MICROBIOLOGY OF AQUATIC SYSTEMS

Species Composition of Bacterial Communities Influences Attraction of Mosquitoes to Experimental Plant Infusions

Loganathan Ponnusamy · Dawn M. Wesson ·
Consuelo Arellano · Coby Schal · Charles S. Apperson

Abstract In the container habitats of immature mosquitoes, catabolism of plant matter and other organic detritus by microbial organisms produces metabolites that mediate the oviposition behavior of *Aedes aegypti* and *Aedes albopictus*. Public health agencies commonly use oviposition traps containing plant infusions for monitoring populations of these mosquito species, which are global vectors of dengue viruses. In laboratory experiments, gravid females exhibited significantly diminished responses to experimental infusions made with sterilized white oak leaves, showing that attractive odorants were produced through microbial metabolic activity. We evaluated effects of infusion concentration and fermentation time on attraction of gravid females to infusions made from senescent bamboo or white oak leaves. We used plate counts of heterotrophic bacteria, total counts of 4′,6-diamidino-2-phenylindole-stained bacterial cells, and 16S ribosomal DNA (rDNA) polymerase chain reaction–denaturing gradient gel electrophoresis (DGGE) to show that changes in the relative abundance of bacteria and the species composition of bacterial communities influenced attraction of gravid *A. aegypti* and *A. albopictus* mosquitoes to infusions. DGGE profiles showed that bacterial species composition in infusions changed over time. Principal components analysis indicated that oviposition responses to plant infusions were in general most affected by bacterial diversity and abundance. Analysis of bacterial 16S rDNA sequences derived from DGGE bands revealed that Proteobacteria (Alpha-, Beta-, Delta-, and Gamma-) were the predominant bacteria detected in both types of plant infusions. Gravid *A. aegypti* were significantly attracted to a mix of 14 bacterial species cultured from bamboo leaf infusion. The oviposition response of gravid mosquitoes to plant infusions is strongly influenced by abundance and diversity of bacterial species, which in turn is affected by plant species, leaf biomass, and fermentation time.

Introduction

Immature stages of container-inhabiting mosquitoes, such as *Aedes aegypti* and *Aedes albopictus*, live in aquatic habitats characterized by diverse microbial communities [32]. These microbes play key roles in nutrient cycling and breakdown of large molecules, such as cellulose, chitin, and lignin, into smaller compounds that can be more easily assimilated [19, 47]. The microbes themselves and catabolism of leaf detritus produce volatile and nonvolatile metabolites that mediate the oviposition behavior of gravid mosquitoes [31]. Bacterial biomass [29, 37] and species composition [38, 53] have been suggested to be important environmental determinants of the occurrence and abundance of mosquitoes.

Each *Aedes* female normally lays eggs in multiple water-filled containers [7], but addition of organic material can significantly increase the number of eggs laid in target containers [36, 51] because more gravid females are attracted and induced to lay eggs [5, 51]. Infusions made

L. Ponnusamy · C. Schal · C. S. Apperson (✉)
Department of Entomology, North Carolina State University,
Campus Box 7613, Raleigh, NC 27695-7613, USA
e-mail: charles_apperson@ncsu.edu

D. M. Wesson
Department of Tropical Medicine, Tulane University,
New Orleans, LA 70112-2699, USA

C. Arellano
Department of Statistics, North Carolina State University,
Campus Box 8203,
Raleigh, NC 27695-8203, USA

from a variety of grass species [6, 34, 42] and from white oak leaves (*Quercus alba*) [51] have been used in ovitraps (oviposition traps) for monitoring the egg-laying activity of *Aedes* mosquitoes in the field. Similarly, gravid females of *Culex* spp. [1, 16, 17, 23, 39] are attracted to hay and grass infusions. However, the attractiveness of infusions to gravid mosquitoes changes over the fermentation period [16, 42]. These changes are thought to be caused by variations in bacterial metabolic activity [16, 22].

Notably, few investigations have cultured bacteria from natural or experimental mosquito habitats [5, 12, 31, 52]. In recent years, new molecular biology technologies have overcome the culturing process and they not only can detect cultured microorganisms but also the uncultured ones. Specifically, denaturing gradient gel electrophoresis (DGGE) has emerged as a useful tool for separating polymerase chain reaction (PCR)-amplified DNA fragments of 16S ribosomal DNA (rDNA) of the same length but different base pair sequences [27]. PCR–DGGE has been widely used to analyze microbial species diversity of samples from soil, water, rhizosphere, and leaf surfaces [9, 10, 14]. Recently, we used PCR–DGGE to analyze the species composition of bacterial communities in container habitats of mosquitoes in the field [32]. Proteobacteria and Bacteroidetes were the predominant heterotrophic bacteria identified. Bacterial communities in the containers consisted largely of undescribed species, and a phylogenetic analysis based on 16S rDNA sequences indicated that bacterial species composition was independent of container type and the spatial distribution of containers.

