ABSTRACT

TRAVANTY, NICHOLAS VINCENT. Interaction of Bacteria and *Aedes albopictus* Larvae: Differential Effects of Bacterial Species on Larval Growth and Transstadial Movement of Bacteria. (Under the direction of Dr. Charles Apperson.)

Laboratory microcosm experiments were conducted to evaluate effects of five bacterial species, isolated from infusions made with senescent white oak (*Quercus alba*) leaves, on the growth and survivorship of larval *Aedes albopictus*. Larvae hatched from surface-sterilized eggs were reared in microcosms containing individual bacterial isolates (*Porphyrobacter* spp., *Enterobacter asburiae*, *Acidiphilium rubrum*, *Pseudomonas syringae*, and *Azorhizobium caulinodans*), a treatment composed of a combination of all five bacterial isolates, a positive control containing a microbial community from an infusion of white oak leaves, and a sterile media control. Experiments were conducted for 21 days after which microcosms were deconstructed and larval survivorship, larval size (head capsule width), estimated time to pupation, and pupal size (cephalothorax length) were analyzed. Additionally, pupae and adults that developed during the study were analyzed for bacterial content to quantify rates of transstadial transmission of bacteria. Positive control microcosms containing diverse microbial communities had an average pupation rate of 89.3% and average survivorship to 21 days of 96%. Pupation events in microcosms with individual or combined bacterial isolates were scarce (twice among all experimental replications), and average survivorship to 21 days ranged from 20% to 56% depending on bacterial isolate treatment. Larval development outcomes (larval growth and survivorship) were not found to be dependant of bacterial isolate species or bacterial isolate density. Potential mechanisms for failed development of larvae in microcosms with bacterial isolates are discussed.
Interaction of Bacteria and *Aedes albopictus* Larvae: Differential Effects of Bacterial Species on Larval Growth and Transstadial Movement of Bacteria.

by

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A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Master of Science

Entomology

Raleigh, North Carolina

2015

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DEDICATION

I very much appreciate the assistance and insight I received from all members of my thesis committee. I am especially appreciative of all the direction and support offered by Dr. Loganathan Ponnusamy during my time working on this and other recent projects. I would like to express my tremendous thanks to Dr. Charles Apperson for giving me this opportunity to be part of his team and develop knowledge under such an enriching environment full of talented, honest, and diligent individuals. I dedicate this thesis to my committee and Dr. Apperson.
BIOGRAPHY

Nicholas Travanty was born and raised in the mid-western city of Kenosha, Wisconsin located on the shores of the great Lake Michigan. His interest in entomology emerged when introduced to the subject while studying at the University of Wisconsin-Madison. In 2012, he descended on North Carolina State University to study the field of medical entomology under the guidance of Dr. Charles Apperson. He hopes to continue to develop his knowledge of how arthropods in our environment affect public health and use this knowledge to improve lives of people.
ACKNOWLEDGMENTS

Haley Sutton frequently provided assistance in preparation of reagents and culturing materials used for these assays.

Dr. Madhavi Kakumanu provided direction on DGGE methods and assisted in development of the marker ladder.
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EFFECTS OF BACTERIAL ISOLATES ON LARVAL DEVELOPMENT

Introduction

*Aedes albopictus* (Skuse) is native to Southeast Asia where it originated as a tree hole-inhabiting species in forests. In the presence of people, its notable adaptation to container-dwelling has allowed the species to gain worldwide distribution following establishment of human settlements and accumulation of refuse. *Ae. albopictus* has invasion through parts of Asia, Africa, the Pacific, Europe and the Americas (Hawley, 1988). Because rainwater-filled tires serve as common production sites for *Ae. albopictus*, the used tire trade has been implicated as a major factor in *Ae. albopictus* distribution, including its subsequent introduction to the United States. The first discovery of the species in the United States occurred in 1985 in the port city of Houston, Texas (Hawley, 1987).

*Aedes albopictus* is an important vector because of its ability to transmit pathogens of public health importance, extensive geographic distribution, and cohabitation with humans. The species is known to carry and transmit 22 different arboviruses, including all four serotypes of dengue fever virus, yellow fever and chikungunya viruses. Additionally, *Ae. albopictus* has been demonstrated to carry the filarial nematode *Dirofilaria immitis* that causes dog heartworm (Gratz, 2004). Though the closely-related species *Ae. aegypti* is more susceptible to dengue virus infection and transmits dengue virus more commonly and efficiently, *Ae. albopictus* has also been responsible for several dengue fever epidemics. During recent outbreaks of dengue fever in Hawaii, La Reunion Island, the Seychelles Islands, and the Maldives Islands, *Ae. albopictus* has been considered the primary vector
responsible for virus transmission (Lambrechts et al., 2010). Over the past several decades, the global expansion of chikungunya virus has been associated with Ae. albopictus; some strains of the virus have adapted to Ae. albopictus and caused massive outbreaks in areas previously unaffected (Nasci, 2014). The continued global expansion of Ae. albopictus is a major public health issue, and the species is considered to likely increase its threat as a public health nuisance in the future (Lambrechts et al., 2010).

*Aedes albopictus* larvae develop in naturally-occurring phytotelmata, such as tree holes, and in artificial containers such as tarps, buckets, and automotive tires (Richards et al., 2008). In both tree holes and artificial containers, inputs of allochthonous vegetation and other organic material serve as the principal carbon sources and nutritive basis for the habitat’s food web (Carpenter, 1983; Kling et al., 2007; Bara et al., 2014). *Aedes* larvae graze and consume the microbial community that is supported by decomposition of allochthonous organic matter (Merritt et al., 1992). The quality and quantity of organic material present in these habitats influences larval productivity, adult survivorship, and size of mosquitoes (Fish and Carpenter, 1982; Leonard and Juliano, 1995; Walker et al., 1997). Because mosquitoes are highly dependent on bacteria as food for larval development, bacteria influences the behavior and biology of mosquitoes at all life stages. To find habitats rich in bacteria, gravid adult *Aedes* females recognize volatile bacterial semiochemicals as cues that guide them to oviposition sites (Allan and Kline, 1995). Less volatile semiochemicals present in aquatic habitats arrest and stimulate gravid females to oviposit (Ponnusamy et al., 2008a). Different bacterial species have been observed to variably
influence oviposition-related behaviors, with some bacteria having positive (attraction) and others negative (repellant) effects (Trexler et al., 2003; Ponnusamy et al., 2010). Bacteria present in experimental microcosms have also been shown to stimulate Aedes egg hatching (Ponnusamy et al., 2011) and mid gut bacteria aid in blood digestion of adult mosquitoes (Gaio et al., 2011) and digestion of food by larvae (Coon et al., 2014).

Bacterial communities associated with container habitats of Aedes mosquitoes are comprised of diverse species (Ponnusamy et al., 2008b; Yee et al., 2012). Some bacterial taxa occur commonly in mosquito-positive containers, suggesting an ecological association with Aedes mosquitoes (Ponnusamy et al., 2008b). The oviposition behavior of gravid Aedes females is influenced by bacteria isolated from experimental habitats containing an infusion of white oak leaves (Ponnusamy et al., 2008a), though knowledge of how individual bacterial species affect larval development is lacking.

Recently, there has been interest in the biological functions performed by gut bacteria in various insect vectors. Modification of the gut microbiome is a novel approach to influence insect host behavior and biology as a means to disrupt pathogen transmission by a process referred to as paratransgenesis (Beard et al., 1992; Favia et al., 2007; Weiss and Askoy, 2011; Djadid et al., 2011; Chavsin et al., 2014). Both wild-type and recombinant strains of bacteria have been introduced into adult mosquito microbiomes to alter vector competence (Dong et al., 2009; Ramirez et al., 2012; Wang et al., 2012) by expression of antimicrobial peptides or stimulation of host insect immune responses. Development of paratransgenesis techniques for Ae. albopictus requires knowledge of how bacteria present in
mosquito larval habitats may persist and impact the biology of adults. Even though several bacterial taxa have been found to be associated with *Ae. albopictus* habitats (Ponnusamy et al., 2008b), it is not well-understood if these species persist transstadi ally from the larval into the pupal and adult stages of the mosquito life cycle.

White oak leaves are a common basis for food webs that support *Aedes* larval development in the Eastern United States. The objective of our research was to evaluate *Aedes albopictus* larval performance in the presence of bacterial isolates cultured from senescent white oak leaves (Ponnusamy et al., 2008a). Specifically, we determined effects of individual bacterial species on larval survivorship, larval development time, and adult mosquito size. Pupae and adults that developed during the experiments were evaluated for transstadi ally-transmitted bacteria using culture-based methods. We hypothesized that bacterial species have differential effects on the rate of *Aedes albopictus* larval development, and it was further hypothesized that rates of transstadial transmission of bacteria from larvae to pupae and adults vary among bacterial species.

**Materials and Methods**

**Experiment I: Development of procedures for bacterial cultivation in white oak leaf extracts**

The following experiment evaluated the performance of filter-sterilized WOL extract as bacterial growth media. This preliminary assay was conducted to determine the effects of filter-sterilized WOL extract concentration and supplemental bacterial growth media on
bacterial isolate densities. Two bacterial isolates (*Enterobacter asburiae* and *Pseudomonas syringae*) were evaluated in this assay.

*Sources of bacterial isolates*

Bacterial cultures were prepared from isolates made from white oak leaf (*Quercus alba*) infusions in a previous study (Ponnusamy et al., 2008a). Bacterial isolates were maintained by Dr. L. Ponnusamy on R2A culture plates (Reasoner and Geldreich, 1985) (Difco™, BD, Franklin Lakes, NJ, USA) and kept at 4°C. Single colonies from each isolate were inoculated into 25 mL of sterile 1X R2A broth (1X = 3.15 g/liter) contained in sterile 50 µL polycarbonate conical tubes (Corning®, Tewksbury, MA, USA). Inoculated media were placed on a shaker operating at 200 rpm at room temperature. After 48 hours tubes were visually inspected for turbidity to confirm bacterial growth and bacterial density was determined by hemocytometer.

*White oak leaf (WOL) extract*

Filter-sterilized WOL extract provided the nutrient base for bacteria in this study. WOL extract preparation, formulation, and concentration are based on Trexler et al. (1998). Senescent white oak leaves were collected in Raleigh, North Carolina and stored indoors inside plastic yard waste bags for approximately 1 year prior to use in extract preparation. Well water was acquired from North Carolina State University Lake Wheeler Poultry Research Station in Raleigh, North Carolina. Well water was held indoors in carboys and
used for extract preparation within 14 days of acquisition. Briefly, 16.8 g of dried senescent leaves were ground to a fine powder in a commercial blender (Waring® Commercial, Stamford, CT, USA), added to 500 mL of well water (= 4X concentration) and shaken at 200 rpm for 60 minutes. Leaf debris was removed from the extract by decanting, followed by course mesh and paper filtration, and finally filter-sterilized with a 0.22 µm nylon filter membrane (47 mm dia.) (Sigma-Aldrich®, St. Louis, MO, USA).

**WOL extract dilution and bacterial inoculation**

Serial dilutions of the sterile 4X WOL extract stock were prepared by addition of filter-sterilized well water. 100 mL volumes of 4X, 2X, 1X, and 0.5X WOL extracts were placed in sterile 250 mL glass bottles. An additional 100 mL volume of WOL extract including sterile R2A broth was prepared to a final dilution of 0.5X WOL/0.1X R2A (0.315 g/L). *Pseudomonas syringae* and *Enterobacter asburiae* isolates were prepared in R2A broth as previously described and served as a source of inocula for WOL extract. Each treatment was inoculated separately with each of the two bacterial species then assessed for density of viable bacteria after 7 days. Two replicates of each treatment were prepared and evaluated. Inoculum bacteria densities were estimated using a hemocytometer then added to each WOL extract concentration to achieve an initial density of approximately $5 \times 10^4 \ (4.70 \ \log_{10})$ bacteria/mL. After inoculation, sterile bottle caps were loosened to allow for gas exchange and the culture bottles were held at 28°C. After 7 days, CFU/ml was determined by serial dilutions and plating by the drop method (Herigstad, *et al.* 2001) on R2A agar plates. Drop
plates were incubated at 30°C and colonies enumerated after 48 hours. Measured CFU
densities were converted to $\log_{10}$ values prior to analysis as described below.

**Experiment II: Bacterial growth in experimental microcosms**

*Microcosm setup and bacterial inoculation*

Glass jars were used as experimental microcosms. Gravel, sand, water and 6 mm dia.
glass beads were added to eight 2-liter glass jars and swirled on a shaker operating at 300
rpm for 2 hours to lightly etch the interior sides of the jars and the surfaces of the glass balls
to facilitate bacterial biofilm formation. Jars were then washed, and 21 of the glass beads
were placed into each of the eight microcosm jars, which were then sterilized by autoclaving.
A 250 mL volume of filter-sterilized 4X WOL extract was prepared as previously described.
Separate 31.25 mL volumes of the stock extract were placed into eight sterilized 2-liter glass
jars containing the sterilized glass beads. Filter-sterilized well water was added to each jar to
dilute the WOL extract to 250 mL (= 0.5X WOL extract). Each of the eight microcosm jars
was fitted with a sterile lid bearing a 47 mm dia. nylon filter membrane (0.22 µm pore size)
attached over a hole in the lid to allow for gas exchange while preventing colonization of the
microcosms with exogenous microorganisms (Figure 1).

Inocula for the experimental treatments consisted of five bacterial isolates
(*Pseudomonas syringae*, *Enterobacter asburiae*, *Acidovorax avenae*, *Curvibacter gracilis,*
and *Bacillus thuringiensis*), and a 0.5X native WOL infusion (Trexler et al., 1998) provided
inoculum for the positive control. Bacterial inocula of each of the 5 isolates were prepared
from stored cultures as described in Experiment I. The native 0.5X WOL infusion was prepared by soaking 4.2 g non-sterile senescent white oak leaves in 1000 mL of non-sterile well water, which was fermented for 7 days in a covered 2-liter glass jar modified to allow for gas exchange as previously described. After 48 hours of bacterial isolate incubation or 7 days of native infusion fermentation, bacterial densities of each inoculum were determined by hemocytometer.

