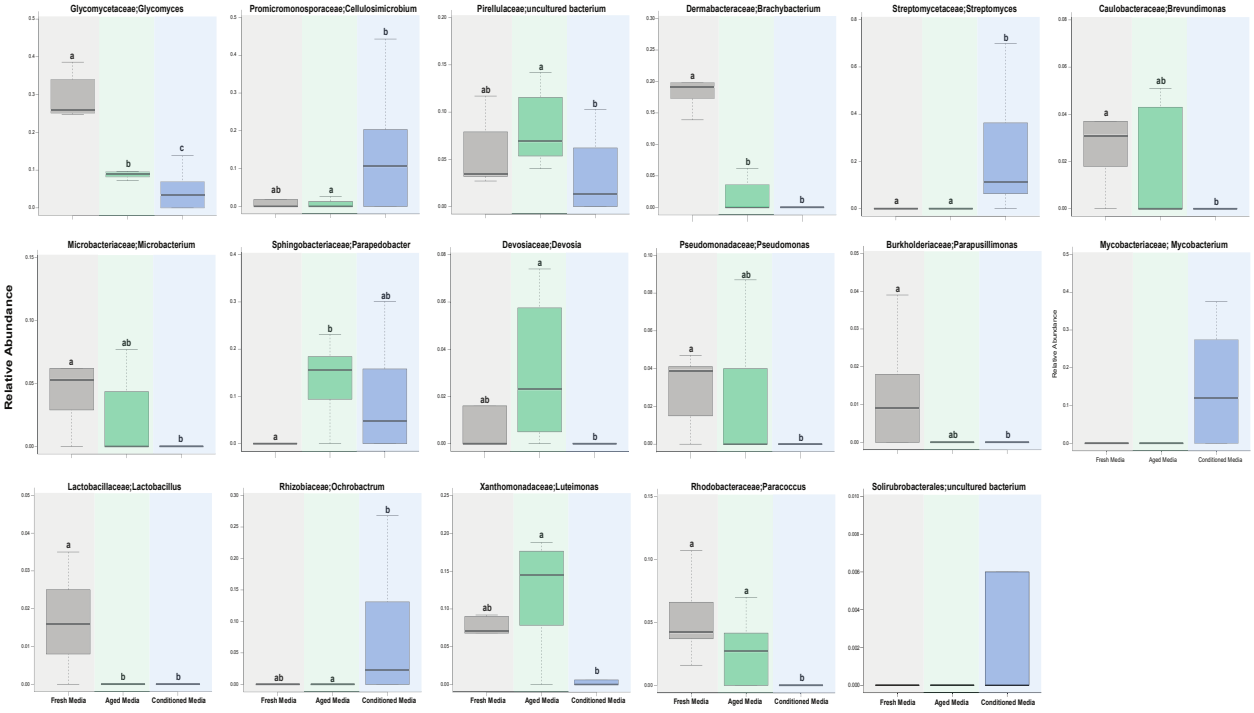


Figure 8: Differences in relative abundance of individual taxa found between fresh larval food, conditioned and aged media regardless of time point. Letters signify p-value <0.05, Wilcoxon rank sum tests with Bonferroni correction. If no letters are present on a graph no significant differences were found.

Aim 2. Determine the effects of larval and post larval stages on bacterial community structure and composition.

COMMUNITY LEVEL EFFECT

To determine the effects of larval and post-larval stages on the bacterial community structure and succession, the timepoints associated with larval development (weeks 2/4) and post-larval (weeks 6/9) were grouped, respectively, to evaluate if there were any differences in taxa. In terms of taxon presence/absence, (Figure 9 & Appendix A) the only taxon found across fresh larval food, larval and post-larval stages of the conditioned media, and the corresponding grouped time points of aged media was *Pirellulacea*. *Parapedobacter* uncultured bacterium was found in aged and conditioned media across larval and post-larval stages but not in fresh larval food. Another interesting finding was that two taxa (*Cellulosimicrobium* sp.*,* and *Galbibacter* sp.) were found in fresh larval food and larval and post-larval stages of conditioned media, but not in aged media. Additionally, when comparing bacterial community composition between the fresh larval food to that of aged medium at weeks 2/4, nine taxa differed (are not shared). Comparison of community composition of fresh larval food and that of weeks 6/9 (corresponding to the post-larval stage of the conditioned medium) revealed a difference in the presence/absence of 13 taxa. Interestingly, 12 taxa differed between fresh larval food and larval stage conditioned media (weeks 2/4), and 21 taxa differed between fresh larval food and the post larval stage conditioned media (weeks 6/9) (Figure 9, see supplementary Appendix A). Last, when the bacterial composition of the larval stage of the conditioned medium (weeks 2/4) and the corresponding period of the aged medium (weeks 2/4) were compared, they were found to differ in 13 taxa, while the post larval stages of the conditioned media (weeks 6/9) and the corresponding period of the aged media (weeks 6/9) differed in 15 taxa. (Figure 9, see supplementary Appendix A).