Our aim in the present study was to describe changes in the abundance of bacteria and diversity of species in bacterial communities in bamboo (BL) and white oak leaf (WOL) infusions during the fermentation process. Using a combination of culture-dependent and culture-independent methods and behavioral assays, we then related the changes in bacterial community structure to the oviposition response of gravid mosquitoes. We hypothesized that changes in microbial community structure would correlate with oviposition responses of mosquitoes to plant infusions. In addition to providing new insights into the molecular ecology of bacterial communities in plant infusions, results of our investigation have practical value in demonstrating the dynamic effects of leaf concentration and fermentation time on the attraction of gravid females to plant infusions that are used in mosquito surveillance programs.

Materials and Methods

Preparation of Plant Infusions

Since the main objective of our research was to characterize bacterial diversity in plant infusions that elicited oviposition responses from gravid mosquitoes, we initially screened in behavioral assays infusions made from plant species that were locally abundant or that had been used previously to make bioactive plant infusions [36, 42, 51]. These plant species included bamboo (*Arundinaria gigantea*), white oak (*Q. alba*), live oak (*Quercus virginiana*), pecan (*Carya illinoensis*), hackberry (*Celtis occidentalis*), red maple (*Acer rubrum*), redtop panic grass (*Panicum rigidulum*), and harvested Bermuda grass (*Cynodon dactylon*). Senescent leaves, gathered from the landscape of separate residential areas in the city of New Orleans, LA, USA or Raleigh, NC, USA, were used to produce 1× concentration infusions, each made by fermenting 33.6 g of senescent leaves in 4 L of well water in a Teflon® bag (TFM Modified PTFE homopolymer inert bag, No. P-00021-2, 63.5-μm thickness, Big Science, Inc.; Huntersville, NC, USA) after Reiter et al. [36], with modifications described by Ponnusamy et al. [31]. Gravid mosquitoes exhibited the highest oviposition responses to two plant species, bamboo and white oak. Consequently, further research was carried out only with these plant species. To optimize production of oviposition semiochemicals, we varied leaf mass and/or fermentation time in preparing infusions. WOL infusions (0.5×, 1×, 2×, and 4×) and bamboo leaf (BL) infusion (1×) were sampled and tested at 7-day intervals for 28 days. When sampled, the infusion was vigorously homogenized for approximately 15 s by shaking the Teflon bag, and a sample was collected with a sterile glass beaker for bacterial community analyses and testing in behavioral assays as described below.

Enumeration of Cultivable Bacteria by Plate Culture

Just prior to bioassay, samples of both plant infusions were taken for bacterial culture and enumeration of colony-forming units (CFU). Duplicate subsamples (1 mL) from each sample of infusion were separately mixed in 9 mL of 0.1% peptone (in sterile water, wt/vol) and serially diluted (tenfold) in 0.1% peptone. After dilution, 100 μL of each of the 10^{-2} to 10^{-5} dilutions was separately spread onto the surface of duplicate plates that contained R2A medium (Difco; Spark, MD, USA) [35] for enumeration of the total heterotrophic bacterial population, and the plates were incubated at 28°C. The number of CFUs was counted on dilution plates that developed between 30 and 300 colonies per plate 4–5 days after spread plating, and the count data were converted to CFU per milliliter of infusion.

Total Bacterial Counts

The DNA-intercalating fluorescent dye 4′,6-diamidino-2-phenylindole (DAPI) was used to estimate the number of bacterial cells in the infusions [33]. Two subsamples (5 mL)

were taken from each infusion sample. Cells were fixed with 1% final concentration of formaldehyde solution (Sigma; St. Louis, MO, USA). After staining for 10 min in darkness, the cell suspensions were filtered through black polycarbonate filters (pore size, 0.2 μm; 25-mm diameter; GE Water & Process Technologies, USA; Minnetonka, MN, USA), and the filters were mounted on glass slides with immersion oil [20, 33]. Total number of cells per milliliter in infusions was estimated from counts of DAPI-stained cells within ocular grids at 1,000×.