Five microcosms containing filter-sterilized 0.5X WOL extract were each inoculated with separate bacterial isolates to achieve an estimated density of approximately $5 \times 10^4$ (4.70 log$_{10}$) bacteria/mL in each microcosm. A sixth microcosm containing filter-sterilized 0.5X WOL extract was inoculated with a combination of the 5 isolates at a density of approximately $5 \times 10^4$ bacteria/mL with each isolate representing an equal portion of the total cell density. A seventh microcosm containing filter-sterilized 0.5X WOL extract was inoculated with the native 0.5X WOL infusion to achieve a density of approximately $5 \times 10^4$ bacteria/mL to serve as positive control. The volume of each treatment inoculum varied slightly between 20 to 40 µL per 250 ml microcosm, reflecting different bacterial concentrations in culture. Inoculum levels were determined by hemocytometer counts, and inoculation volume was standardized by the addition of sterile 1X R2A media. An eighth microcosm containing filter-sterilized 0.5X WOL extract without bacterial inoculation served as a negative control. Microcosms were kept stationary at 28°C and 70% relative humidity on a 14:10 light/dark cycle and were sampled as described below.
**Bacterial sampling**

Within a laminar flow hood, the water column and biofilm from each microcosm were sampled 6 days following inoculation. Each jar was lightly swirled to uniformly distribute the bacterial cells in the water column. A single water column sample (1 mL) from each microcosm was removed, added to 9 mL of sterile 0.1% peptone water (Fisher Scientific, Waltham, MA USA), vortexed for 60 sec, and serially diluted up to 8 times in sterile 0.1% peptone water. Biofilms on glass beads were sampled. From each microcosm jar, using sterilized long-handled steel forceps, three beads were removed and placed in 10 mL of sterile 0.1% peptone water. The beads were vortexed (VWR® Mini Vortex, Radnor, PA, USA) at 2000 rpm for 60 sec and the suspension was serially diluted as described previously. CFU/ml in the water column and CFU/cm² in biofilm samples were determined by the drop plate method on R2A plates and colonies enumerated after 48 hours at 30 °C. Drop plates were re-observed after 96 hours for additional colonies slow growing bacteria. Bacteria densities, estimated by extrapolating the densities of CFUs on drop plates, were converted to $\log_{10}$ values prior to statistical analysis. The sterile media control was sampled last to detect potential contamination occurring between microcosms during sampling.

**Experiment III: Effects of R2A supplementation and steam-sterilization of WOL infusion on bacterial growth**

An assay was performed to determine if 0.5X WOL extract supplemented with R2A would increase growth of the bacterial isolates. A 12.5 mL volume of filter-sterilized 4X
WOL extract was prepared (as previously described) and combined with 5 mL of sterile 1X R2A broth and 82.5 mL of filter-sterilized well water to produce sterile 0.5X WOL/0.05X R2A media. An aliquot (7.5 ml) of this medium was added to each of 13 sterile 50 mL polycarbonate conical tubes. A separate 0.5X WOL extract was produced by mixing 0.42 g of powdered senescent white oak leaves in 100 mL well water followed by shaking at 200 rpm for 60 min. The resulting suspension was filtered through a mesh screen followed by paper filtration and the extract was autoclaved (121°C) for 15 min. The resulting steam-sterilized extract was divided into 7.5 mL volumes in 13 separate sterile 50 mL polycarbonate centrifuge tubes.

Inocula were prepared from bacterial cultures as previously described. Isolates included those utilized in Experiment II (E. asburiae, P. syringae, A. avenae, C. gracilis, and B. thuringiensis) and Rhizobium huautlense. Bacterial cell densities were estimated by hemocytometer and two replicates of each media type were inoculated with individual isolates, each at approximately 5x10^4 (4.70 log_{10}) bacteria/mL. Media in the thirteenth tube of each medium type were inoculated with a sterile loop to serve as negative controls. Tubes were capped and placed on a shaker operated at 200 rpm at room temperature. Tubes were visually inspected after 5 days for bacterial growth.

**Experiment IV: Effects of nutrient additives to WOL extract on bacterial growth**

Further experiments were performed to determine if addition of R2A and other nutrients to filter-sterilized 0.5X WOL extract supported growth of 12 species of bacteria. A
50 mL portion of filter-sterilized 4X WOL extract was prepared as previously described and 12.5 mL was added to four different steam-sterilized media: (i) 87.5 mL well water and 1.5 g agar (0.5X WOL); (ii) 77.5 mL well water, 10 mL R2A broth (1X), and 1.5 g agar (0.5X WOL/0.1X R2A); (iii) 87.5 mL well water, 0.1 g sucrose (0.5X WOL/0.1% sucrose), and 1.5 g agar; and (iv) 87.5 mL well water, 0.1 g microcrystalline cellulose (Acros Organics, Morris Plains, NJ, USA), and 1.5 g agar (0.5X WOL/0.1% cellulose). The resulting agar plates were inoculated by streaking with one of the following bacterial species that were originally isolated from WOL infusion (Ponnusamy et al., 2008a) and prepared as inocula in R2A broth as previously described: *Porphyrobacter* spp., *Pseudomonas lanceolata*, *Rhizobium huautlense*, *Enterobacter asburiae*, *Acidiphilium rubrum*, *Acidovorax avenae*, *Pseudomonas syringae*, *Curvibacter gracilis*, *Brevundimonas vesicularis*, *Azorhizobium caulinodans*, and *Bacillus thuringiensis*. Additionally, a negative control for each medium involved streaking an agar plate with sterile R2A broth. Inoculated plates were incubated at 30°C and assessed for colony formation after 96 hours.

**Experiment V: Effects of bacterial isolates on development of larvae**

*Microcosm setup and bacterial inoculation*

Following the procedures described for Experiment II, eight microcosms were prepared, each containing 250 mL of sterile 0.5X WOL extract and 21 sterile glass beads. Inocula were prepared following previously described methods and included five bacterial isolates (*Porphyrobacter* spp., *E. asburiae*, *A. rubrum*, *P. syringae*, and *A. caulinodans*) and
a native WOL infusion. The five bacterial species included were selected based on growth results from previous experiments. Five microcosms were each inoculated with a separate bacterial isolate, a sixth microcosm was inoculated with a combination of the 5 isolates, a positive control was inoculated with the native WOL infusion, and a sterile media control was prepared following previously described methods. Microcosms were kept at 28°C and 70% relative humidity on a 14:10 light/dark cycle for 6 days to allow for bacterial growth and development prior to larval introduction.

Bacterial sampling

The methods used for bacterial sampling of the water column and biofilms were as previously described in Experiment II. Sampling occurred on the third and sixth day following microcosm inoculations. After the sixth day, larvae were introduced (day 0) and bacterial sampling occurred on each subsequent third day, including immediately prior to microcosm deconstruction (day 21).

Larval introduction

*Anopheles albopictus* eggs were acquired from colonies established from eggs collected in New Orleans, LA in 2003 and maintained in the Medical Entomology Laboratory, North Carolina State University. Eggs were surface-sterilized by washing with 1% bleach for 60 seconds and then rinsed with sterile distilled water five times. To verify that eggs had been surface-sterilized, a 500 µL portion of the last rinse water was spread-plated on R2A agar
and monitored for bacterial growth after 48 hours and again after 96 hours. Sterilized eggs remained in sterile distilled water and were allowed to hatch for 2 hours. Twenty-five 1st instars were transferred to each of the 8 microcosms, including the sterile media control jar. Microcosms were held in at 28°C and 70% relative humidity on a 14:10 light/dark cycle. To determine if 1st instars harbored culturable bacteria, a sample of 25 newly hatched larvae was homogenized, serially diluted in peptone water, and plated on R2A agar. Plates were observed for CFUs every 24 h for 96 h at 30 °C.

Processing pupae and adults

After larvae were introduced, microcosms were inspected for pupae every 24 h up to 21 days when experiments were terminated. Half of the pupae in each microcosm were to be reserved for rearing to adults after which the wing length of each specimen would be measured to derive a surrogate estimate of mosquito size as subsequently described. Preliminary experiments showed that pupae would survive and successfully molt to adults following the sterilization procedures described below. The remaining half of pupae from each microcosm was to be individually surface-sterilized, homogenized, and used to culture bacteria on R2A media. Because males generally pupate faster than females, each successive pupa to develop in a given microcosm was alternately allocated for rearing or culturing. This method would assure that an approximately equivalent number of males and females were subject to rearing and bacterial culture.
Within 24 h of pupation, sterile wide-bore pipette tips were used to remove pupae along with a portion of the water column (<1 mL). Individual samples were placed into separate sterile watch glasses and excess water was removed. Pupae were surface-sterilized by washing in 1% bleach for 60 sec, and then rinsed 5 times with sterile PBS. Pupae reserved for bacterial assessment were individually placed in 200 µL sterile PBS in sterile 1.5 mL centrifuge tubes and homogenized. Serial dilutions of the homogenate were prepared in sterile PBS and spread plated on R2A agar. Plates were incubated at 30°C and colonies counted after 48 and 96 hours. After surface-sterilization, pupae that had been reserved for rearing to adults were placed into shell vials containing 5 mL of sterile distilled water and capped with a sterile cotton ball. Vials were kept at 28°C and 70% relative humidity and monitored every 24 hours for adult emergence. Shell vials containing emerged adults were refrigerated at 4°C for 30 min to immobilize mosquitoes. Mosquitoes were transferred to sterile watch glasses containing 1 mL of cold sterile PBS, examined under a stereo microscope (50X) to determine its sex (Rattanarithikul, 1982), and then a single wing was removed, mounted on a glass slide, and the length measured (Heuvel, 1963) as a surrogate measure of adult size. The length of each slide-mounted wing was measured using a stereo microscope (50X) fitted with a calibrated ocular micrometer. Mosquitoes were then surface-sterilized in 90% ethanol for 60 s, rinsed 5 times with sterile PBS, homogenized in 200 µL of sterile PBS, spread plated and monitored for colony formation following the same procedures described previously for pupae.
**Microcosm deconstruction**

Twenty-one days after introduction of larvae, the final sampling of microcosms occurred and was immediately followed by deconstruction. The contents of each microcosm were poured separately through fine mesh netting fitted into a funnel to recover larvae. Recovered larvae were then placed into distilled water and the numbers of living and dead larvae from each microcosm were counted. Larval survivorship was calculated as the percentage of larvae that had been introduced at day 1 \((n = 25)\) that had either survived to day 21 or had pupated prior to day 21.

**Transstadial transmission of bacteria**

Bacterial CFUs on agar plates inoculated with pupae or adult homogenates were considered to have resulted from transstadial bacteria transmission. Frequency of transstadial transmission of bacteria was calculated as the percentage of cultured pupae or adults that contained culturable bacteria. For each case of transstadial transmission, the number of CFUs from a single pupa or adult was calculated. From these data, average numbers of CFUs in each pupa and adult were estimated. CFUs were converted to \(\log_{10}\) values prior to statistical analysis.

**Experiment VI: Addition of R2A to WOL extract**

Similar to Experiments II and V, microcosms containing sterile WOL extract were prepared and inoculated with bacterial isolates followed by introduction of *Ae. albopictus*
larvae. However, the WOL extract was augmented with 0.1X R2A broth to increase the growth of bacteria. Experiment VI was performed over a 4 month period from September to December, 2013. Three replications of the assay were performed at separate times with different WOL extract preparations. The experimental treatments are described below.

**Microcosm setup and bacterial inoculation**

As described previously in Experiments II and V, 8 microcosm jars were prepared, but the medium was augmented with sterile R2A broth. Each sterile microcosm contained 250 mL of 0.5X WOL/0.1X R2A (37.5 mL 4X WOL extract, 25 mL 1X R2A broth, 187.5 mL distilled water). Microcosms included glass beads to provide a substrate for biofilm production that would be consistent with previous experiments. However, in this experiment the glass beads remained in the microcosms for the duration of the assays without being sampled for bacteria.

Inocula consisted of five bacterial isolates (*Porphyrobacter* spp., *E. asburiae*, *A. rubrum*, *P. syringae*, and *A. caulinodans*) and a native WOL infusion. Bacterial inocula were prepared in R2A broth media as described in previous experiments. The native WOL infusion described in Experiments II and V served as the source for positive control inoculation. Bacterial inocula were freshly prepared for each experimental replicate, and the same preparation of the native WOL infusion was the source material for all positive control inoculations in experimental replications. Test microcosms were prepared and inoculated
with the isolates individually and in combination, and control microcosms were as previously described in Experiments II and V.

_Bacterial sampling_

Bacterial sampling of the water column followed methods and a schedule as described in Experiment V. Results of Experiment IV indicated that densities of water column and biofilm bacteria exhibited concordant trajectories. Consequently, biofilm sampling was not included in this experiment. Water column sampling and measurements of CFUs occurred on every third day following bacterial inoculation. Bacterial CFUs were converted to \( \log_{10} \) values prior to statistical analyses.

_Larval introduction_

*Anopheles albopictus* eggs were surface sterilized, hatched and introduced into the microcosms as previously described. Additional larvae from surface-sterilized eggs were assessed for culturable bacteria as previously described.

_Processing pupae and adults_

Pupae that developed in microcosms were collected as previously described with the following exceptions. Because no cultivable bacteria were found in the adults in Experiment V, it was thought that wing removal could have potentially ruptured the cuticle and allowed sterilant to enter the mosquito and kill any bacteria present. Consequently, the wing removal
procedure was not included in the current series of experiments. Rather, to estimate adult size, cephalothorax length of pupae was instead measured (Koenraadt, 2008). Prior to bacterial assessment or pupal rearing, each pupa was measured for cephalothorax length using a stereo microscope (50X) fitted with an ocular micrometer, and sex was determined. Pupae being reared to adults were placed in sterile watch glasses with glass lids rather than shell vials stoppered with cotton balls as previously described. CFU densities for pupae and adults were estimated following the previously described procedures. Wings of adults were not removed but their lengths were measured \textit{in situ}.

\textit{Microcosm deconstruction and processing of mosquitoes}

Deconstruction of the microcosms was performed as described in the previous experiment with the following exceptions. The head capsule widths of all larvae (alive and dead at time of deconstruction) were measured (Teng and Apperson, 1996) using a stereo microscope (50X) fitted with a calibrated ocular micrometer. The head capsules (viewed dorsally) of individual larvae were measured transversely across the eyes at the broadest point of head width. Measured head capsule widths from all treatments and replicates were used to produce a frequency distribution (class interval = 0.02 mm). The resulting modes within the distribution were correlated with larval instars and used to classify instar development of individual living larvae (Teng and Apperson, 1996). After being measured for head capsule width, living larvae recovered from the positive control were then assessed for cultivatable bacteria using methods previously described for pupae and adults.
Mosquito development indices (MDIs) (Kaufman et al., 2001) were calculated using the counts of living larvae of each instar and pupae for each microcosm. Individual living larvae recovered at the time of microcosm destruction were assigned the following integer values based on their level of development. First instar larvae = 1, second instar larvae = 2, third instar larvae = 3, and fourth instar larvae = 4. Pupae that developed prior to deconstruction were included in MDIs and given a value equal to 5. Each microcosm’s larvae and pupae values were summed and divided by the number of larvae and pupae included in the summation to produce an MDI. MDIs were calculated separately for each treatment for each experimental replication.

Larval survivorship for each microcosm was assessed based on the number of living larvae recovered at the time of deconstruction and pupae that had developed prior to deconstruction. Survivorship was calculated as a percentage of larvae introduced into each microcosm (n = 25) that were living at day 21 or that had pupated.