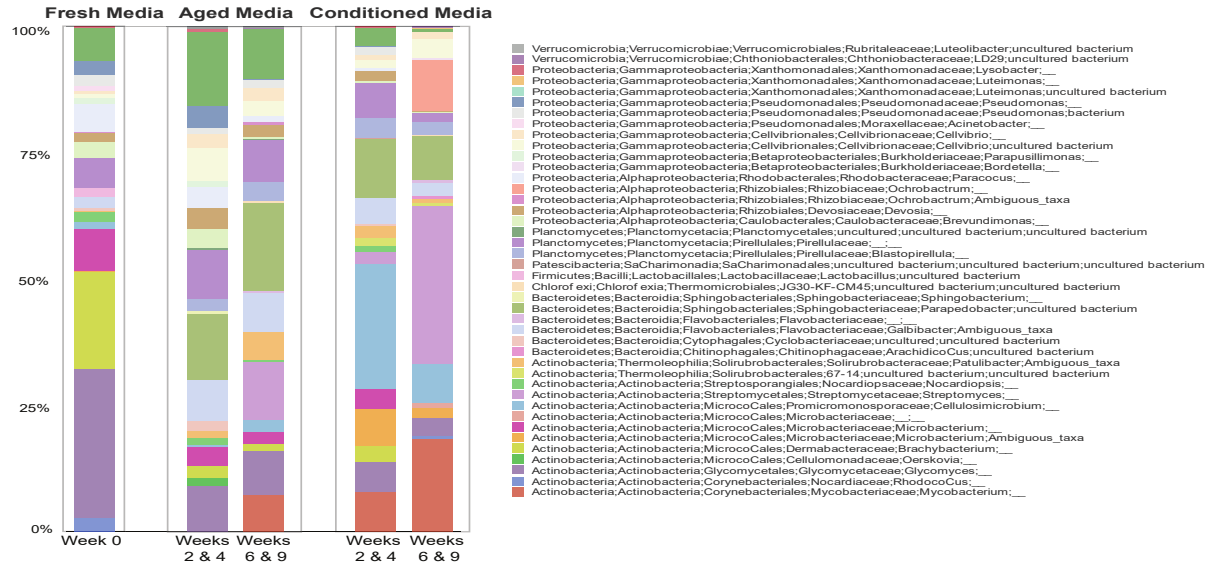


Figure 9: Relative abundance of the microbial communities of fresh larval food (week 0), aged media and conditioned media grouped as larval (weeks 2 & 4) and post larval stages (weeks 6 & 9). Each bar represents the average relative abundance of taxa for all samples at the given timepoint. Colors represent the relative abundance of each taxon.

ALPHA DIVERSITY

To compare the bacterial diversity within the grouped samples, alpha diversity was analyzed (Figure 10) and again only significant differences were found in the phylogenetic diversity analysis (Figure 10C). Phylogenetic diversity did not differ significantly between week-0 and weeks 2/4 for neither aged nor larval conditioned media. Although only the aged media showed a significant difference between the grouped time periods (weeks 2/4 aged to weeks 6/9 aged media), for conditioned medium, similar yet non-significant increases in richness and phylogenetic diversity from the larval to post-larval stages of conditioned media were observed (Figure 10 A and C). According to these results, the increase in the diversity appears to be associated with the taxonomic divergence found in my substrates from weeks 2/4 to weeks 6/9.

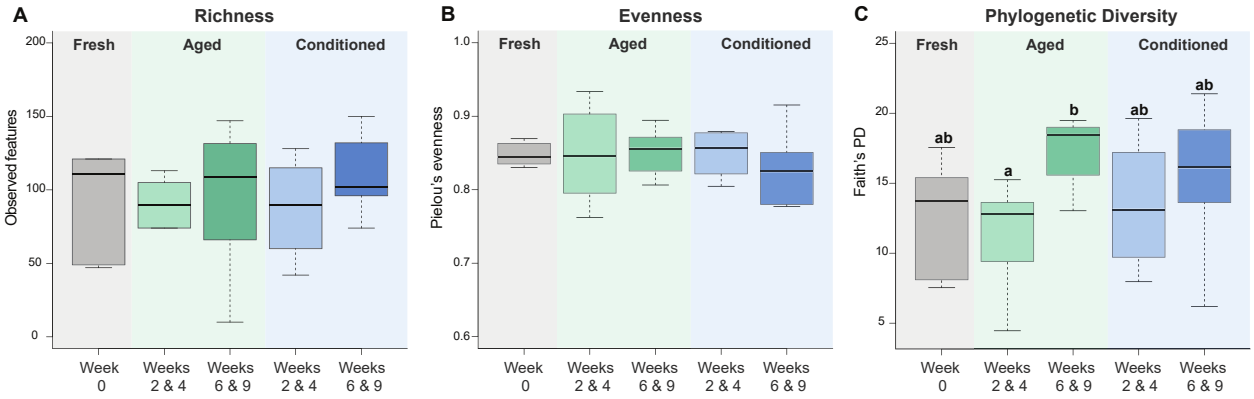
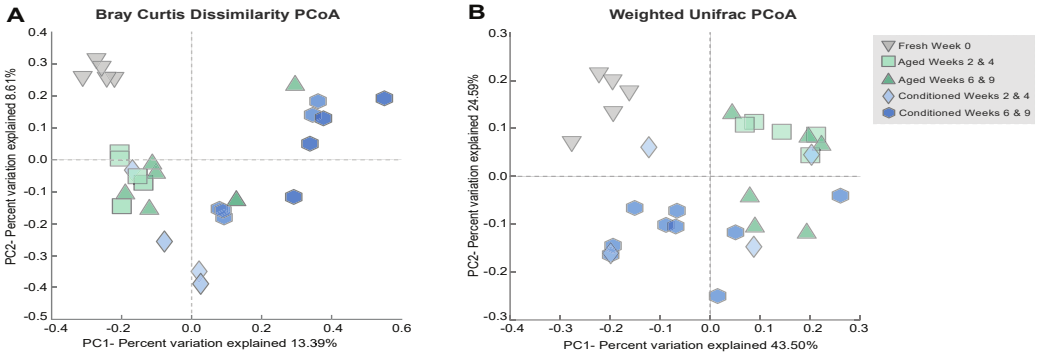


Figure 10: Alpha diversity measures regarding larval and post-larval analysis of fresh larval food, aged media and conditioned media. A) richness based on the observed number of features. B) evenness measured by Pielou's evenness index and C) and phylogenetic diversity measured by Faith's index. Letters signify p-value <0.05, Kruskal Wallis test. If no letters are present on a graph no significant differences were found.