Sterile egg bacterial analysis

Following completion of all 3 experimental trials, further tests were performed to identify culturable bacteria harbored in Ae. albopictus eggs. Additional eggs were acquired from the lab stock utilized in the experimental trials. Eggs were surface-sterilized and allowed to hatch into sterile water; neonate larvae were homogenized, diluted, plated on R2A agar, and incubated as previously described. After 48 and 96 hours, plates were inspected for colony development. Colonies with unique morphologies were sub-cultured on R2A agar and bacterial isolates were prepared. Isolates were purified by re-streaking 5 times, and
individual isolates were identified by PCR amplification of the 16S ribosomal RNA gene fragments (approximately 1450 bp) using universal bacterial primers 27F (5’-AGAGTTTGATCMTGGCTCAG-3’) and 1494R (5’-ACGGCTACCTTGTTACGACTT-3’) (Ponnusamy et al., 2008a). Individual colonies were suspended in 50 µL of DNA-grade water (Thermo Fisher Scientific Inc., Grand Island, NY, USA), and boiled for 5 minutes. The resulting suspensions were centrifuged at 8000 rpm for 1 min, and 1 µL portions of the supernatants were used as templates in 50 µL PCR assays. The PCR reaction mix for each sample included 25 µL of AmpliTaq Gold 360® DNA polymerase (Thermo Fisher Scientific Inc., Grand Island, NY, USA), 2 µL of each 27F and 1494R universal bacterial primers (each 10 µM), and 20 µL of DNA-free PCR water. A negative control was prepared using all components of the reaction mix with 1 µL of DNA-free PCR water as template. PCR was performed on a Bio-Rad PTC-100 thermocycler (Bio-Rad Laboratories Inc., Hercules, CA, USA) using the following program: initial denaturation at 95 °C for 10 min, 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 90 s, and a final extension step at 72 °C for 10 min. PCR products (2.5 µL) were analyzed by agarose gel electrophoresis to confirm amplification and verify that correct fragment lengths were generated. Sanger sequencing (Eton Bioscience Inc., Morrisville, NC, USA) was performed on the PCR products with the 27F primer. Sequence results were then analyzed with the NCBI sequence database using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1997) to determine putative bacterial phylotypes.
Because bacterial CFU concentrations varied among the bacterial species treatments and over the time of the experiment, bacterial density was considered to be an independent variable that mediated larval development. Bacterial CFU density data collected from day 0 though day 21 for each bacterial isolate treatment were included in statistical analyses to determine if differences in CFU densities among the bacterial treatments were statistically significant. Using SPSS® Statistics software (ver. 22.0.0.0, IBM®, Armonk, NY, USA), a linear mixed model (α = 0.05) was used to analyze bacterial treatment and the combined effects of bacterial treatment and time (as repeated measures) as fixed effects with replication as a random effect on bacterial CFU densities.

Following the linear mixed model analysis, two approaches were developed to describe bacterial density as an independent variable. Primarily, pair-wise Sidak analyses were performed to identify sources of significant mean (α = 0.05) differences among bacterial densities of the bacterial isolate treatments. Bacterial isolate treatments that did not have significantly different densities were grouped together, and the two resulting groups of treatments were categorized as “low density” or “high density” treatments. Secondarily, bacterial densities for all time points for each trial of each bacterial treatment were averaged. Subsequent statistical analyses (described below) were performed using both descriptions of bacterial density as either a categorical value (low or high density) and as a continuous variable (average bacterial density).
In the final statistical analyses, bacterial treatment and bacterial density were considered to be independent variables. Initially, because the bacterial density level (low or high density) was directly associated with each bacterial isolate treatment, the combined effects of the two factors were assessed by nesting bacterial treatment within bacterial density. Dependent variables included survivorship and MDI. Linear mixed model analyses ($\alpha = 0.05$) were performed to test for effects of bacterial density and bacterial isolate treatment nested within bacterial density as fixed factors and replication as a random factor on survivorship and MDI using SPSS® software. Pair-wise Sidak comparisons were included in the statistical analyses to identify significantly different means ($\alpha = 0.05$) among the independent variables.

Additionally, LMM tests were performed to test for the effects of bacterial treatment, average bacterial density (as a continuous variable), and the combined effects of bacterial treatment and average bacterial density on survivorship and MDI using SPSS® software.

**Results**

**Experiment I: Development of procedures for bacterial cultivation in white oak leaf extracts**

After 7 days of incubation average CFU densities of *P. syringae* were highest in the 0.5X WOL/0.1X R2A and 0.5X WOL treatments ($7.80 \log_{10} \text{CFU/mL}$ and $7.60 \log_{10} \text{CFU/mL}$, respectively) and lowest in the 4X WOL treatment ($3.18 \log_{10} \text{CFU/mL}$). Similarly, average CFU densities of *E. asburiae* were highest in the 0.5X WOL/0.1X R2A
and 0.5X WOL treatments (7.90 log_{10} CFU/mL and 7.76 log_{10} CFU/mL, respectively) and lowest in the 4X WOL treatment (4.29 log_{10} CFU/mL). Complete results are listed in Table 1 and illustrated in Figure 2. Because the 0.5X WOL treatment achieved relatively high CFU densities for both bacterial species and the medium was composed of naturally-occurring materials (white oak leaves, well-water), it was considered to realistically represent mosquito larval habitat and was chosen as the media for future assays.

**Experiment II: Bacterial growth in experimental microcosms**

Six days after inoculation with bacteria, cultivable colony-forming bacteria were found in only four of the eight microcosms, including the positive control. Estimated water column CFU densities were as follows: *E. asburiae* = 7.76 log_{10} CFU/mL, *P. syringae* = 8.34 log_{10} CFU/mL, combined isolates = 8.37 log_{10} CFU/mL, and positive control = 7.98 log_{10} CFU/mL. Estimated biofilm CFU densities were as follows: *E. asburiae* = 6.54 log_{10} CFU/cm^2, *P. syringae* = 7.13 log_{10} CFU/cm^2, combined isolates = 6.95 log_{10} CFU/cm^2, and positive control = 6.85 log_{10} CFU/cm^2. No cultivable bacteria were found in the water columns or biofilms of the *A. avenae, C. gracilis, and B. thuringiensis* treatments. The sterile media control was free of cultivable bacteria.

It was originally intended that larvae be introduced into these microcosms and assessed for their rate of development and survivorship to pupae and adults. The assay was terminated after the initial determination of bacteria densities because not all the bacteria grew in the WOL extract. It was suspected that the filter-sterilized WOL extract did not
provide adequate nutrients to support growth of the selected bacterial species. A follow-up assay was performed to test viability of bacterial isolates in 0.5X WOL extract media supplemented with nutrients.

Experiment III: Effects of R2A supplementation and steam-sterilization of WOL infusion on bacterial growth

Results of the previous experiment indicated that some bacterial species did not grow in filter-sterilized 0.5X WOL media. It was suspected that the media was lacking required nutrients. Accordingly, we compared bacterial growth on two variations of 0.5X WOL extract media, namely, steam-sterilized 0.5X WOL extract and filter-sterilized 0.5X WOL extract, both augmented with R2A culture broth.

Steam-sterilized 0.5X WOL extract did not support growth of any bacterial isolate used in this study. Bacterial isolates (*E. asburiae* and *P. syringae*) that had previously been observed to grow prolifically in Experiments I and II in filter-sterilized WOL media did not grow in steam-sterilized WOL extract (data not shown). Steam-sterilization through autoclaving of WOL extract had been considered as a potential method to sterilize media while maintaining many of the particulate nutrients that would otherwise be removed by filter-sterilization. However, the complete lack of growth in steam-sterilized media for all bacterial isolates indicated that steam-sterilization was not a viable method to be utilized in future assays.
Acidovorax avenae previously did not grow in filter-sterilized 0.5X WOL media (Experiment II). With the addition of diluted R2A broth (0.05X) in this experiment, both replicates of *A. avenae, E. asburiae* and *P. syringae* were observed to have growth 5 days after inoculation. However, replicates of *C. gracilis, B. thuringiensis,* and *R. huautlense* treatments failed to grow in filter-sterilized 0.5X WOL/0.05X R2A media. No bacterial growth was observed in the negative control indicating that bacterial growth in the experimental treatments did not result from contamination.

Results of this assay showed that *C. gracilis, B. thuringiensis* and *R. huautlense* failed to grow in filter-sterilized WOL extract augmented with dilute R2A. It was therefore concluded that these three candidate bacterial species were not suitable for future mosquito larval feeding experiments. Additional isolates to be used in larval feeding experiments would have to be identified through screening of candidate bacterial isolates for growth in filter-sterilized 0.5X WOL media and filter-sterilized 0.5X WOL media supplemented with additional nutrients.

**Experiment IV: Effects of nutrient additives on bacterial growth in WOL extract**

Growth of 12 bacterial species was evaluated in four variations of filter-sterilized 0.5X WOL media. Complete results summarizing bacterial growth are shown in Table 2. Bacterial growth was observed in filter-sterilized 0.5X WOL extract for bacterial isolates including: *Porphyrobacter* sp., *R. huautlense, E. asburiae, A. rubrum, A. avenae, P. syringae,* and *A. caulinodans.* Colony formation was observed in the *R. huautlense* and *A. avenae*
treatments; however, the colonies developed by these two species were very small in comparison to the other isolates. Growth was observed on 0.5X WOL/0.1X R2A media for *Porphyrobacter* sp., *R. huautlense*, *E. asburiae*, *A. rubrum*, *P. syringae*, and *A. caulinodans*. Growth was observed on 0.5X WOL/0.1% sucrose media for *Porphyrobacter* sp., *R. huautlense*, *E. asburiae*, *A. rubrum*, and *P. syringae* and on 0.5X WOL/0.1% cellulose media for *Porphyrobacter* spp., *R. huautlense*, *E. asburiae*, *A. rubrum*, *P. syringae*, *B. vesicularis*, and *A. caulinodans*. There was no bacterial growth in the negative control agar plates showing that growth observed on the culture plates was not a result of contamination. Because 5 bacterial isolates (*Porphyrobacter* sp., *E. asburiae*, *A. rubrum*, *P. syringae*, and *A. caulinodans*) exhibited robust colony formation on filter-sterilized 0.5X WOL extract without supplemental nutrients, these isolates were chosen to be used in for future larval feeding experiments.

**Experiment V: Effects of bacterial isolates on development of larvae**

*Egg sterilization*

Following surface-sterilization of eggs, a sub-sample of the hatched neonate larvae was homogenized and plated on R2A media. Additionally, a sample of the last rinse water used in the egg sterilization procedure was plated on R2A media to verify egg sterilization. Both the last rinse and the homogenized neonate larvae yielded colonies of the same morphology suggesting that the same bacterial species was being introduced into the microcosms. However, subsequent samples from the treatment microcosms did not include
colonies with this particular morphology, suggesting that this bacterial species did not persist in the experimental microcosms. Additionally, the sterile media control was also not found to contain any viable bacteria at any time following inoculation of the microcosms.

*Comparative growth of bacterial isolates*

Cultivable bacteria were detected in all inoculated microcosms both in the water column and in biofilm samples throughout the 21 day duration of this experiment. The sterile media control remained without detectable bacterial growth. With the exception of the *A. rubrum* treatment, all isolate treatments were observed to maintain unique colony morphologies on drop plates. On day 3 and all subsequent sampling occasions, the *A. rubrum* microcosm’s water column and biofilm samples were observed to contain multiple bacterial colony morphologies, indicating contamination had occurred. Day 9 water column density of the *A. caulinodans* treatment, day 6 biofilm density of the positive control, and day 6 biofilm density of the *E. asburiae* treatment are not reported due to lab errors in drop plate culturing assays.

Water column and biofilm CFU densities varied between treatments and time points as shown in Figure 3. Three days after inoculation (3 days prior to larval introduction), *E. asburiae, P. syringae*, and combined isolates treatments each achieved highest estimated water column CFU densities of $7.90 \log_{10} \text{CFU/mL}$, $7.93 \log_{10} \text{CFU/mL}$, and $8.08 \log_{10} \text{CFU/mL}$, respectively. Similarly, biofilm densities for these treatments tended to be highest at the early time points - $6.88_{10} \log \text{CFU/cm}^2$ on day 3 for *E. asburiae*, $6.55_{10} \log \text{CFU/cm}^2$
on the third day following inoculation for *P. syringae*, and 6.72 log\(_{10}\) CFU/cm\(^2\) on day 0 for the combined isolates. For each of these treatments, measured water column and biofilm bacterial densities generally decreased on subsequent sampling occasions. The lowest measured water column CFU densities for each treatment were: 6.83 log\(_{10}\) CFU/mL on day 15 for *E. asburiae*, 7.15 log\(_{10}\) CFU/mL on day 9 for *P. syringae*, and 7.00 log\(_{10}\) CFU/mL on day 21 for the combined isolates. The lowest measured biofilm CFU densities for each were: 5.20 log\(_{10}\) CFU/cm\(^2\) on day 12 for *E. asburiae*, 5.81 log\(_{10}\) CFU/cm\(^2\) on day 21 for *P. syringae*, and 4.67 log\(_{10}\) CFU/cm\(^2\) on day 21 for the combined isolates. These three treatments generally produced much higher water column and biofilm bacterial CFU densities compared to the other isolate treatments.

Bacterial densities in water columns for the *Porphyrobacter* spp., *A. rubrum* and *A. caulinodans* treatments each decreased below the initial inoculation density (4.70 log\(_{10}\) bacteria/mL). The *Porphyrobacter* sp. and *A. rubrum* water column bacterial densities decreased to 4.08 log\(_{10}\) CFU/mL and 4.34 log\(_{10}\) CFU/mL, respectively, three days following inoculation, and the lowest water column bacterial density of *A. caulinodans* was 4.00 log\(_{10}\) CFU/mL on day 7. Biofilm densities were also lowest 3 days prior to larval introduction for the *Porphyrobacter* sp. treatment (2.62 log\(_{10}\) CFU/cm\(^2\)) and the *A. rubrum* treatment (2.97 log\(_{10}\) CFU/cm\(^2\)). The biofilm density of the *A. caulinodans* treatment was lowest on day 3 at 3.07 log\(_{10}\) CFU/cm\(^2\).

After larvae were introduced, estimated CFU densities for the *Porphyrobacter* spp. and the *A. caulinodans* treatments fluctuated for the remainder of the 21 day experiment, and
on no occasion did the water column densities for each exceed inoculation densities. The biofilm of the *Porphyrobacter* sp. and *A. caulinodans* treatments eventually elevated to peak densities of $4.29 \log_{10} \text{CFU/cm}^2$ and $3.77 \log_{10} \text{CFU/cm}^2$, respectively, on day 18. These two treatments generally had the lowest CFU densities in this experiment. Water column and biofilm bacterial densities of the *A. rubrum* treatment, however, increased to $6.45 \log_{10} \text{CFU/mL}$ and $5.25 \log_{10} \text{CFU/cm}^2$ on day 9. However, the increase was likely due to contaminating microbes. The *A. rubrum* treatment was found to contain contaminant bacteria on day 3 and subsequently had an increase in water column and biofilm CFU densities through the end of the experiment.

Initially (day 0), the bacteria densities measured in the positive control were $8.26 \log_{10} \text{CFU/mL}$ and $6.37 \log_{10} \text{CFU/cm}^2$, respectively. Bacterial densities at each sampling time generally decreased for the remainder of the experiment. The lowest water column density was measured on day 18 at $5.86 \log_{10} \text{CFU/mL}$, and the lowest biofilm density was on day 21 at $4.67 \log_{10} \text{CFU/cm}^2$.

Bacterial densities in water column and biofilm samples exhibited similar trends over the 21 day experiment (Figure 3). Previously, it had been anticipated that microcosms would develop different growth trajectories for populations of planktonic and biofilm bacteria. Because microcosm contamination of the *A. rubrum* microcosm was thought to occur during biofilm sampling, and because population fluctuations of water column and biofilm bacterial populations exhibited the same temporal patterns, assessment of biofilms on glass beads was discontinued in subsequent experiments.
Mosquito development

Larvae introduced into the sterile WOL extract control were observed to actively swim and browse for food, but did not grow past the 1st instar. All larvae died within 48 hours of their introduction into the sterile microcosm. These results indicate that the media alone did not provide nutrition sufficient to support larval development. Microcosms containing bacteria were each observed to extend survival and initiate larval development showing that the presence of bacteria was necessary for larval survival and growth.

Besides the rapid mortality observed in the sterile media control, larval death at a relatively early development stage was also observed in the Porphyrobacter sp. and the A. caulinitans treatments. On day 6, it was noted that approximately half of the larvae in these microcosms had died and the remaining larvae had not advanced past the 1st instar. All larvae from these two treatments were dead on day 8 with no evidence of 2nd instars having developed. Larval mortality from starvation was thought to have resulted from the low bacterial density in these treatments.

Larvae in the E. asburiae treatment appeared to have developed beyond 1st instars. On day 6, it was noted that the larvae were comparably larger than 1st instars in other microcosms, and on day 16 many of the larvae had again molted and appeared to be 3rd instars. However, all larvae in this treatment had died by day 19.

By day 16 larvae in the P. syringae treatment were observed to have molted to 2nd and 3rd instars similarly to those in the E. asburiae treatment. Only 3 of 25 larvae in the P. syringae treatment were alive at day 21. In the combined isolate treatment, larvae developed
to 2nd and 3rd instars by day 16, and by day 21 some appeared to develop to the 4th instar stage. Six of 25 larvae survived to day 21 in the combined isolates treatment. Survivorship results for all treatments are shown in Figure 4.

Larvae in the *A. rubrum* treatment initially grew at a rate comparable to larval development observed in the *Porphyrobacter* sp. and *A. caulinodans* treatments. On day 3, all larvae remained as neonates with several having died. However, contamination was observed on day 3, and by day 15, although approximately half of the larvae had died, the remaining larvae were observed to have increased in size. On day 18 pupae began to develop, and 6 pupae developed by day 21. On day 21, four living larvae remained in this microcosm with overall survivorship of 40%. Results for this microcosm are questionable because contamination obfuscated interpretation of the experimental outcomes.

The only treatment to have high larval survivorship and larval development to the pupal stage was the positive control (inoculated with native WOL infusion). Mosquitoes exhibited 100% survivorship with 23 larvae in this microcosm pupating, and the remaining 2 larvae surviving to day 21. The average time (± SE) to pupation (males and females) was 15.3 (± 0.9) days (*n* = 23). Of the positive control pupae that were reserved for rearing to adults, average wing length (± SE) measured among males (*n* = 6) was 1.82 (± 0.03) mm, and among females (*n* = 5) was 1.81 (± 0.17) mm the average wing length.

Water column bacterial densities for most treatments decreased prior to introduction of larvae into the microcosms. After larvae were introduced densities tended to decrease, with particularly low bacterial densities measured in the *Porphyrobacter* sp. and *A.*
caulinodans treatments. It is likely that the bacterial density in the A. rubrum treatment would have remained comparably low had contamination not occurred.

Transstadial movement of bacteria to pupae and adults

Of the 23 pupae that developed in the positive control microcosm, 11 were immediately assessed for cultivable bacteria. The 12 remaining pupae were reared to adults, 11 of which were subsequently assessed for cultivable bacteria, while one adult escaped under the laminar flow hood. Unidentified bacteria were cultured from 7 of the 11 pupae. An average (± SE) of 182.9 (± 136.9) CFU/pupa (n = 7, independent of sex) was found among pupae containing cultivable bacteria. Cultivable bacteria were not detected in any of the 11 adults examined.

This experimental design was not replicated because of low survivorship of larvae. Consequently, the experimental design was modified in an attempt to increase growth of bacteria in the microcosms. In Experiment I it was demonstrated that the addition of 0.1X R2A to filter-sterilized 0.5X WOL extract resulted in an average 2.1% increase in the density of P. syringae and E. asburiae. Experiment IV demonstrated that a medium composed of 0.5X WOL and 0.1X R2A could support the growth of Porphyrobacter sp., E. asburiae, A. rubrum, A. avenae, P. syringae, and A. caulinodans isolates. Accordingly, another microcosm experiment was carried out that included WOL extract augmentation with R2A.
Experiment VI: Addition of R2A to WOL extract

Egg sterilization

R2A agar plates were inoculated with last-rinse water from the egg sterilization procedure to verify that eggs were sterilized. Bacterial growth was observed on agar plates from two of the three replications of the assay. Similarly, evaluation of neonate larvae homogenate revealed presence of viable bacteria among the same two of three replicates. Colonies from last rinse and neonate plates were similar in both replicates and sources and likely included a single bacterial species. These bacteria were likely introduced into all microcosms for these two replicates; however, sampling of microcosms, including the sterile media control, revealed no evidence of growth of this bacterial species at any subsequent time point. Therefore the presence of this bacteria species was considered to not have affected the outcome of mosquito growth experiments. On no occasion were viable bacteria found in samples of the sterile media control indicating that cross-contamination between microcosms and microcosm contamination from an extrinsic source did not occur in this experiment.

Comparative growth of bacterial isolates

Bacteria were consistently cultured from water column samples taken from experimental microcosms and the positive control microcosm. Visual examination of colony morphologies on drop plates showed that isolates were of uniform size and shape for the duration of the experiment, suggesting that treatment microcosms supported the single bacterial species with which they were inoculated. An assortment of colony morphologies
were observed for microcosms containing mixed bacteria species (combined isolate treatment and the positive control treatment).

The initial (three days following microcosm inoculation) water column bacterial density of the combined isolate microcosm for the first replication was not included in this study’s data as the drop plate was inadvertently smeared. Also, sampling and assessment of the water column for day 15 of the second replication was not performed.

At day 0, the *Porphyrobacter* sp. treatment contained its lowest average (± SE) CFU density measured at 7.76 (± 0.28) log$_{10}$ CFU/mL, and the greatest measured average bacterial density was on day 18 (8.22 (± 0.02) log$_{10}$ CFU/mL). The *E. asburiae* treatment was measured with lowest CFU density on day 12 (8.29 (± 0.16) log$_{10}$ CFU/mL) and highest on day 21 (8.80 (± 0.24) log$_{10}$ CFU/mL). Lowest average CFU density for the *A. rubrum* treatment was measured on day 3 (7.89 (± 0.06) log$_{10}$ CFU/mL) and highest on day 15 (8.32 (± 0.32) log$_{10}$ CFU/mL). For the *P. syringae* treatment, lowest average CFU density was measured on day 0 (8.37 (± 0.29) log$_{10}$ CFU/mL) and highest on day 3 (8.72 (± 0.10) log$_{10}$ CFU/mL). The *A. caulinodans* treatment was measured with lowest average CFU density on day 0 (7.74 (± 0.07) log$_{10}$ CFU/mL) and highest on day 15 (8.30 (± 0.04) log$_{10}$ CFU/mL). The combined isolate treatment was measured with lowest average CFU density on day 12 (8.54 (± 0.06) log$_{10}$ CFU/mL) and highest on day 18 (8.90 (± 0.30) log$_{10}$ CFU/mL). The positive control was measured with lowest average CFU density on day 21 (7.18 (± 0.26) log$_{10}$ CFU/mL) and highest on day 0 (8.38 (± 0.16) log$_{10}$ CFU/mL). CFU densities for each
treatment are illustrated in Figure 5 and the mean densities for each treatment are provided in Table 3.

A linear mixed model analysis for effects of bacterial treatment, time (repeated measure) and the combined effects of bacterial treatment and time on water column CFU densities was performed (Table 4). Bacterial treatment \((P < 0.001)\) and the interaction of bacterial treatment and time \((P = 0.007)\) each had significant effects on CFU densities. Time alone was not found to have significant effects \((P = 0.435)\). Effects of bacterial treatment on CFU densities were separated by Sidak pair-wise comparisons (Table 5). Significant differences were found between the following treatments: *Porphyrobacter* sp. and *E. asburiae* \((P < 0.000)\), *Porphyrobacter* sp. and *P. syringae*, \((P < 0.000)\), *Porphyrobacter* sp. and combined isolates \((P < 0.000)\), *A. rubrum* and *E. asburiae* \((P < 0.000)\), *A. rubrum* and *P. syringae*, \((P < 0.000)\), *A. rubrum* and combined isolates \((P < 0.000)\), *A. caulinodans* and *E. asburiae* \((P < 0.000)\), *A. caulinodans* and *P. syringae*, \((P < 0.000)\), and *A. caulinodans* and combined isolates \((P < 0.000)\). Because no significant differences of CFU densities were found between the *Porphyrobacter* sp., *A. rubrum*, and *A. caulinodans* bacterial treatments, and no significant differences of CFU densities were found between the *E. asburiae*, *P. syringae*, and the combined isolates treatments, each group of three bacterial treatments were classified as “low bacterial density” (*Porphyrobacter* sp., *A. rubrum*, and *A. caulinodans*) or “high bacterial density” (*E. asburiae*, *P. syringae*, and the combined isolates). The density classification was considered as an independent variable in subsequent LMM analysis of larval development outcomes.
Mosquito development

For each replication, larvae in the sterile media control did not molt and all larvae died within 96 hours of introduction. No dead larvae were recovered on day 21, presumably because neonate corpses were too small to be retained by the screen employed to filter the media at the time of deconstruction.

Larval survivorship in the high bacterial density treatment (*E. asburiae*, *P. syringae*, and the combined isolates) averaged (± SE) 33.3% (± 9.6), 20% (± 2.3), and 40% (± 26.2), respectively, at day 21. Among treatments classified as having low bacterial densities, larvae in the *Porphyrobacter* sp. had an average survivorship of 18.7% (± 5.3), in the *A. rubrum* treatment, larval survivorship averaged 24% (± 6.1), and larvae in the *A. caulinodans* treatment had an average survivorship of 56% (± 4.6). In comparison, survivorship on the positive control microcosm averaged 96% (± 4.0) for the three replications. A mixed model analysis showed no significant effects of bacterial treatment (nested within bacterial density) (*P* = 0.202) or bacterial density alone (*P* = 0.860) on larval survivorship (Table 6).

Head capsule width measurements for all living and dead larvae recovered on day 21 from all three replicates (*n* = 314) of the bacterial treatments (excluding the positive control) were used to construct a frequency distribution for mosquito size (Figure 6). Based on this distribution, instar intervals were determined as follows: first instars (≤ 0.30 mm), second instars (> 0.30 – 0.44 mm), third instars (> 0.44 – 0.68 mm), and fourth instars (> 0.68 mm). The head capsule width frequency distribution of each treatment is presented in Figure 7.
Larvae generally failed to complete development to pupae or adults in the bacterial isolate conditions of this experiment. On only 2 occasions was there a pupation event among larvae developing in bacterial isolates. One male pupa developed in the *E. asburiae* treatment on day 19 (cephalothorax length = 1.68 mm), and another male pupa developed in the *A. rubrum* treatment on day 17 (cephalothorax length = 1.68 mm). In contrast, almost all larvae from the three positive control replications pupated, and the average (± SE) pupation rate in the positive control was 89.3% (± 5.8). Over the three replications of the positive control a total of 67 pupae developed. Females represented 47.8% of the pupae (n = 32), and males represented 52.2% (n = 35). The cephalothorax length of pupae generated in the positive control averaged (± SE) 1.85 (± 0.02) mm for females and 1.65 (± 0.02) mm for males. The average (± SE) time to pupation for males was 10.6 (± 0.7) days for females and 8.2 (± 0.3) days for males for pupae generated in the positive control.

Using the instar of individual living larvae recovered at day 21 and the number of pupae that had developed for each treatment, MDIs were calculated. The index reflects the average larval development for the three replicates of each bacterial treatment. The average MDI values (± SE) for high density CFU treatments were as follows: *E. asburiae* treatment = 3.16 (± 0.32), *P. syringae* treatment = 3.48 (± 0.26), and combined isolates treatment = 3.13 (± 0.13). Index values among the low density bacteria treatments were: *Porphyrobacter* sp. = 2.44 (± 0.50), *A. rubrum* = 3.32 (± 0.17), *A. caulinodans* = 2.47 (± 0.20), and positive control = 4.93 (± 0.05).
A mixed model analysis for the effects of bacterial treatment (nested within bacterial
density) and bacterial density on mosquito development index outcomes (Table 7) showed no
significant effects of bacterial treatment (nested within bacterial density) \( (P = 0.215) \) or from
bacterial density \( (P = 0.051) \).

Additionally, the average bacterial density for the 21-day experimental period was
determined for each treatment of each experimental trial. LMM were performed to test for
the effects of bacterial treatment, average bacterial density, and the combined effects of
bacterial treatment and average bacterial density on larval survivorship and MDI. Bacterial
treatment \( (P = 0.997) \), average bacterial density \( (P = 0.932) \), and the combined effects \( (P =
0.997) \) were not found to have significant effects on larval survivorship. Bacterial treatment
\( (P = 0.593) \), average bacterial density \( (P = 0.870) \), and the combined effects \( (P = 0.581) \) were
not found to have significant effects on MDI outcomes. Results are reported in Table 8 and
Table 9, respectively.

*Transstadial movement of bacteria to pupae and adults*

As discussed above, unlike larvae developing in single or mixed bacteria isolate
conditions, larvae persisting in a full microbial community of the positive control
microcosms were consistently able to pupate and achieve adulthood (89.3%, overall). Living
larvae recovered from the positive control microcosm \( (n = 4) \) were surface-sterilized and
assessed for cultivable bacteria with an average (± SE) density of \( 3.48 \times 10^5 \) (± 1.85 x \( 10^5 \))
CFU/larva. Of the 31 positive control pupae surface-sterilized and assessed for bacterial
content, 27 pupae contained cultivable bacteria. The average (± SE) frequency of transstadial transmission of bacteria from larvae to pupae in the positive control microcosms was 85.7% (± 7.5). Bacteria density was considerably reduced in pupae, averaging 487.5 (± 98.9) CFU/pupa for the 27 pupae containing cultivable bacteria. Twenty-seven adult mosquitoes were surface-sterilized and assessed for cultivable bacteria content, and on no occasion was any bacterial growth observed on culture plates. Therefore, no transstadial transmission of bacteria from pupae to adults was detected.

Identification of egg bacteria

Three bacterial isolates were purified from neonate larvae that hatched from surface-sterilized eggs. Sequencing of the 16S rRNA gene target was performed for each sample. The sequence returned from one of the samples contained ambiguous nucleotide positions, was thought to have contained multiple bacterial species, and was discarded. The two remaining samples had 97% and 98% nucleotide sequence homology matches to *Paenibacillus chondroitinus* (GenBank accession no. NR043415) and *Paenibacillus glycanilyticus* (GenBank accession no. JQ647886), respectively.

Discussion

The experiments performed, including preliminary assays designed to optimize experimental conditions, were designed to determine if *Aedes albopictus* larvae could complete development when provided only bacteria as food. Experiment VI is considered
the thesis’ main experiment as it provided data that directly tested the hypothesis that the bacterial species would achieve differential effects on the growth and survivorship of larvae.