BETA DIVERSITY

Analysis of beta diversity using Bray Curtis (Figure 11 A), indicated that the bacterial communities at weeks 2/4 of both larval conditioned (*P* = 0.005) and aged media (*P* = 0.001), as well as weeks 6/9 of both larval conditioned (*P* = 0.001) and aged media (*P* = 0.003) differed significantly from the community structure of fresh larval medium (Figure 11A). Bacterial community structure of aged and conditioned medium at weeks 2-4 differed significantly (*P* = 0.02). Interestingly, community structure of aged and conditioned media remained different even past the larval stage (weeks 6/9) (*P* = 0.022). However, when comparing community structure of weeks 2/4 to that of weeks 6/9 a significant change was only found for the conditioned medium (*P* = 0.003) but not for the aged medium (*P* = 0.851). When phylogenetic distances were incorporated, using weighted UniFrac (Figure 11 B), results remained consistent. First, both conditioned (*P* = 0.002) and aged (*P* = 0.006) media differed significantly from the baseline fresh medium at weeks 6/9, but conditioned media differed significantly as well at weeks 2/4 (*P* = 0.028). Second, bacterial community structure did not differ between the larval and post-larval stage of the conditioned medium. In contrast to the Bray Curtis analysis, weighted UniFrac did not reveal differences in community structure between

conditioned and aged medium from the same time period. Overall, these results show that the community structure of larval and post-larval stages of the conditioned media differ significantly, but they do not differ between the corresponding time points of the aged medium.



Statistically significant groups (PERMANOVA)

Group 1	Group 2	Permutations	pseudo-F	p-value	q-value
A_week2_4	C_week2_4	999	1.2315	0.02	0.0244
A_week2_4	C_week6_9	999	1.6024	0.001	0.0033
A_week2_4	F_week0	999	1.3359	0.001	0.0033
A_week6_9	C_week2_4	999	1.1290	0.008	0.0114
A_week6_9	C_week6_9	999	1.2507	0.022	0.0244
A_week6_9	F_week0	999	1.3150	0.003	0.0060
C_week2_4	C_week6_9	999	1.5035	0.003	0.0060
C_week2_4	F_week0	999	1.4921	0.005	0.0083
C_week6_9	F_week0	999	1.8550	0.001	0.0033

Statistically significant groups (PERMANOVA)

Group 1	Group 2	Permutations	pseudo-F	p-value	q-value
A_week2_4	C_week6_9	999	1.8349	0.014	0.0467
A_week6_9	F_week0	999	2.0603	0.006	0.0300
C_week2_4	F_week0	999	1.6697	0.028	0.0700
C_week6_9	F_week0	999	2.8079	0.002	0.0200

Figure 11: Beta diversity comparisons of fresh, aged, and conditioned media evaluating the effect of larval and post larval stages. Principal coordinate analysis (PCoA) based on A) Bray Curtis dissimilarity B) Weighted unifrac. Significance was tested using PERMANOVA with 999 permutations. Pairwise comparisons that were significantly different (P<=0.05) are shown in the tables below each PCoA plot.

SPECIES SIGNIFICANCE

Despite obvious differences in presence/absence of taxa, comparison of individual taxon relative abundance between the substrates when grouped into larval and post-larval stages only revealed nine taxa to be significantly differentially abundant (Figure 12): *Glycomyces* sp., *Pirellulaceae* uncultured bacterium, *Brachybacterium* sp., *Streptomyces* sp., *Microbacterium* sp., *Pseudomonas* sp., *Lactobacillus* sp., *Ochrobactrum* sp., and *Paracoccus* sp.