*Impacts of bacterial food source on larval development*

In Experiment VI, effects of the various bacterial treatments on larval development outcomes (survivorship and MDIs) were not significantly different. However, significantly more larvae in the control WOL infusion microcosms survived and developed into adults compared to microcosms containing bacterial isolate treatments. In the sterile media control, all larvae perished within 4 days from hatching without developing beyond the neonate stage. Larvae in bacterial isolates treatments generally survived longer and often developed to second, third, or fourth instar larvae. These results show that a diet of WOL infusion combined with dilute R2A broth and absent of bacteria does not provide adequate nutrition to support larval development, but bacteria do provide a nutritive resource for developing *Aedes albopictus* larvae. The hypothesis that bacterial isolates would have varying effects on development of *Aedes albopictus* larvae was not supported by the experimental results. Larval survivorship at 21 days and MDIs were not significantly different between bacterial isolate treatments.

The average time (± SE) to pupation for larvae in the positive control was 9.4 (± 0.4) days and is comparable with pupation development times previously described. *Ae. albopictus* larvae developing in maple leaf infusion-based microcosms at a density of approximately 0.1 larvae/mL were reported to have average times to pupation between 9 and
12 days (Dieng et al., 2002). Gomes et al. (1995) reported average times to pupation of 7.7 days, 14.3 days, and 13.5 days of larvae developing in experimental tree holes, bamboo shoots, and tires, respectively. *Ae. albopictus* cephalothorax lengths have not been previously reported. However, average cephalothorax lengths of *Ae. aegypti* reared in a lab-based study were reported to be approximately 1.5 mm to 1.7 mm (Bara et al., 2014). The similarity of these results to those of the present study indicates that conditions of the positive control were comparable to those in natural mosquito habitats. The nutritive basis of the 0.5X WOL extract/0.1X R2A media provided a sufficient pool of nutrition to support what can be considered normal larval development, provided the microcosm were colonized by a diverse/natural microbiota.

Larvae in the positive control microcosms had higher survival and pupation rates compared to larvae in the bacterial isolates. Most larvae developing in the positive control pupated prior to habitat deconstruction. Over the three replications, few fourth instar larvae ($n = 4$) remained in the microcosms at the time of deconstruction. In general, the size (head capsule widths) of these larvae and larvae surviving in the bacterial treatments were smaller that the size of *Ae. albopictus* larvae reported by Teng et al. (1996), which were reared in a lab setting under similar temperature and density conditions on a diet composed of a mixture of brewer’s yeast, lactalbumin, and ground rabbit food. The average head capsule width for each larval instar was reported to be approximately 14% larger on average than the head capsule width intervals described in the current study. The larger size of larvae developing
on an artificial diet compared to bacterial isolates indicates that the bacterial isolates provided inadequate nutrition.

*Aedes albopictus* larvae are reported to be well-adapted for resistance to starvation (Barrera, 1996). However, in the present experiment, starvation was pronounced, as bacterial isolates were insufficient to support larval development to the pupal stage. Rates of survivorship were very low and pupation rarely occurred in any of the bacterial isolate treatments. Had the experiment continued for a period much beyond 21 days, it is unlikely that any larvae would have survived and few, if any, would have pupated. Starvation likely occurred because of the absence of a wider community of microbes (bacteria and fungi) and protozoans. It is also possible that the bacterial species used in this bioassay were either indigestible or provided an insufficient nutritional profile required by *Ae. albopictus* larvae.

Although bacterial CFU densities in the isolate microcosms remained relatively static over the course of the experiment, bacteria in the positive control decreased over the 21 day period. Similar results were reported by Kaufmann *et al.* (2002) for microbial communities in *Aedes triseriatus* microcosms. A comparable pattern of reduced bacterial density followed by reduction in protozoan abundance was seen in microcosms that contained grazing mosquito larvae. Reduction of bacteria over time was described to result from a combination of direct larval grazing and protozoan feeding on bacteria. Protozoans were shown to operate as an intermediate by consuming bacteria while providing a food source for larvae. WOL infusions prepared following the same procedures as the positive control inoculant used in the current study were observed to carry a diverse community of protozoans (unpublished
The positive control microcosm of the current study likely included a diverse microbial community, and multitrophic feeding effects of protozoans and larvae may have operated as described in the Kaufmann et al. (2002) study.

Marine detritivore invertebrates have been shown to have a diverse diet, consuming algae, fungi, protozoans, and bacteria (Phillips, 1984). Analysis of marine invertebrate detritivore diets has shown that bacteria generally do not provide a sufficient source of nutrients, such as long chain poly-unsaturated fatty acids (PUFAs) and sterols. In these food chains, protozoans, and other meiofauna that are able to synthesize PUFAs and sterols are the primary consumers of bacteria. Macroinvertebrates consume the meiofauna and acquire these essential nutrients (Phillips, 1984). It is probable that Aedes larvae require a diverse diet that includes a variety of microbes to obtain essential nutrients similarly to marine detritivores.

Sterols are required for insect development and cannot be synthesized by insects (Clark and Bloch, 1959). The synthesis of sterols by most bacterial taxa rarely occurs, and the bacteria included in the current study (alphaproteobacteria, gammaproteobacteria) cannot produce sterols (Volkman, 2003). Both plant vegetation, such as white oak leaves, and yeast (as a component on the R2A media) contain various sterols (Volkman, 2003). Accordingly, vital sterols were present in the bacterial isolate microcosms, but the dilute concentrations of these compounds may have been insufficient to support the development of larvae or were not provided in the form needed for assimilation.
In previous research, Horn and Lichtwardt (1981), provided *Aedes aegypti* larvae, hatched from surface-sterilized eggs, a diet that lacked a sterol source. Larvae developed to fourth instars in an average of 11 days, but failed to pupate. This outcome is generally similar to that observed for *Ae. albopictus* larvae in the current study, and indicates that a lack of sterols may have been the limiting factor in the development of the larvae. In the Horn and Lichtwardt study, it was further demonstrated that Trichomycete fungi (gut-commensalists) acquired sterols from the environment, which allowed mosquitoes to develop normally (1981). This observation supports the concept that sterol-producing eukaryotic microbes are a critical limiting factor in the growth and survivorship of larvae.

Similar to the current study, several other studies have examined effects of bacterial isolate diets on dipteran larval development. Hollis *et al.* (1985) fed sterilized cattle feces inoculated with individual bacterial isolates from cattle rumen to *Musca autumnalis* (Muscidae) larvae. Average rates of survivorship to pupae were reported to vary between 8% and 57.2% depending on bacterial isolate treatment, and *Escherichia coli* and *Lactobacillus plantarum* were demonstrated to promote the highest survivorship (57.2% and 32.8%, respectively). Contrastingly, positive controls composed of unsterilized feces averaged 100% survivorship to pupae between replications. Perotti *et al.* (2001) provided surface-sterilized eggs of *Haemotobia irritans* (Muscidae) with various treatments of sterilized cattle manure each inoculated with a single bacterial species isolated from manure or the gut of *H. irritans*. Survivorship rates to pupae ranged between 0% and 53% and were dependent on bacterial isolate treatment. The species *Pseudomonas mendocina* provided the
highest survivorship rate (53%), and a non-sterilized manure control supported an average survivorship rate of 74%. Zurek *et al.* (2000) purified several bacterial isolates from the gut of *Musca domestica* (Muscidae) larvae. Neonate larvae from surface-sterilized eggs of *M. domestica* were allowed to develop on trypticase soy egg yolk agar plates inoculated with individual bacterial isolates. Survivorship to adults was reported to vary from 0% to 91.5% depending on bacterial isolate treatment, and the *Lactococcus garviae* treatment had the highest reported survivorship (91.5%) to adults. Fitt and O’Brien (1985) isolated bacteria, including *Enterobacter* spp. and *Serratia* spp, from the decomposed fruits and bodies of Tephritid fruit flies. Diets composed of sterilized fruit inoculated with these individual isolates resulted in normal fly development. Taken together, these studies demonstrated that fly larvae could successfully complete development when fed bacterial monocultures, though it should be noted that feeding assays included bacterial growth media derived from eukaryotic sources (egg yolk, cattle manure, and tree fruit). Sterile media controls (sterile media not inoculated with bacteria) included in the Hollis *et al.*, Perotti *et al.*, and Zurek *et al.* had low survivorship outcomes (8%, 0%, and 0%, respectively) showing that the media alone provided insufficient or reduced nutritional support of larval development. It is possible that in each of these studies, specific combinations of media and bacteria (dependent on species) were required to complete larval development. In these studies, the underlying mechanisms that lead to variation in fly development were generally regarded to be differences in the nutritional profiles of the individual bacterial species. The Fitt and O’Brien (1985) study goes further to question variation in the digestibility of bacterial
species. It was demonstrated that the digestibility of *Enterobacter cloacae* and *Serratia liquefaciens* was modulated by changes in pH, and it was concluded that acidification of the midgut allowed these bacteria to be more readily digested resulting in enhanced larval development. Kaufmann *et al.* (2008), observed bacterial communities in tree holes inhabited by *Aedes triseriatus* larvae. Bacterial communities underwent increases in the proportion of Betaproteobacteria and *Flavobacteria* when grazing larvae were present. The increased density of *Flavobacteria* was later found to result from grazing-resistance through indigestibility of the bacteria (Chen *et al.*, 2014). It is possible that in the current study, starvation observed among larvae developing in microcosms containing bacterial isolates can be attributed to indigestibility of the bacterial species. The bacterial isolates used in Experiment VI included two gammaproteobacteria (*E. asburiae* and *P. syringae*) and three alphaproteobacteria (*Porphyrobacter, A. rubrum*, and *A. caulinodans*). In the Kaufmann *et al.* (2008) study, both of these sub-phylum level taxa were monitored for taxonomic shifts correlated with larval grazing with no significant effects being reported. The current study did show that all five of these species contributed to larval nutrition as indicated by larval growth, suggesting that they were digested by *Ae. albopictus* larvae. Additionally, comparative studies that assess the densities of viable bacteria in larval feces would be needed to determine differences in the digestibility of the bacterial species used in the present microcosm experiments.

Sota and Kato (1994) showed that *Ae. albopictus* larvae completed development achieving 40% pupation on a diet that was composed exclusively of bacteria. In these
experiments, *Pseudomonas fluorescens* bacteria were rinsed and purified of extrinsic media and then centrifuged into a thick pellet at the bottom of a sterilized vessel. Larvae produced from non-sterilized eggs were allowed to feed on the bacteria until the supply was depleted at which point pupation occurred and adults successfully emerged. However, adults were of reduced sizes when compared to a control group fed on a standard lab diet. This outcome indicates that *Ae. albopictus* development can be completed when the only food source is a single bacterial species contrasts with results of the current study. No controls to detect extrinsic microbial contaminants were included in the Sota and Kato’s experiments. Therefore, it is possible that extrinsic microbes introduced into Sota and Kato’s feeding assay from unsterilized eggs contributed nutrients essential to larval development.

More recently Coon *et al.* (2014) examined the impacts of dietary bacterial isolates on the development of *Ae. aegypti*. Bacterial isolates were obtained on agar plates from homogenates of lab-reared *Ae. aegypti* eggs, larvae, and adults. Individual colonies were used to directly inoculate radiation-sterilized standard diet (rat chow, lactalbumin, and brewer’s yeast) combined with sterile water contained in culture flasks. Axenic larvae were then introduced to the culture flask treatments, allowed to feed, and monitored for survivorship and time to pupation. Larvae fed bacterial isolates generally completed development similarly to non-sterile control larvae fed a standard diet, but axenic larvae maintained on a sterile standard diet did not complete development. All but one (*Microbacterium spp.*) of the 7 bacterial isolates used in the experiment supported larval development, suggesting that bacteria are, indeed, an important component of larval
nutrition either by directly providing nutrition or facilitating digestion and nutrient acquisition.

The *A. caulinodans* treatment outcome resulted in the highest survivorship at day 21 compared to the other isolate treatments. This treatment was peculiar in that the development of surviving larvae was limited to third instars with no fourth instar larvae developing in any of the experimental replications. *Azorhizobium* is different than the remaining bacterial isolates used in this study because of its nitrogen-fixing capabilities (Dreyfus et al., 1988). Little is known about the contributions of nitrogen-fixing bacteria to insect nutrition, though there is some evidence that various nitrogen-fixing bacterial species provide nutrition by recycling nitrogenous waste. *Tetraponera* ants have been found to harbor in their gut nitrogen-fixing bacteria that are thought to metabolize ant waste products (uric acid) into amino acid precursor compounds (Van Borm et al., 2002). Nitrogen-fixing bacteria associated with Cerambycid beetle larvae have also been found to recycle nitrogenous waste into essential and non-essential amino acids (Ayayee et al., 2014). It is possible that *A. caulinodans* has a role in nitrogenous nutrient recycling in larval mosquito habitats. Further studies are needed to investigate how *Azorhizobium* and other aquatic nitrogen-fixing bacterial species affect *Aedes* larval growth and survivorship.

*Transstadial and vertical transmission of bacteria*

Larvae developing in the bacterial isolates failed to pupate and therefore it was not possible to analyze transstadial transmission of individual bacterial species used in this study.
However, larvae in the positive control successfully completed development to pupae and adults. The bacterial load of fourth instar larvae was several orders of magnitude larger than that of pupae and adults. Pupal bacterial densities were reduced to several hundred bacteria per pupa while cultivable bacteria were not recovered from adults.

The finding of reduced bacterial densities in mature stages of mosquito development using culture-based methods in the present study is similar to those found in previous studies. Moll et al. (2001) reared larvae from non-sterilized eggs of three mosquito species, including *Ae. aegypti* on a standard lab diet. Developing larvae, pupae and adults were sampled and assessed for bacterial density independent of sex using culture-based methods on trypticase soy agar. Briefly, surface-sterilized *Ae. aegypti* fourth instars contained approximately 43,000 CFUs/larva, <48 hour-old surface-sterilized pupae contained approximately 3,000 CFUs/pupae, and surface-sterilized adults contained approximately 50 CFUs/mosquito. The remaining non-*Aedes* species had comparable counts of bacterial CFUs for each stadium. Likewise, Zouche et al. (2009) recovered relatively low densities of bacterial CFUs from surface-sterilized adult *Ae. albopictus* that had been reared in a lab. Measured CFU densities ranged between 3 to 100 CFUs per surface-sterilized adult using a combination of both modified Luria Bertani and peptone-yeast-calcium chloride media for cultivation of bacteria. Future growth experiments may include additional bacterial growth media to increase chances of culturable bacteria detection, and culture-independent methods may be employed to detect transstadial bacteria.
The egg hatching procedure included surface-sterilization to eliminate contamination from exogenous bacteria. However, in Experiment VI the plating of neonate larval homogenates resulted in bacterial colony formation on R2A agar plates in two of the three replications. Additionally, cultured neonate homogenate in Experiment IV resulted in colony formation. The homogenous morphology of the bacteria that were found among these occurrences suggested that they were either developed from vertically transmitted bacteria or represented bacterial contamination that was resistant to the surface-sterilization methods used. Subcultures were prepared on R2A agar media with the intent of identifying the species by PCR amplification and DNA sequencing. Unfortunately, the culture plates were inadvertently discarded. It should be noted, however, that the unidentified bacteria were introduced into the sterile microcosms along with the neonate larvae. In subsequent sampling and plating of the water column bacteria these bacteria were not recovered.