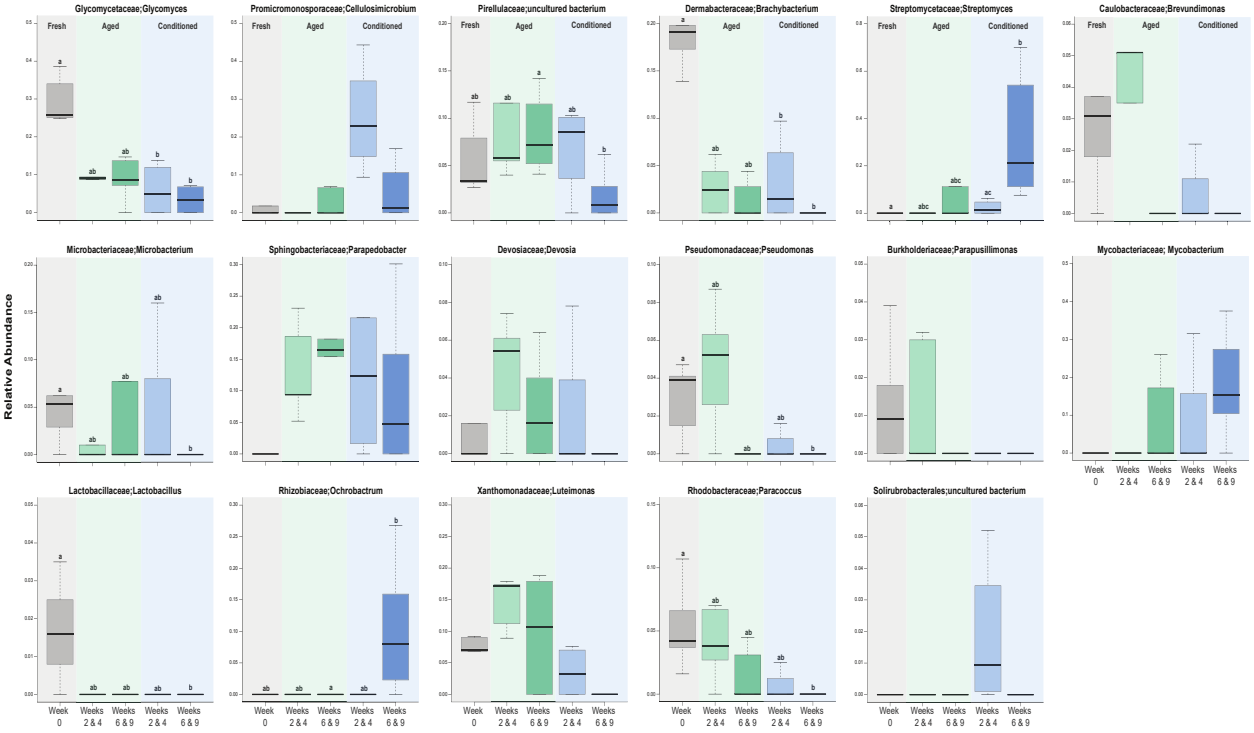


Figure 12: Differences in the relative abundance of taxa across fresh larval food, aged and conditioned media when grouped in larval (weeks 2 & 4) and post larval stages (weeks 6 & 9). Letters signify p-value <0.05, Wilcoxon rank sum tests with Bonferroni correction. If no letters are present on a graph no significant differences were found.

CHAPTER IV: DISCUSSION

To investigate the effect of aging and larval conditioning on the bacterial community structure and succession of sand fly rearing media, I characterized the bacterial community of aged and conditioned substrates at different time points. I hypothesized that the aging and larval conditioning processes would impact the bacterial community structure of the larval rearing media. I predicted that: (1) Bacterial community structure would differ over time for both aging and larval-conditioned media; and that (2) Bacterial community structure would differ between larval and post-larval stages of the conditioned medium and the same time-period of the aged media. Overall, my results were consistent with these hypotheses showing successional changes in bacterial community structure over time for both rearing media types and demonstrating that the bacterial community of these media types differ both during the larval stage and post the larval stage time points.

Alpha diversity comparisons of the fresh larval food, conditioned media and aged media revealed significant differences in phylogenetic diversity, indicating temporal phylogenetic divergence that increases over time. However, no significant differences were observed between aged and conditioned media when compared to fresh larval food (Week-0). Beta diversity analyses showed that fresh larval food, aged, and conditioned media all differed from each other, and time significantly impacted the community structure within each substrate type. When evaluating the effects of larval and post-larval stages on the bacterial community structure and succession, alpha diversity analyses only showed significant differences in phylogenetic diversity, which again provides evidence of a temporal taxonomic divergence. Regarding beta diversity, I found that conditioned media at larval and post-larval stages significantly differed from aged media and fresh larval food suggesting a larval driven community that drifts

between the larval and post-larval stage, further supporting that larvae induce changes in the bacterial community of the media.

I identified 42 taxa (based on 97% sequence identity clustering) from fresh larval food, conditioned media, and aging media. The three substrates shared 19 taxa, and each one had at least one taxon that was not present in the other substrates. *Acinetobacter* sp. was only present in fresh larval food, *Oerskovia* sp. was unique to aged media, and there were six taxa that were only present in conditioned media (*Luteimonas* sp.*, Ochrobactrum* sp.*, Bordetella* sp.*, Arachidococcus* sp.*, 67-14* uncultured bacterium, and *Microbacteriaceae*).

Of the 42 taxa observed in my samples, P*seudomonas* sp.*, Sphingobacterium* sp.*, Flavobacteriaceae, Devosia* sp.*, Paracoccus* sp.*, Streptomyces* sp.*, Rhodococcus* sp.*,* and *Luteolibacter* sp.*,* have been previously identified as farming soil microorganisms (Armalytė *et al.* 2019). Two genera that I identified, *Streptomyces*, and *Acinetobacter,* have been found in the microbiome of *Lu. longipalpis* (McCarthy *et al.* 2011). Another taxon that I found, *Ochrobactrum* sp. was also found, among other species, in unfed *Lu. longipalpis* females and males (Sant'Anna *et al.* 2014). *Brevundimonas* sp., and *Ochrobactrum* sp., which were identified in all three substrates, have been previously isolated from the midgut of laboratory reared *P. papatasi* (Karakuş *et al.* 2017). I found *Ochrobactrum* sp*.* and *Pseudomonas* sp*.* in all three of my substrates, which have been previously found in unfed, fed, and gravid *Lu. Intermedia* females (Monteiro *et al.* 2016). In summary, several of the taxa that I characterized in my sand fly rearing substrates have been associated with the breeding sites and habitats that support sand fly development as well as found in the gut microbiome of sand flies. These findings indicate that at least some of the identified bacteria are biologically relevant to sand flies and thus might work as components of biological control strategies.

Previous behavioral studies suggest that the presence of bacteria impacts sand fly attraction and oviposition responses. Peterkova-Koci *et al.* (2012) showed that sand flies from the new world (*Lu. Longipalpis*) are more attracted to lay eggs on substrates that contain bacteria, and that certain bacteria affect the sand fly's developmental time. Marayati *et al.* (2015) demonstrated that sand flies are more attracted to media where larvae have been reared to the 2nd/3rd instar followed by 4th/ pupae. The work we (Faw *et al*. 2021) performed on attraction and oviposition of conditioned (2nd/3rd) and unconditioned mediums demonstrated that there was a significant attraction of sand flies to the conditioned media over aged media, and a significant preference for attraction and oviposition on conditioned and aged media in comparison with their respective baseline attraction and oviposition levels.