Further attempts were made to recover this bacterial species by culturing homogenates of 1st instar *Ae. albopictus* hatched from surface-sterilized eggs. Three bacterial subcultures were prepared, and two of the three subcultures were bacterial isolates identified as *Paenibacillus chondroitinus* and *Paenibacillus glycanilyticus*. The colonies identified as *P. chondroitinus* appeared remarkably similar to colonies that had emerged from larvae of surface-sterilized eggs in Experiment IV and Experiment V, and it is suspected that the same species was present among the surface-sterilized eggs for both experiments.

The genus *Paenibacillus* is ubiquitous and diverse with species found in the environment, among plants, and associated with arthropods. Species have been found in peat
bogs in Northern China (Ming et al., 2012), and have been demonstrated to operate as plant-growth-promoting rhizobacteria by reducing the effects of environmental stress factors on plants (Timmusk and Wagner, 1999). A *Paenibacillus* species closely related to *P. chondroitinus* has been found as a gut symbiont of the insect *Diestrammena apicalis* (Orthoptera) (Park et al., 2009). *Paenibacillus larvae* produces highly infectious endospores that are the causative agent of the disease American Foulbrood, a highly destructive disease affecting honeybees (*Apis mellifera*) and other native pollinators (Genersch, 2010).

*Paenibacillus* spp. have previously been found in association with various mosquito species and mosquito habitats. A *Paenibacillus* species was isolated from *Anopheles gambiae* oviposition sites in Kenya (Lindh et al., 2012) and found to produce semiochemicals associated with oviposition attraction of the mosquito, though despite production of these semiochemicals, behavior assays did not demonstrate significant attraction by gravid females to this unidentified *Paenibacillus* species. Rani et al. (2009) reported that gut bacteria of field-collected male *Anopheles stephensi* in India were dominated by Gram-positive firmicutes, including several *Paenibacillus* species. An unspecified *Paenibacillus* strain was isolated from field-collected *Aedes aegypti* adult populations in Panama, and adult female *Ae. aegypti* infected by this bacterial strain had significantly reduced dengue virus titers (Ramirez et al., 2012). It is possible that the isolates generated in the current study (identified as *P. chondroitinus* and *P. glycanilyticus*) could affect the susceptibility of *Ae. albopictus* to dengue and other arboviruses. These isolates may be considered for use in
future assays testing the effects of bacterial species on oviposition attraction or vector competence of *Ae. albopictus*.

In the final egg hatching assay, no viable bacteria were found in the last rinse water, though it was noted that the eggs remained unhatched when the last rinse water was sampled. The results imply that these bacteria likely resided inside the eggs, though; it is unknown whether the bacteria were located within the larvae or on the interior surface of the egg chorion. To more precisely ascertain the location of these bacteria, future culture-based assays should compare colony development of cultured homogenates of surface-sterilized neonate larvae and cultured homogenates of un-hatched surface-sterilized eggs. Additionally, molecular tests of surface-sterilized unhatched and hatched larvae from surface-sterilized eggs could be performed to detect unculturable bacteria species that may be present in *Ae. albopictus* eggs.

*Future assays/methods review*

Several questions resulted from the outcomes of the present study. Experimental results suggested that bacteria provide nutrition that contributes to larval development, but bacteria alone are unlikely to provide sufficient nutrition to complete larval development. A larger microbial community that includes fungi and other eukaryotic organisms is required to provide the adequate nutrition for *Ae. albopictus* larvae to develop to pupae and adults. To test effects of fungal species on larval development, an assay similar to Experiment VI should be performed using fungal isolates cultured from senescent white oak leaf infusion
and introduced into sterile WOL extracts. Similarly, larvae can be introduced and monitored for development. Additionally, by preparing combinations of fungal and bacterial isolates together, the interaction of bacteria and fungi on larval nutrition can be determined. These tests would resolve whether fungi alone or a combination of fungi and bacteria are needed to support larval development. If future assays were to demonstrate that the addition of isolated fungi to bacterial isolates can support larval development, the hypothesis of the current study could be re-examined to see if variation in larval development is dependent on various bacterial isolates in combination with fungi.

The independent variables tested in the current study included bacterial species and bacterial density. The accuracy of bacterial density enumeration was limited by culture-based methods on R2A media. The bacterial isolates used in this experiment are known to be culturable on R2A media, and the density measurements for these isolates are considered to be accurate. However, the positive controls contained diverse microbial communities. It is likely that some of the bacterial species in the positive control microcosms were not culturable on R2A media or may require other conditions for growth, and thus density measurements for the positive controls may be less accurate. In future assays, enumeration of bacteria should include culture-independent methods, such as cell staining, as was accomplished in Walker et al. (1991). Further, as in the aforementioned study of Kaufmann et al. (2002), additional microbes should be monitored in future assays. It is likely that in natural habitats, fungi and protozoans contribute to Aedes larval nutrition.
The MDI was conceived and employed to measure differences in larval development outcomes. Values for the index were produced using counts of living larvae recovered on day 21, and did not include larvae that had died prior to day 21. The time course of larval mortality would provide additional information about the suitability of bacterial species as food for larvae. In the present experiments, the day that larvae died could not be determined. An alternative experimental design would involve placing larvae and 10 mL portions of bacterial growth media into individual vials rather than collectively into a microcosm similar to the previous mosquito development study of Rueda et al. (1990). Using this experimental design, time to mortality of individuals can be ascertained, and more accurate larval development outcomes can be measured.
REFERENCES CITED


Table 1. Average bacterial isolate densities ($\log_{10}$ CFUs/mL) in various WOL infusion dilutions 7 days following inoculation (Experiment I).

<table>
<thead>
<tr>
<th></th>
<th>Media</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4X WOL</td>
</tr>
<tr>
<td>Enterobacter asburiae</td>
<td>4.29</td>
</tr>
<tr>
<td>Pseudomonas syringae</td>
<td>3.18</td>
</tr>
</tbody>
</table>
Table 2. Positive or negative growth of white oak leaf-associated bacteria on variations of filter–sterilized WOL extracts
(Experiment IV).

<table>
<thead>
<tr>
<th>Bacterial isolate treatment</th>
<th>0.5X WOL</th>
<th>0.5X WOL/0.1X R2A</th>
<th>0.5X WOL/0.1% sucrose</th>
<th>0.5X WOL/0.1% cellulose</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Porphyrobacter</em> sp.</td>
<td>+*</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Pseudomonas lanceolata</em></td>
<td>-*</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Rhizobium huautlense</em></td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Enterobacter asburiae</em></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Acidiphilium rubrum</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Acidovorax avenae</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Pseudomonas syringae</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Curvibacter gracilis</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Brevundimonas vesicularis</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Azorhizobium caulinodans</em></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Bacillus thuringiensis</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*+ and – indicate presence and absence of bacterial growth, respectively, 2 days after plates were inoculated.*
Table 3. Average bacterial densities (± SE) ($\log_{10}$ CFUs/mL) of the water columns for each treatment at each sampling interval (Experiment VI).

<table>
<thead>
<tr>
<th></th>
<th>Day 3</th>
<th>Day 6</th>
<th>Day 9</th>
<th>Day 12</th>
<th>Day 15</th>
<th>Day 18</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Porphyrobacter</em> sp.</td>
<td>7.67</td>
<td>7.93</td>
<td>8.14</td>
<td>8.06</td>
<td>8.08</td>
<td>8.14</td>
<td>8.22</td>
</tr>
<tr>
<td><em>Enterobacter asburiae</em></td>
<td>8.51</td>
<td>8.55</td>
<td>8.61</td>
<td>8.56</td>
<td>8.29</td>
<td>8.80</td>
<td>8.46</td>
</tr>
<tr>
<td><em>Acidiphilium rubrum</em></td>
<td>7.44</td>
<td>7.89</td>
<td>8.04</td>
<td>8.09</td>
<td>8.13</td>
<td>8.32</td>
<td>8.14</td>
</tr>
<tr>
<td><em>Pseudomonas syringae</em></td>
<td>8.15</td>
<td>8.72</td>
<td>8.48</td>
<td>8.67</td>
<td>8.51</td>
<td>8.68</td>
<td>8.53</td>
</tr>
<tr>
<td><em>Azorhizobium caulinodans</em></td>
<td>6.91</td>
<td>8.14</td>
<td>8.10</td>
<td>8.00</td>
<td>8.11</td>
<td>8.30</td>
<td>7.93</td>
</tr>
<tr>
<td>Combined isolates</td>
<td>8.45</td>
<td>8.63</td>
<td>8.56</td>
<td>8.56</td>
<td>8.54</td>
<td>8.74</td>
<td>8.90</td>
</tr>
<tr>
<td>Positive control</td>
<td>8.37</td>
<td>7.89</td>
<td>7.52</td>
<td>7.22</td>
<td>7.42</td>
<td>7.46</td>
<td>7.28</td>
</tr>
</tbody>
</table>
Table 4. Mixed model analysis of effects of bacterial treatment, time, and the combined effects (bacterial treatment and time) on measured CFU densities.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>F</th>
<th>P &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial treatment</td>
<td>6</td>
<td>61.938</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Time</td>
<td>7</td>
<td>1.003</td>
<td>0.435</td>
</tr>
<tr>
<td>Bacterial treatment x time</td>
<td>42</td>
<td>1.868</td>
<td>0.007*</td>
</tr>
</tbody>
</table>

* Significant at $\alpha = 0.05$
Table 5. Sidak significance values for pair-wise comparisons of bacterial treatments for effects on CFU densities.

<table>
<thead>
<tr>
<th></th>
<th>Enterobacter asburiae</th>
<th>Acidiphilium rubrum</th>
<th>Pseudomonas syringae</th>
<th>Azorhizobium caulinodans</th>
<th>Combined isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Porphyrobacter</em></td>
<td>&lt; 0.001*</td>
<td>0.990</td>
<td>&lt; 0.001*</td>
<td>1.000</td>
<td>&lt; 0.001*</td>
</tr>
<tr>
<td><em>E. asburiae</em></td>
<td>-</td>
<td>&lt; 0.001*</td>
<td>1.000</td>
<td>&lt; 0.001*</td>
<td>0.788</td>
</tr>
<tr>
<td><em>A. rubrum</em></td>
<td>-</td>
<td>-</td>
<td>&lt; 0.001*</td>
<td>1.000</td>
<td>&lt; 0.001*</td>
</tr>
<tr>
<td><em>P. syringae</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>&lt; 0.001*</td>
<td>0.667</td>
</tr>
<tr>
<td><em>A. caulinodans</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>&lt; 0.001*</td>
</tr>
</tbody>
</table>

* Significant at $\alpha = 0.05$
Table 6. Mixed model analysis for effects of bacterial density and bacterial treatment (nested within bacterial density) on survivorship of *Aedes albopictus* larvae.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>$F$</th>
<th>$P &gt; F$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial treatment (bacterial density)</td>
<td>4</td>
<td>1.758</td>
<td>0.202</td>
</tr>
<tr>
<td>Bacterial density</td>
<td>1</td>
<td>0.033</td>
<td>0.860</td>
</tr>
</tbody>
</table>
Table 7. Mixed model analysis for effects of bacterial density and bacterial treatment (nested within bacterial density) on mosquito development index of *Aedes albopictus* larvae.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>F</th>
<th>P &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial treatment (bacterial density)</td>
<td>4</td>
<td>1.700</td>
<td>0.215</td>
</tr>
<tr>
<td>Bacterial density</td>
<td>1</td>
<td>4.711</td>
<td>0.051</td>
</tr>
</tbody>
</table>
Table 8. Mixed model analysis for effects of bacterial treatment, average bacterial density, and combined effects on survivorship of *Aedes albopictus* larvae.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>F</th>
<th>P &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial treatment</td>
<td>6</td>
<td>0.073</td>
<td>0.997</td>
</tr>
<tr>
<td>Average bacterial density</td>
<td>1</td>
<td>0.008</td>
<td>0.932</td>
</tr>
<tr>
<td>Bacterial treatment x average bacterial density</td>
<td>6</td>
<td>0.076</td>
<td>0.997</td>
</tr>
</tbody>
</table>
Table 9. Mixed model analysis for effects of bacterial treatment, average bacterial density, and combined effects on mosquito development index of *Aedes albopictus* larvae.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>F</th>
<th>P &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial treatment</td>
<td>6</td>
<td>0.811</td>
<td>0.593</td>
</tr>
<tr>
<td>Average bacterial density</td>
<td>1</td>
<td>0.024</td>
<td>0.870</td>
</tr>
<tr>
<td>Bacterial treatment x average bacterial density</td>
<td>6</td>
<td>0.832</td>
<td>0.581</td>
</tr>
</tbody>
</table>
Figure 1. Photograph of microcosm lid adapted with 0.22 μm nylon filter membrane.
Figure 2. Mean CFU densities of *P. syringae* and *E. asburiae* grown in white oak leaf extracts of varying concentrations (Experiment I).
Figure 3. Water column and biofilm bacterial CFU densities in microcosms inoculated with various bacterial treatments; *Aedes albopictus* larvae introduced at day 0 (Experiment V).
Figure 4. Mean survivorship after 21 days of *Aedes albopictus* larvae introduced into microcosms inoculated with various bacterial treatments. (Experiment V).
Initial data point of each treatment is the inoculation event ($5 \times 10^4$ bacteria/mL); not shown due to graph scaling limitations.

Figure 5. Water column bacterial CFU densities ($\pm$ SE) of microcosms inoculated with various bacterial treatments; *Aedes albopictus* larvae introduced at day 0 (Experiment VI).
Figure 6. Head capsule width distribution of living larvae recovered from all treatments at day 21 (Experiment VI).
Figure 7. Head capsule width distributions of living larvae at day 21 from bacterial isolate treatments and positive control microcosms (Experiment VI).
APPENDIX
Appendix A. Effects of *Aedes albopictus* larvae on water column and leaf biofilm bacterial communities and detection of transstadial bacteria by denaturing gradient gel electrophoresis.

**Introduction**

Chapter one of the current thesis (*Interaction of Bacteria and Aedes albopictus Larvae: Differential Effects of Bacterial Species on Larval Growth and Transstadial Movement of Bacteria*) describes a series of culture-based experiments intended to investigate the role of individual bacterial species on *Aedes albopictus* development. It was hypothesized that different bacterial species present in larval habitats have differential effects on larval development. It was demonstrated that isolated bacterial species associated with white oak leaves are insufficient to support larval development of this arthropod species, and results of the previous study showed that the bacterial isolate treatments used in larval microcosm experiments did not have significant effects of larval development outcomes. It is probable that the lack of variation in outcomes between the treatments was largely attributed to the overwhelming impact of starvation and high rates of larval mortality. In the current approach, larvae are allowed to develop in microcosms containing a native microbial community. By tracking the progression of bacterial communities associated with microcosm water columns, microcosm leaf biofilms, and mosquitoes persisting within the microcosms by use of DGGE fingerprinting, additional insight regarding the feeding habits of larvae can be obtained, and investigation of the hypotheses of the previous study can continue. In the current study we intend to determine effects that the presence of larvae have
on bacterial communities and whether *Ae. albopictus* larvae have differential effects on water column and leaf biofilm bacterial communities.

The previous study also hypothesized that different bacterial species vary in transstadial transmission to pupae and adults of *Ae. albopictus* mosquitoes. Because larvae failed to develop to adults, the study inadequately determined which isolated bacterial species persisted transstadially from the larvae to the adults, and the use of culture-based methods failed to detect viable transstadial bacteria in adult *Ae. albopictus* mosquitoes that developed in laboratory microcosms containing native microbial communities. Detection of transstadial bacteria was limited to only those species that are viable on the growth media that had been utilized in the previous assays. In the current experiment, molecular methods using universal bacterial primers allow the detection of un-culturable bacterial taxa.

This study uses paired control and experimental microcosms to survey how bacterial communities associated with white oak leaves change in the presence of developing mosquito larvae. Native white oak leaf infusions were allowed to ferment and then divided into paired equal portions; the control was absent of larvae while the other received larvae treatment. Prior to the introduction of *Ae. albopictus* larvae, paired control and treatment microcosms shared similar bacterial community profiles. Observations of contrasts between bacterial community progression in microcosms populated with larvae and those absent of larvae was accomplished by using PCR amplification of a variable region of the 16S rRNA gene using universal bacterial primers and denaturing gradient gel electrophoresis (DGGE).
Materials and Methods

White oak leaf infusion preparation

Recently-senesced white oak leaves were collected at North Carolina State University in Raleigh, North Carolina. Circular leaves disks (23 mm dia.) were cut using a #15 cork borer centered on the leaves’ primary veins with the intention of making each disk to be of similar size, surface area, and nutrient content. Average leaf disk mass was determined by sampling and weighing 25 random leaf disks at a time, repeated 10 times. Average individual leaf disk mass (± SD) was measured to be 54.8 (± 2.8) mg.

Based on a previously resolved formulation (Trexl et al., 1998), 3 one-liter volumes of 0.5X WOL infusion (each with approximately 4.2 g WOL disks ($n = 76$ leaf disks) and 1 L of well water) were prepared in sterile glass jars, and allowed to ferment for 6 days. After the fermentation period, each volume of infusion was gently homogenized by mixing and divided into 2 similar 500 mL portions. Division was accomplished by alternatively placing 100 mL aliquots of the parent infusion into two separate sterilized 2-liter glass jars. Leaf disks from the parent infusion were randomly selected and divided into equal quantities ($n = 38$) and placed separately into the two paired infusions. Each pair of infusions was intended to have a similar bacterial community of both the water column and among biofilms present on the leaf disks. Randomly, one jar from each pair was chosen to operate as a control microcosms and the other as a treatment microcosm to which mosquito larvae were added. Each of the six microcosm jars was fitted with a sterile plastic lid that included a nylon filter
membrane (0.45 µm pore size, 47 mm dia., Sigma-Aldrich®, St. Louis, MO, USA) attached to a hole in the lid to allow for gas exchange.

Larval introduction

Mosquito eggs were acquired from *Ae. albopictus* colonies kept at North Carolina State University. Colonies were developed from field-collected mosquitoes originating in New Orleans, Louisiana in 2003. Eggs were surface-sterilized with 1% bleach solution for 60 seconds, then with 95% ethanol for 60 seconds, and then rinsed 5 times with sterile distilled water. A 1 mL portion of last rinse water was reserved for assessment of sterility. Sterilized eggs were allowed to hatch in sterile distilled water for 120 minutes; then, emergent first instar larvae were allotted to the three treatment microcosms at 0.25 larvae/mL densities (125 larvae in each 500 mL microcosm). Additionally, two pools of 25 first instar larvae to be used for subsequent bacterial community analysis were reserved in 200 µL of sterile TNE buffer in capped 1.5 mL centrifuge tubes and then stored at -80°C. From the 1 mL of last rinse water, 200 µL was spread-plated on R2A (Reasoner and Geldreich, 1985) to detect viable bacteria. The plate was incubated at 30°C and checked for colony formation after 96 hours.

Water column, biofilm, and mosquito sampling

Water columns and leaf biofilms were sampled from the control (larvae-negative) microcosms, and water columns, leaf biofilms, and mosquitoes (larvae, pupae, adults) were
sampled from the treatment (larvae-positive) microcosms. Sampling occurred regularly at 6
day intervals for 18 days (day 0, day 6, day 12, and day 18). At each sampling occasion, two
samples of each type were obtained from each microcosm to provide primary and backup
samples for bacterial community analyses. Initial sampling (day 0) of the water column and
leaf disks occurred immediately prior to larval introduction. Day 0 samples included first
instar larvae that had been reserved following the egg sterilization and hatching procedure
previously described. Day 6, day 12, and day 18 sampling included water column, leaf disks,
and larvae; day 12 and 18 included pupae; and day 18 included adults. All larvae, pupae, and
adults were selected randomly and were chosen independently of size or sex. Adults that
emerged prior to day 18 were vacated from the microcosms daily. Adults collected on day
18 and used for subsequent bacterial community analyses were less than 24 hours old.

Prior to sampling, each microcosm was gently swirled to homogenize the water
column. Water column samples (5 mL), absent of larvae, were withdrawn using sterile
plastic 5 mL syringes (Fisher Scientific, Pittsburg, PA, USA). As syringes were discharged,
bacteria suspended in water column samples were retained on sterile polycarbonate filter
membranes (0.22 µm pore size, 22 mm dia., Sigma-Aldrich®, St. Louis, MO, USA)
contained in sterile filter elements fitted to the terminal of each syringe. Filter membranes
were quartered and submerged in 200 µL of sterile TNE buffer contained in a sterile 1.5 mL
centrifuge tube. Leaf biofilms were then sampled by removal of a single leaf disk using
sterilized long-handled forceps. Each disk was quartered and submerged in 200 µL of sterile
TNE buffer contained in a sterile 1.5 mL centrifuge tube. Water column and leaf biofilm samples were kept at -80°C for storage prior to DNA extraction.

Individual larvae and pupae were removed from microcosms using sterile 1 mL pipettes. Watch glasses containing pooled groups of 3 larvae or pupae (and approximately 3 mL of extracted water column) were immediately transferred to refrigeration at 2 °C for 30 minutes to immobilize the insect and prevent defecation of gut contents. After immobilization, water column fluid present among each pooled sample was removed by pipetting. Pooled groups of larvae \( n = 3 \) or combined larvae and pupae sampled on the same day from the same microcosms \( n = 3 + 3 = 6 \) were surface-sterilized by washing in 1% bleach for 60 seconds, then rinsed with sterile distilled water 5 times. For each pooled sample, a 1 mL aliquot of last-rinse water was retained in a sterile 1.5 mL centrifuge tube and stored at -80 °C. Pooled groups of 3 larvae or pupae were then submerged in 200 µL of TNE buffer in 1.5 mL capped centrifuge tubes and stored at -80 °C.

Adults collected on day 18 were released from individual treatment microcosms into a plexiglass box, then collected by aspirator, and secured in glass vials. Vials were refrigerated at 2 °C for 30 minutes to immobilize adults, after which groups of 3 adults were pooled in watch glasses and surface-sterilized as previously described.

Because leaf infusion, leaf disks, and mosquitoes were sampled from control and experimental microcosms though the duration of the experiment, the quantity of larvae per water column volume, the quantity of larvae per number of leaf disks, and the ratio between water column volume and leaf disk numbers changed over time. The volume and number of
components removed were designed to conserve similar ratios of microcosm components for the duration of the experiment. Table 1 describes the estimated densities and ratios of the microcosm components.

DNA extraction and purification

Genomic DNA extraction followed previously described protocol (Smith et al., 2001). Individual sample vials (containing water column, leaf disks, or mosquitoes) were thawed to which 160 µL lysis buffer I, 20 µL Protease K, and 20 µL lysozyme were added. Sample materials were manually pulverized with sterile Teflon pestles, and then incubated at 37°C for 60 minutes while being agitated every 20 minutes. 200 µL of lysis buffer II was added, mixed and incubated at 56 °C for 60 minutes while being agitated every 20 minutes. Extraction was carried out using phenol-chloroform. 200 µL of each phenol and chloroform were added followed by centrifugation (10 min, 10K rpm). The aqueous layer was isolated to which 400 µL of chloroform was added followed by centrifugation (10 min, 10K). The aqueous layer was again isolated from which DNA was precipitated using 700 µL cold ethanol and 35µL sodium acetate and stored at -20 °C for 24 hours. Samples were then thawed and pellets isolated by centrifugation (15 min, 12K), dried, and dissolved in 50 µL of sterile DNA-free water (Thermo Fisher Scientific Inc., Grand Island, NY, USA). Final purification was accomplished with Wizard DNA Clean Up System (Promega, Madison, WI, USA) and re-suspended in 50 µL of sterile DNA-free water. The DNA concentration of purified samples was measured by Nanodrop (Nanodrop Products, Wilmington, DE, USA).
The DNA concentrations of all samples were normalized to concentrations between 25 ng/µL and 50 ng/µL. Samples having DNA concentrations below 25 ng/µL were dehydrated by lipolysis and then resuspended in sterile DNA-free water, and samples having concentration above 50 ng/µL were diluted with sterile DNA-free water.

**DNA amplification**

Polymerase chain reaction was used to amplify the variable V3 region of 16S ribosomal subunit genes using universal bacterial primers F327-GC (5’-GC-clamp +CCTACGGGAGGCAGCAG-3’) and 518R (5’-ATTACCGCGGTGCTGG-3’); the forward primer including a 40-bp GC clamp. PCR was performed in 50 µL reactions, and reactants for each sample included: AmpliTaq Gold® 360 Master Mix (25 µL), forward primer (2 µL), reverse primer (2 µL), sterile DNA-free water (20 µL), and template DNA (1 µL) was provided by genomic DNA extractions. Following the reaction setup of each sample, a negative control was prepared that included DNA-free water as the reaction template. A PCR touchdown program used was as follows: initial denaturation at 95 °C for 10 min, followed by 10 touchdown cycles consisting of denaturation at 95 °C for 1 min, annealing period (initially at 65 °C then decreasing by 1 °C each cycle to touchdown temperature of 55 °C on tenth cycle) for 1 min, and extension period at 72 °C for 3 min, then 40 cycles of denaturation (95 °C), annealing (55 °C), and extension (72 °C), with the final extension step extended to 10 min. PCR products were then stored at -20 °C.
PCR products (including the negative control) were checked for amplification using agarose gel electrophoresis. Individual sample product amplicons (2 µL) with loading dye (1 µL) were run in individual lanes on a 1.2% agarose gel containing ethydium bromide. Each agarose gel included a Hi-Lo Marker (Minnesota Molecular, Minneapolis, MN, USA) as a reference for fragment length. Gels were electrophoresed at 100 vdc for 30 minutes, after which, gels were photographed using UVP Doc-It®LS Image Acquisition (UVP, Upland, CA, USA), and amplification of approximately 160 bp fragments from each sample was verified.

**Denaturing gradient gel electrophoresis**

DGGE performed in this study utilized the Dcode™ universal mutation detection system (Bio-Rad Laboratories Inc., Hercules, CA, USA). Preliminary testing of DGGE gradient concentrations identified a 45 to 55% denaturing gradient as an effective spectrum for denaturation of 16S rRNA gene fragments generated by the F327/518R universal primer set. Acrylamide denaturant gels (16 mL, 8% wt/vol, 45 to 55% denaturing gradient) were poured using 0.75 mm spacers and a 25-well comb. The limitation of 25 samples per gel required development of 4 gels to complete analysis of all study samples.

PCR amplicons (12 µL) from each sample were combined with loading dye (3 µL) and each loaded into a single lane. Every fourth or fifth lane contained a ladder composed of mixed products 16S rDNA amplification products from 6 known bacterial isolates (Ponnusamy et al., 2008): Enterobacter asburiae, Acidivorax avenae, Pseudomonas.
syringae, Brevundimonas vesicularis, Azorhizobium caulinodans, and Bacillus thuringiensis. Each gel was electrophoresed at 50 vdc for 18 hours in 0.5X TAE running buffer heated to 60 °C followed by staining with GelStar™ nucleic acid stain (Lonza, Allendale, NJ, USA) for 15 minutes. Gels were then photographed using UVP Doc-It®LS Image Acquisition.

**OTU re-amplification and identification**

Immediately following gel photography, gels were observed on a UV light plate. Individual bands representing distinct operational taxonomic units (OTUs) were cut from gels using individual sterile scalpel blades and placed into 25 µL of sterile DNA-free water in sterile 1.5 mL centrifuge tubes. Tubes were kept at 4 °C for 24 hours to elute DNA. PCR amplification of 16S rRNA gene fragments was performed in 25 µL volumes using AmpliTaq Gold® 360 Master Mix and universal bacterial primers (F327 and 518R) as previously described. Template material (1 µL per reaction) was provided by eluted gel DNA, and a negative control included 1 µL of DNA-free water as a template. As previously, a PCR touchdown program was followed, and PCR products were then stored at -20 °C. Amplification of products was confirmed by gel electrophoresis following the procedures as previously stated. 10 µL portions of band amplification products were then sequenced by Sanger sequencing (Eton Bioscience, Research Triangle Park, NC). Resulting sequences were analyzed and abbreviated to the target primer regions using BioEdit Sequence Alignment Editor (Ibis Biosciences, Carlsbad, CA, USA). Putative bacterial phylotypes were
determined using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1997) by comparison with sequences in the NCBI sequence database.

*DGGE image processing and analysis*

Separate acrylamide gel preparations produced variations in denaturing gradients between four gel images. “Smiles” (horizontal waves associated with poured gradient inconsistencies) were corrected, and vertical re-proportioning of gel images to align ladder bands was accomplished using the image transformation tool (Adobe® Photoshop® ver. 6.0.1, San Jose, CA, USA). The four gel images were combined into a single image so that band analysis of all samples was accomplished simultaneously.

Analyses of presence of bands, band intensities, and band positions were performed using 1D Analysis Software (UVP Visionworks LS ver. 8.6, Upland, CA, USA). Following automatic detection of lanes and bands by the software, additional bands were identified and added manually based on visual inspection. Software analysis converted band positions to \( R_f \) values ranging from 0.0 to 1.0, each representing the proportional distance of the gel length that DNA fragments migrated before denaturation. 1D analysis also provided individual band intensities which were converted to relative intensities as values ranging from 0.0 to 1.0. Relative intensity is the proportion each band’s intensity of the total intensity of all bands detected within its sample lane. Bands with relative intensities measured at less than 0.05 were excluded from subsequent analyses.
Following 1D analysis, equivalent R<sub>f</sub> values of OTU bands common between different sample lanes was corroborated. OTU bands that were determined to be similar based on visual inspection of gel images but regarded with small variation in R<sub>f</sub> values (as determined by the 1D analysis software) were manually corrected to have equivalent R<sub>f</sub> values.