Attraction to larval conditioned media has been suggested to involve the larvae's gut microbiome and the changes in the chemical cues emanating from the bacteria in the substrate (Marayati *et al.* 2015). It has been described that sand fly larvae obtain their gut microbiome from their surrounding environment (Volf *et al.* 2002, Maleki-Ravasan *et al.* 2015). Since larvae are coprophagic and they forage and defecate in the rearing substrate, this process could alter the media and provide an environment that is selective for particular microbes that benefit more from these larval secretions. My results support this hypothesis, as they depict clear changes in the bacterial community structure of the media when larvae are present and show that the community changes significantly once they are no longer present following adult eclosion.

Based on the results presented here, the attraction to conditioned 2nd/3rd could possibly be due to the presence of different taxa such as *Arachidicoccus* sp. and *Chthoniobacteraceae LD29* uncultured bacterium, as these were found to be unique to larval conditioned media. Other alternatives that could explain the attraction can be based on the increment of relative abundance of certain taxa after two weeks of conditioning. For example, the relative abundance of *Microbacterium* sp., and

Mycobacterium sp. highly increased in the 2nd/3rd instar conditioned media when compared to the fresh larval food week-0. The reduction of the relative abundance of taxa or the lack of some taxa (*Flavobacteriaceae, Parapedobacter* uncultured bacterium*, JG30 KF-CM45* uncultured bacterium*, Saccarimonadales* uncultured bacterium*, Planctomycetales* uncultured bacterium*, LD29* uncultured bacterium*,* and *Luteolibacter* sp.) could also be related to sand fly attraction to particular substrates as the presence or abundance of these bacteria might cause repellence to the media.

In another study, Kakumanu *et al*. (2020) cultured and isolated 18 taxa from the most attractive conditioned medium (2nd/3rd instar larvae rearing medium). They used the isolates to distinguish which ones were the most attractive. They found that there are some dose related effects, where female sand flies are attracted to six specific bacteria (*Leucobacter holotrichiae, Microbacterium sorbitolivorans, Sphingobacterium daejeonense, Sphingobacterium* sp*., Bacillus zhangzhouensis, Pseudomonas nitrititolerans*), and a mix of the twelve bacterial isolates at low cell density but were significantly repelled at high cell concentrations. They also found some bacteria (*Cellulosimicrobium cellulans, Luteimonas padinae, Alcaligenes faecalis, Brevundimonas olei*) cause no effect with respect to the attraction of sand flies (Kakumanu et al. 2020). Here, I identified seven taxa that might relate to the taxa previously isolated by Kakumanu *et al*. (2020). I identified *Luteimonas* uncultured bacterium*, Cellulosimicrobium* sp., and *Brevundimonas* sp*.* which were found to have no attraction effect, *Pseudomonas* sp*.* which was found to be attractive at high cell densities, and *Microbacterium* sp*.,* and *Sphingobacterium* sp. which were found to be attractive at low cell densities (Kakumanu *et al*. 2020). Therefore, the taxa that were found to be attractive by Kakumanu *et al.* (2020) and are also found in this study suggest that these bacteria might be responsible for sand fly attraction to 2nd/3rd larval stage conditioned media.

Preliminary results from a study by Mathew Miller (2017, unpublished data) suggested that the bacterial communities of conditioned and aged media did not appear to differ much, but both conditioned and aged media appeared to differed in comparison to their respective baseline samples (Figure 1). my results contrast this finding. Here I showed that the bacterial community of aged and larval conditioned media significantly differed from each other. Specifically, five taxa (*Mycobacterium* sp*., Microbacterium* ambiguous taxa*, Streptomyces* sp*., 67-14* uncultured bacterium*, Arachidococcus* sp.) were only present in the conditioned media at week 2 (based on my sequencing depth). While Miller was only able to identify 17 taxa, I was able to characterize 42 taxa, including six taxa shared in both conditioned and aged media (*Parapedobacter* sp*., Flavobacteriaceae, Pseudomonas* sp*., Sphingobacterium* sp*., Brevundimonas* sp*., Cell vibrio* sp.) that were also found by Miller. It is worth mentioning that the discrepancies between Miller's findings and mine might be related to the number of replicates analyzed, as Miller only had one replicate per substrate and I had three for aged and two for conditioned (week-2). Another possibility is that it could be due to the initial substrate (i.e. fresh larval food) being different, as I cannot assume that it contained the same microbial community as the one I used (*i.e.* feces from different rabbits are expected to contain different microbes).

Some limitations of this research project are that I only characterized bacteria and did not account for other microorganisms like yeasts and fungi that are expected to inhabit these substrates. Also, due to the difficulties that I had during library preparation, I ended with a small sample size for some of the groups resulting in a lack of power for testing significance. Due to this limitation, I plan to re-sequence my data in order to increase my sequencing depth, which hopefully will allow us to have more samples for the analysis and significance testing. Also, 16S rRNA sequencing can only reliably provide genus level data (at best), which prevented us from characterizing the community at the species level. Moreover, the available 16S reference databases are not complete so many taxa could not even be classified to the genus-level, limiting my

ability to accurately classify most of the identified taxa and compare them to other studies. It is also important to note that I did not test the sand fly's preference for the media that I analyzed microbially. Hence, my inference is correlational as it is based on the assumption that the patterns observed by Faw *et al* (2021) would be consistent here as well. Hence, sand fly attraction to conditioned media versus sterile media needs to be tested in order to confirm if the bacteria (or other microbes) in the substrate are causing the attraction rather than other components of the substrate.

CHAPTER V: CONCLUSION

The goal of this study was to determine how the conditioning and aging processes of sand fly rearing medium impact the microbiome structure of sand fly rearing substrates. I found that when the substrate was left to age over time without larvae, changes in the bacterial community structure were not significant. Interestingly, there was a significant change in the bacterial community structure when larvae were introduced to the substrate. Further studies need to be performed to test how the taxa identified in this study impact sand fly attraction, oviposition, and development. Additionally, future studies should characterize fungi and other microorganisms (mites, helminths) to determine if they are related to the attractiveness of the substrate and whether they contribute or delay larval development and overall sand fly fitness.

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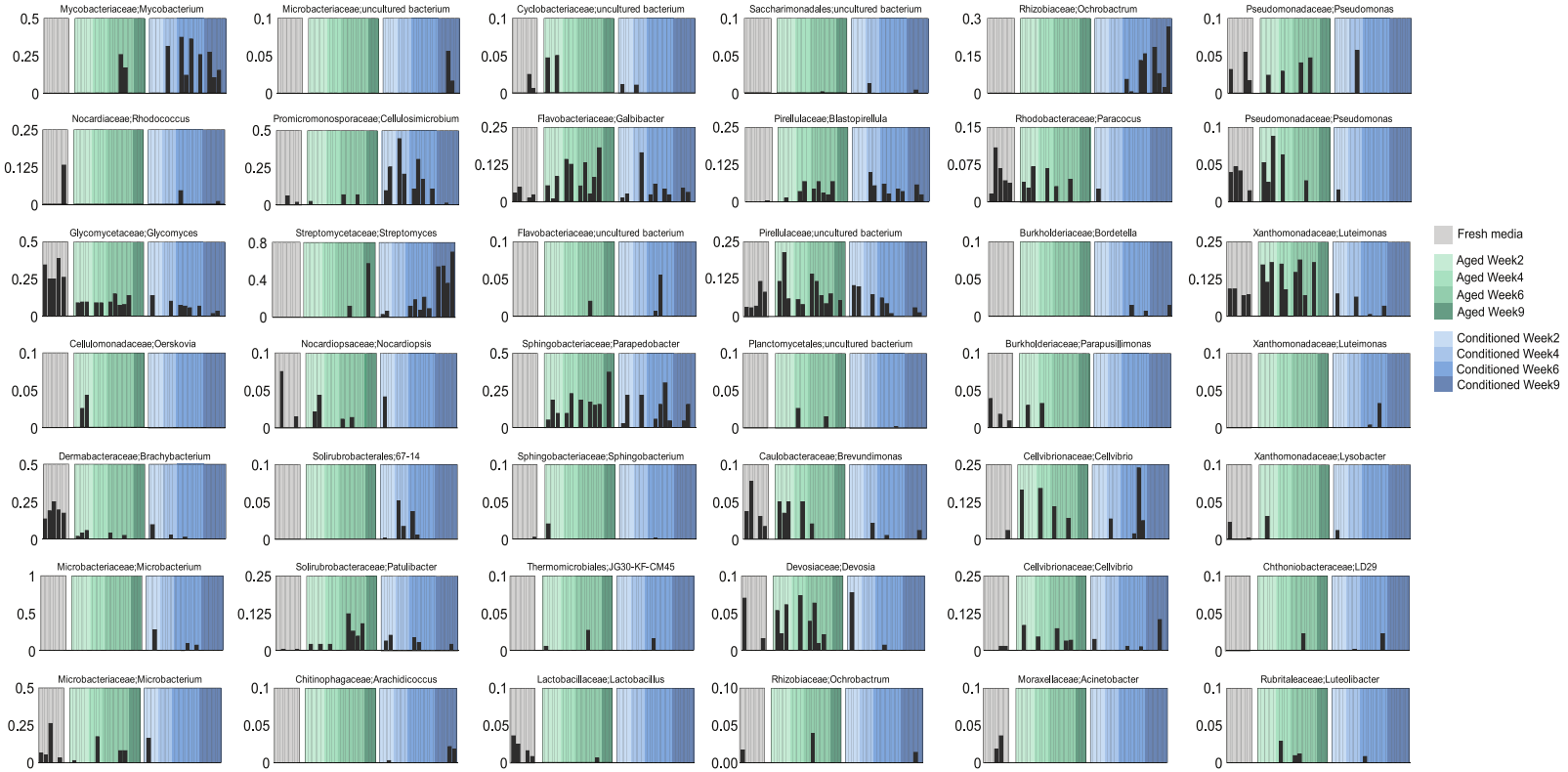
APPENDIX A: RAW DATA.