**Statistical analysis**

Shannon-Weaver index (Shannon and Weaver, 1963) and Pielou’s evenness of species index (Washington, 1984) were calculated using Microsoft Excel (Microsoft Office Excel 2007, Redding, WA, USA), and Jaccard’s similarity measurements were completed using Community Analysis Package (ver. 3.1, Pieces Conservation Ltd., Limington, Hants., UK). All statistical analyses were performed using JMP® Pro (ver. 12.0.1, SAS Institute, Cary, NC, USA).

Shannon-Weaver diversity (\(H'\)) and evenness indices (\(E\)) were evaluated for each sample based on individual DGGE OTU band frequencies and intensity values. Shannon-Weaver diversity was calculated using the equation

\[
H' = -\sum P_i \ln P_i,
\]

where \(P_i\) describes the intensity of a each OTU band in a sample as a proportion of the total intensity of all bands in a sample and is calculated as

\[
P_i = n_i / N,
\]
where $n_i$ is the measured intensity of OTU band $i$, and $N$ is the total intensity of all bands from the given sample. Evenness ($E$) was calculated as

$$E = H' / \ln S,$$

where $S$ is the total number of OTU bands from a given sample. Measures of Jaccard’s similarity between samples is calculated as

$$J = a / (a + b + c),$$

where $a$ is the number of OTU bands present in both samples, $b$ is the number of OTU bands absent in the first sample though present in the second sample, and $c$ is the number of bands absent in the first sample though present in the second sample.

Individual linear mixed model (LMM) analyses ($\alpha = 0.05$) were performed among water column and biofilm samples to test for the effects of presence of larvae and time on diversity (Shannon-Weaver diversity) and evenness (Pielou’s evenness of species).

Jaccard’s similarity of bacterial communities was calculated for pairs of water column samples and pairs of leaf biofilm samples for days 0, 6, 12, and 18. LMM analyses were performed to test for the effects of locality in microcosm, time, and the combined effects of these two factors on similarity of paired microcosms. The ‘locality in microcosm’ variable was considered as a fixed factor and refers to whether the paired samples are from the water column of leaf biofilms. Time was also considered as a fixed factor, and replication was included in the model as a random factor.
Results and Discussion

Microcosm setup and larval development

Prior to introduction, larvae were surface-sterilized to eliminate exogenous bacteria that would obfuscate detection of transovarial bacteria. Colony formation was not observed on R2A culture plates inoculated with last rinse water from the egg sterilization procedure suggesting that adequate sterilization had been accomplished. Among each experimental trial, larvae emerging from surface-sterilized eggs persisted in experimental microcosms through the course of the experiment. Larvae were consistently seen actively feeding and developing in the microcosms with no obvious larval mortality having occurred through the experimental period. Generally, through the course of the experiment, the number of larvae decreased (through sampling or development to adults) at a rate below that of water column and leaf disk removal. Because of this, the larvae persisting in microcosms on days 6 and 12 were at a slightly lower density that those on day 0. The larval density further decreased at day 18, though the microcosm experiments were immediate terminated following the day 18 sampling, and the relatively low larval density at this time would have no impact on development outcomes.

DGGE gel analyses

16S rRNA gene fragments were amplified from all microcosm water column and leaf biofilm genomic DNA extractions and from all genomic DNA extractions of surface-sterilized larvae, pupae, and adult mosquitoes as demonstrated by agarose gel electrophoresis
of PCR products. A faint band was detected on the negative control of the 16S rRNA gene amplification suggesting that some contamination occurred during the PCR assay. PCR negative control amplification products were carried through to visualization on the DGGE. The banding pattern paralleled the adjacent ladder lane, suggesting that some overflow occurred during DGGE gel loading. However, a distinct additional band was present that is thought to represent the source of PCR contamination (discussed below).

To test for the effects of *Ae. albopictus* larvae on water column and leaf biofilm bacterial communities, Shannon-Weaver diversity and Pielou’s evenness of species for each sample was determined based on OTUs recognized following DGGE analysis. Separately, among water column and biofilm samples, the effects of time and presence of larvae on bacterial diversity were tested by linear mixed model analyses. Both factors (time and presence of larvae) and the combined effects of these two factors were found to have no significant effects of either Shannon-Weaver diversity and evenness of species in the water column samples. Likewise, these two factors and the combined effects of these two factors were not found to have significant effects on bacterial community diversity and evenness of leaf biofilm bacterial communities. LMM results for diversity and evenness outcomes of water column samples are located on Table 2 and Table 3, respectively, and leaf biofilm results are located on Table 4 and Table 5.

Jaccard’s similarity values were calculated for each paired microcosm at each of the four sampling times. A LMM analysis was performed to test for the effects of microcosm locality (water column or leaf biofilm) and time (with replication as a random variable) on
similarity values of the paired microcosms. Both time ($P < 0.001$) and microcosm locality ($P = 0.010$) were found to have significant effects on similarity values, while the combined effects of these two factors did not have significant effects (Table 6). The mean ($\pm$ SE) Jaccard similarity values of water column samples was 0.48 ($\pm$ 0.08) and 0.63 ($\pm$ 0.07) for leaf biofilm samples. Comparisons of means between the two microcosm localities showed that leaf biofilms have significantly greater bacterial community similarity compared to those of water column samples.

Tukey HSD pairwise comparisons of time points (Table 7) showed that time points concurrent to one another generally had comparable similarity values while, and time points with greater temporal distance tended to have significantly different similarity values. Jaccard similarity values are further diagrammed in Figure 1. Both water column and leaf biofilm bacterial communities of paired microcosms progressively decreased in similarity over time.

**OTU identification**

Ten OTU bands were cut from DGGE gels, re-amplified by PCR, and sequenced by Sanger sequencing. Two of the results included multiple sequences and failed to match to any known sequences in the NCBI database. Two additional sequences matched non-target arthropod 18S rRNA gene sequences. The remaining six sequences partially matched 16S ribosomal gene sequences of various culturable and uncultured bacterial taxa. Results are delineated on Table 8.
The negative control sample was found to contain contamination of Lepidoptera DNA. The source of this DNA is unknown, though OTU bands of corresponding R\textsubscript{f} values are absent from the remaining samples. Additionally, mosquito samples amplified 18S rRNA of *Ae. albopictus*. The universal primers used in the PCR assay appear to amplify non-target arthropod DNA, and reevaluation of primer sequence specificity may be pertinent to future studies analyzing bacterial communities associated with arthropods.

*Transovarial and transstadial transmission of bacteria*

Following surface-sterilization of *Ae. albopictus* eggs, bacterial communities of emergent larvae were fingerprinted by DGGE, and two distinct bands were visualized for each sample. The uppermost bands of these samples had the highest average relative abundance (mean relative abundance (± SE) = 0.41 (± 0.12)) was determined to represent non-target arthropod DNA, and corresponded to 18S *Ae. albopictus* gene amplification. The remaining OTU band sequence was 99% similar to *Wolbachia pipiensis*.

Bacterial DNA amplified from surface-sterilized mosquito pupae and adults potentially represents bacterial species that persisted transstadially. Similarly to day 0 larval samples, the most prominent DGGE band in each sample was the uppermost. Though these major bands were not all individually sequenced, they all are of comparable intensity, denatured at a similar distance (equivalent R\textsubscript{f} values), and were exclusive to mosquito samples, and it is likely that they all represent amplification of non-target *Ae. albopictus* 18S rRNA gene. Two additional bands representing transstadial transmission were sequenced
including one from a pupa (Figure 2, band number 9) and one from an adult (Figure 2, band number 10). The OTU band from the pupa was found to include multiple reads preventing specific OTU identification, and the sequenced adult OTU most closely matched an uncultured bacteria. OTUs corresponding to the *Wolbachia pipiensis* band identified in the neonate larvae sample are present in all larvae, pupae and adults. Though no additional sequencing of these corresponding bands was performed, it is likely that these bands also represent the presence of *Wolbachia pipiensis*.

The methods employed in the current study to detect transovarial and transstadial bacteria rely on amplification of bacterial DNA. Detection of DNA and identification of OTUs is regarded as potentially representing transovarial and transstadial bacteria because it is unknown whether the amplified DNA is from viable bacteria. Additional molecular assays that detect bacterial RNA can more accurately and conclusively detect viable bacteria.
References cited


Table 1. Estimated ratios of microcosm elements following periodic water column, leaf biofilm, and mosquito sampling for experimental and control microcosms.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day</th>
<th>Larval density (larvae/mL)</th>
<th>Larvae/leaf disks</th>
<th>Leaf disks/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experimental</strong></td>
<td></td>
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<td></td>
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</tr>
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<td>0.2551</td>
<td>3.4722</td>
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<td></td>
<td>6</td>
<td>0.2511</td>
<td>3.5000</td>
<td>0.0717</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>0.2367</td>
<td>3.3438</td>
<td>0.0708</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>0.2209</td>
<td>3.1667</td>
<td>0.0698</td>
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<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td></td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>0.0735</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>-</td>
<td>-</td>
<td>0.0708</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>-</td>
<td>-</td>
<td>0.0681</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>-</td>
<td>-</td>
<td>0.0652</td>
</tr>
</tbody>
</table>
Table 2. Mixed model analysis for effects of presence of *Aedes albopictus* larvae, time and combined effects on bacterial diversity of WOL infusion water columns.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>$F$</th>
<th>$P &gt; F$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence of mosquito larvae</td>
<td>1</td>
<td>0.118</td>
<td>0.573</td>
</tr>
<tr>
<td>Time</td>
<td>3</td>
<td>0.656</td>
<td>0.593</td>
</tr>
<tr>
<td>Presence of mosquito larvae x time</td>
<td>3</td>
<td>0.690</td>
<td>0.573</td>
</tr>
</tbody>
</table>
Table 3. Mixed model analysis for effects of presence of *Aedes albopictus* larvae, time and combined effects on bacterial evenness of WOL infusion water columns.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>$F$</th>
<th>$P &gt; F$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence of mosquito larvae</td>
<td>1</td>
<td>3.765</td>
<td>0.073</td>
</tr>
<tr>
<td>Time</td>
<td>3</td>
<td>0.206</td>
<td>0.891</td>
</tr>
<tr>
<td>Presence of mosquito larvae x time</td>
<td>3</td>
<td>2.997</td>
<td>0.067</td>
</tr>
</tbody>
</table>
Table 4. Mixed model analysis for effects of presence of *Aedes albopictus* larvae, time and combined effects on bacterial diversity of WOL biofilms.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>$F$</th>
<th>$P &gt; F$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence of mosquito larvae</td>
<td>1</td>
<td>0.0002</td>
<td>0.988</td>
</tr>
<tr>
<td>Time</td>
<td>3</td>
<td>1.594</td>
<td>0.235</td>
</tr>
<tr>
<td>Presence of mosquito larvae x time</td>
<td>3</td>
<td>0.176</td>
<td>0.911</td>
</tr>
</tbody>
</table>
Table 5. Mixed model analysis for effects of presence of *Aedes albopictus* larvae, time and combined effects on bacterial evenness of WOL biofilms.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>$F$</th>
<th>$P &gt; F$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence of mosquito larvae</td>
<td>1</td>
<td>2.821</td>
<td>0.115</td>
</tr>
<tr>
<td>Time</td>
<td>3</td>
<td>1.860</td>
<td>0.180</td>
</tr>
<tr>
<td>Presence of mosquito larvae x time</td>
<td>3</td>
<td>0.443</td>
<td>0.726</td>
</tr>
</tbody>
</table>
Table 6. Mixed model analysis for effects of microcosm locality, time and combined effects on bacterial community similarity of paired microcosms.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>F</th>
<th>P &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microcosm locality (water column or leaf biofilm)</td>
<td>1</td>
<td>8.879</td>
<td>0.010</td>
</tr>
<tr>
<td>Time</td>
<td>3</td>
<td>23.611</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Microcosm locality x time</td>
<td>3</td>
<td>1.560</td>
<td>0.235</td>
</tr>
</tbody>
</table>

* Significant at $\alpha = 0.05$
Table 7. Sidak significance values for pair-wise comparisons of bacterial treatments for effects on CFU densities.

<table>
<thead>
<tr>
<th></th>
<th>Day 0</th>
<th>Day 12</th>
<th>Day 18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>0.136</td>
<td>&lt; 0.001*</td>
<td>&lt; 0.001*</td>
</tr>
<tr>
<td>Day 6</td>
<td>-</td>
<td>0.006*</td>
<td>0.0010*</td>
</tr>
<tr>
<td>Day 12</td>
<td>-</td>
<td>-</td>
<td>0.793</td>
</tr>
</tbody>
</table>

* Significant at $\alpha = 0.05$
Table 8. Identification of DGGE OTU band sequences by matching to NCBI database.

<table>
<thead>
<tr>
<th>Band no.</th>
<th>OTU identity</th>
<th>GenBank accession</th>
<th>Similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lepidoptera sp. partial 18S rRNA</td>
<td>KM975548.1</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td><em>Panteoa vagans</em> partial 16S rRNA</td>
<td>KM068031.1</td>
<td>90</td>
</tr>
<tr>
<td>3</td>
<td>Uncultured α-proteobacteria partial 16S rRNA</td>
<td>AF544953.1</td>
<td>98</td>
</tr>
<tr>
<td>4</td>
<td><em>Wolbachia pipientis</em> partial 16S rRNA</td>
<td>KR026940.1</td>
<td>99</td>
</tr>
<tr>
<td>5</td>
<td><em>Pseudomonas plecoglossicida</em> partial 16S rRNA</td>
<td>JX126810.1</td>
<td>97</td>
</tr>
<tr>
<td>6</td>
<td><em>Klebsiella</em> sp. partial 16S rRNA</td>
<td>KR190283.1</td>
<td>100</td>
</tr>
<tr>
<td>7</td>
<td>Multiple reads</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td><em>Aedes albopictus</em> 18S rRNA partial sequence</td>
<td>AB085210.1</td>
<td>100</td>
</tr>
<tr>
<td>9</td>
<td>Uncultured bacterium partial 16S rRNA</td>
<td>JF218946.1</td>
<td>95</td>
</tr>
<tr>
<td>10</td>
<td>Multiple reads</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 1. DGGE fingerprints of bacterial communities of water column (WC), leaf biofilm (BF), larvae, pupae, and larvae; microcosms with larvae (L+) and absent of larvae (L-)
(-) negative control; M marker of known bacterial species; 1 WC, L-, day 0; 2 WC, L+, day 0; 3 BF, L-, day 0; 4 BF, L+, day 0; 5 Larvae, day 0; 6 WC, L-, day 6; 7 WC, L+, day 6; 8 BF, L-, day 6; 9 BF, L+, day 6; 10 Larvae, day 12; 11 WC, L-, day 12; 12 WC, L+, day 12; 13 BF, L-, day 12; 14 BF, L+, day 12; 15 Larvae, day 12; 16 Pupae, day 12; 17 WC, L-, day 18; 18 WC, L+, day 18; 19 BF, L-, day 18; 20 BF, L+, day 18; 21 Larvae, day 18; 22 Pupae, day 18; 23 Adults, day 18;
Identification of bands is delineated in Table 8.
Figure 2. Comparison of changes in Jaccard’s similarity of paired microcosm samples over time.

Water column samples from paired microcosms
Leaf biofilm samples from paired microcosms