Sample Number	1	2	3	4	5	10	6	9	17	20	26	27	28	29	30	40	11	12	21	25	31	32	33	34	35	41	42	44	45
Description	Fresh_week0	Fresh_week0	Fresh_week0	Fresh_week0	Fresh_week0	Aged_week2	Aged_week2	Aged_week2	Aged_week4	Aged_week4	Aged_week6	Aged_week6	Aged_week6	Aged_week8	Aged_week8	Aged_week8	Cond_week2	Cond_week2	Cond_week4	Cond_week4	Cond_week6	Cond_week6	Cond_week6	Cond_week8	Cond_week8	Cond_week1	Cond_week1	Cond_week1	Cond_week9
Actinobacteria;Actinobacteria;Corynebacteriales;Mycobacteriaceae;_Mycobacterium;_	0	0	0	0	0	0	0	0	0	0	0	0	0.26	0.172	0	0	0	0	0.315	0	0.375	0.12	0.362	0	0.255	0.274	0.104	0.154	0
Actinobacteria;Actinobacteria;Corynebacteriales;Nocardiaceae;_Rhodococcus;_	0	0	0	0	0.131	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.046	0	0	0	0	0	0	0.01	0
Actinobacteria;Actinobacteria;Glycomycetales;Glycomycetaceae;_Glycomyces;_	0.34	0.251	0.248	0.386	0.259	0.091	0.094	0.094	0.067	0.089	0.097	0.147	0.072	0.077	0.137	0	0.138	0	0	0.101	0.071	0.089	0.056	0	0.069	0	0.016	0.034	0
Actinobacteria;Actinobacteria;Micrococcales;Cellulomonadaceae;_Oerskovia;_	0	0	0	0	0	0	0.026	0.044	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Actinobacteria;Actinobacteria;Micrococcales;Dermabacteraceae;_Brachybacterium;_	0.138	0.191	0.25	0.198	0.173	0.024	0.044	0.062	0	0	0.044	0	0	0.028	0	0	0.097	0	0	0.03	0	0.017	0	0	0	0	0	0	0
Actinobacteria;Actinobacteria;Micrococcales;Microbacteriaceae;_Microbacterium;Ambiguous_taxa	0	0	0	0	0.013	0	0	0	0	0	0	0	0	0	0	0	0	0.287	0	0	0	0.002	0.097	0	0.077	0	0	0	0
Actinobacteria;Actinobacteria;Micrococcales;Microbacteriaceae;_Microbacterium;_	0.062	0.053	0.262	0	0.029	0.01	0	0	0	0.17	0	0	0	0.077	0.077	0	0.16	0	0	0	0	0	0	0	0	0	0	0	0
Actinobacteria;Actinobacteria;Micrococcales;Microbacteriaceae;_;_	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.001	0	0	0	0	0.056	0.016	0
Actinobacteria;Actinobacteria;Micrococcales;Promicromonosporaceae;_Cellulosimicrobium;_	0	0	0.063	0	0.018	0.026	0	0	0	0	0.069	0	0	0.066	0	0	0.094	0.253	0.443	0.203	0.106	0.302	0.169	0	0.106	0	0.012	0	0
Actinobacteria;Actinobacteria;Streptomycetales;Streptomycetaceae;_Streptomyces;_	0	0	0	0	0	0	0	0	0	0	0	0	0.113	0	0	0.515	0.031	0.063	0	0	0.112	0.184	0.078	0.211	0.089	0.541	0.551	0.363	0.7
Actinobacteria;Actinobacteria;Streptosporangiales;Nocardiopsaceae;_Nocardiopsis;_	0	0.075	0	0	0.015	0	0.022	0.044	0	0	0.012	0	0.014	0	0	0	0.041	0	0	0	0	0	0	0	0	0	0	0	0
Actinobacteria;Thermoleophilia;Solirubrobacterales;67-14;_uncultured bacterium;_uncultured bacterium	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.002	0	0.052	0.017	0.037	0.005	0	0	0	0	0	0	0
Actinobacteria;Thermoleophilia;Solirubrobacterales;Solirubrobacteraceae;_Patulibacter;Ambiguous_taxa	0	0.006	0	0	0.005	0.023	0	0.022	0.022	0	0	0.123	0.067	0.048	0.09	0	0.033	0.053	0	0	0.044	0.026	0	0	0	0	0	0.023	0
Bacteroidetes;Bacteroidia;Chitinophagales;Chitinophagaceae;_Arachidicoccus;_uncultured bacterium	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.022	0	0	0	0	0	0	0	0	0	0.021	0.019
Bacteroidetes;Bacteroidia;Cytophagales;Cyclobacteriaceae;_uncultured;_uncultured bacterium	0	0	0	0.025	0.007	0.047	0	0.05	0	0	0	0.001	0	0	0	0	0.012	0	0.011	0	0	0	0	0	0	0	0	0	0
Bacteroidetes;Bacteroidia;Flavobacteriales;Flavobacteriaceae;_Galbibacter;Ambiguous_taxa	0.029	0.048	0	0.011	0.022	0.052	0.009	0.084	0.14	0.124	0.053	0.131	0.027	0.082	0.179	0	0.011	0.027	0	0.164	0.023	0.059	0	0.043	0.023	0	0.044	0.032	0
Bacteroidetes;Bacteroidia;Flavobacteriales;Flavobacteriaceae;_;_	0	0	0	0	0	0	0	0	0	0	0	0	0.02	0	0	0	0	0	0	0	0	0.007	0.053	0	0	0	0	0	0
Bacteroidetes;Bacteroidia;Sphingobacteriales;Sphingobacteriaceae;_Parapedobacter;_uncultured bacterium	0	0	0	0	0	0.052	0.186	0.094	0.094	0.231	0.182	0	0.172	0.154	0.156	0.372	0.033	0.216	0	0.215	0	0.069	0.158	0.301	0.049	0	0.049	0.159	0
Bacteroidetes;Bacteroidia;Sphingobacteriales;Sphingobacteriaceae;_Sphingobacterium;_	0	0	0	0	0.003	0.021	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.002	0	0	0	0	0	0	0
Chloroflexi;Chloroflexia;Thermomicrobiales;JG30-KF-CM45;_uncultured bacterium;_uncultured bacterium	0	0	0	0	0	0.006	0	0	0	0	0	0	0.027	0	0	0	0	0	0	0	0	0.016	0	0	0	0	0	0	0
Firmicutes;Bacilli;Lactobacillales;Lactobacillaceae;_Lactobacillus;_uncultured bacterium	0.035	0.025	0	0.016	0.008	0	0	0	0	0	0	0	0	0	0.006	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Patescibacteria;Saccharimonadia;Saccharimonadales;uncultured bacterium;_uncultured bacterium;_uncultured bacterium	0	0	0	0	0	0	0	0	0	0	0	0	0.002	0	0	0	0	0	0.013	0	0	0	0	0	0	0	0.004	0	0
Planctomycetes;Planctomycetacia;Pirellulales;Pirellulaceae;_Blastopirellula;_	0	0	0	0	0.005	0	0.012	0	0.033	0.066	0.042	0.066	0.028	0.022	0.066	0	0	0	0.098	0.052	0.06	0.027	0	0.042	0.035	0	0.057	0.024	0
Planctomycetes;Planctomycetacia;Pirellulales;Pirellulaceae;_;_	0.032	0.027	0.034	0.117	0.079	0.116	0.213	0.058	0.055	0.04	0.142	0.115	0.069	0.041	0.074	0.052	0.103	0.099	0	0.073	0.062	0.042	0.008	0	0	0	0.028	0.013	0
Planctomycetes;Planctomycetacia;Planctomycetales;uncultured;_uncultured bacterium;_uncultured bacterium	0	0	0	0	0	0	0	0	0.026	0	0	0	0	0.015	0	0	0	0	0	0	0	0	0	0.022	0	0	0	0	0
Proteobacteria;Alphaproteobacteria;Caulobacterales;Caulobacteraceae;_Brevundimonas;_	0.037	0.078	0	0.031	0.018	0.051	0.035	0.051	0	0.051	0.021	0	0	0	0	0	0	0	0	0.022	0	0.005	0	0	0	0	0	0.012	0
Proteobacteria;Alphaproteobacteria;Rhizobiales;Devosiaceae;_Devosia;_	0.07	0	0	0	0.016	0.054	0.023	0.061	0	0.074	0.04	0.064	0.01	0.022	0	0	0.078	0	0	0	0	0.008	0	0	0	0	0	0	0
Proteobacteria;Alphaproteobacteria;Rhizobiales;Rhizobiaceae;_Ochrobactrum;Ambiguous_taxa	0.017	0	0	0	0	0	0	0	0	0	0	0.039	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.013	0
Proteobacteria;Alphaproteobacteria;Rhizobiales;Rhizobiaceae;_Ochrobactrum;_	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.055	0.007	0	0.131	0.159	0.185	0.08	0.023	0.269
Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;_Paracoccus;_	0.016	0.107	0.066	0.042	0.037	0.038	0.027	0.07	0	0.067	0.031	0	0	0.045	0	0	0.025	0	0	0	0	0	0	0	0	0	0	0	0
Proteobacteria;Gammaproteobacteria;Betaproteobacteriales;Burkholderiaceae;_Bordetella;_	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.015	0	0	0.007	0	0	0	0.014
Proteobacteria;Gammaproteobacteria;Betaproteobacteriales;Burkholderiaceae;_Parapusillimonas;_	0.039	0	0.018	0	0.009	0	0.03	0	0.032	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Proteobacteria;Gammaproteobacteria;Cellvibrionales;Cellvibrionaceae;_Cellvibrio;_uncultured bacterium	0	0	0	0	0.03	0.165	0	0	0.169	0	0.111	0	0	0.07	0	0	0	0	0.068	0	0	0	0.019	0.238	0.063	0	0	0	0
Proteobacteria;Gammaproteobacteria;Cellvibrionales;Cellvibrionaceae;_Cellvibrio;_	0	0	0	0.016	0.017	0	0.066	0	0.046	0	0.002	0.075	0	0.034	0.036	0	0.038	0	0	0	0	0.017	0	0	0.014	0	0	0.104	0
Proteobacteria;Gammaproteobacteria;Pseudomonadales;Moraxellaceae;_Acinetobacter;_	0	0	0.018	0.035	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadaceae;_Pseudomonas endosymbiont of Orthophagus	0.032	0	0	0.055	0.018	0	0.024	0	0.03	0	0	0.04	0	0.047	0	0	0	0	0	0.057	0.001	0	0	0	0	0	0	0	0
Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadaceae;_Pseudomonas;_	0.039	0.047	0.041	0	0.015	0.052	0.026	0.087	0.063	0	0	0	0.028	0	0	0	0.016	0	0	0	0	0	0	0	0	0	0	0	0	0
Proteobacteria;Gammaproteobacteria;Xanthomonadales;Xanthomonadaceae;_Luteimonas;_uncultured bacterium	0.09	0.092	0	0.068	0.071	0.172	0.112	0.179	0.174	0.089	0.145	0.188	0.068	0	0.179	0	0.076	0	0	0.064	0	0.006	0	0	0.034	0	0	0	0
Proteobacteria;Gammaproteobacteria;Xanthomonadales;Xanthomonadaceae;_Luteimonas;_	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.004	0	0.032	0	0	0	0	0
Proteobacteria;Gammaproteobacteria;Xanthomonadales;Xanthomonadaceae;_Lysobacter;_	0.023	0	0	0	0.002	0	0.031	0	0	0	0	0	0	0	0	0	0.012	0	0	0	0	0	0	0	0	0	0	0	0
Verrucomicrobia;Verrucomicrobiae;Chthoniobacterales;Chthoniobacteraceae;_LD29;_uncultured bacterium	0	0	0	0	0	0	0	0	0	0	0	0	0.023	0	0	0	0	0	0	0.002	0	0	0	0	0.023	0	0	0	0
Verrucomicrobia;Verrucomicrobiae;Verrucomicrobiales;Rubritaleaceae;_Luteolibacter;_uncultured bacterium	0	0	0	0	0	0	0	0	0.029	0	0.009	0.011	0	0	0	0	0	0	0	0	0.008	0	0	0	0	0	0	0	0

APPENDIX B: RELATIVE ABUNDANCE OF TAXA FOUND.



*Relative abundance of individual taxa across all timepoints in each individual sample of fresh larval food, aged media, and conditioned media.