DNA Extraction and Purification

Infusion (15 mL) was filtered through a polycarbonate membrane filter (0.22-μm pore size, 47-mm diameter, Millipore; Billerica, MA, USA), and the filter for each sample was cut into quarters and placed into a microcentrifuge tube (1.5 mL, USA Scientific, Orlando, FL, USA) after which 250 μL of TNE buffer (100 mM Tris; 0.2 M NaCl; 10 mM EDTA; pH 7.4) was added. After vortexing, the tubes were stored at −80°C. Samples were thawed at room temperature and total nucleic acids were extracted and purified using methods described previously [40]. Crude DNA was purified with the WIZARD DNA Cleanup System (Promega, Madison, WI, USA). Purified DNA was subsequently used as a template to amplify the variable V3 region of 16S rDNA with universal bacterial primers 357F-GC (5′-GC-clamp + CCTACGGGAGGCAG CAG -3′) and 518R (5′-ATTACCGCGGCTGCTGG-3′). A 40-bp GC clamp was incorporated into the forward primer to prevent dissociation of the DNA double strand during DGGE analyses [27]. DNA extracted from infusion samples was amplified with a 1× GoTaq Green Master Mix (Promega, Madison, WI, USA), 0.2 μmol of each primer, and 1 μL (~5–15 ng) of template DNA, with sterile deionized water added to achieve a final volume of 50 μL. After initial denaturation of double-stranded DNA for 3 min at 94°C, the following PCR protocol was used: ten touchdown cycles [32] consisting of 1 min at 94°C, 1 min at 65°C, and 1 min at 72°C with a decrease in the annealing temperature of 1°C per cycle; 30 cycles consisting of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C; and extension for 10 min at 72°C. PCR products were electrophoresed in a 1.5% agarose gel followed by ethidium bromide staining. Amplicon size and yield were determined by comparison to molecular weight standards (Low DNA Mass Ladder; Gibco BRL, Carlsbad, CA, USA).

DGGE Analyses

PCR products derived from infusion samples were resolved separately for each of the three trials by DGGE [27], using a *Dcode*™ Universal Mutation Detection System (Bio-Rad,

Hercules, CA, USA). The DNA fragments were separated on a polyacrylamide gel with 8% (wt/vol) acrylamide (acrylamide/*bis*-acrylamide, 37.5:1.0, wt/wt), containing a linear gradient of denaturant that ranged from 40% to 65% (with 100% denaturant defined as 7 M urea plus 40% [vol/vol] formamide). PCR products from time course samples were stored at −80°C and analyzed concurrently for each trial. Approximately 500 ng of the PCR product from each sample was loaded in a separate well of a DGGE gel, which was run for 18 h at 50 V and 60°C. After completion of electrophoresis, gels were stained in 0.5× TAE buffer containing SYBR green I (Sigma) and digitally photographed. PCR–DGGE analyses were replicated for infusion samples, and identical DGGE band profiles were obtained (data not shown). Banding patterns of each gel were analyzed to assess bacterial species diversity and relative abundance in the infusion samples as described below. Each band was considered an operational taxonomic unit (OTU). A potential bias of the methodology should be noted. PCR amplification has been reported to lower apparent bacterial richness by causing a shift in evenness toward a few dominant phylotypes [30], which would reduce the relative abundance of other less common members of the community to below the detection limit of DGGE. Consequently, in our analyses, DGGE likely failed to detect some comparatively rare bacterial species in BL and WOL infusions.

Partial 16S rDNA Sequences from Excised Gel Bands

Representative bands were excised from DGGE gels with a sterile scalpel blade under UV illumination, placed in 50 μL of sterile water, and incubated overnight at 4°C to allow the DNA in the band to diffuse out of the gel piece. A 1-μL aliquot of eluted DNA was reamplified by PCR using the original primers without the GC clamp. Briefly, each PCR mixture contained 1 μL of eluted DNA from the DGGE band, 0.2 μmol of each primer, 5 μL of 10× *Pfx* amplification buffer, 1.5 μL of 10 mM dNTP mixture, 1 μL of 50 mM MgSO$_4$, and 1 U of Platinum® *Pfx* DNA Polymerase (Invitrogen, Inc.; Carlsbad, CA, USA), and sterile deionized water was added to achieve a final reaction volume of 50 μL. Resulting PCR products were purified using the QIAquick PCR Purification Kit (Qiagen; Valencia, CA, USA). Sequencing was performed at Genomic Sciences Laboratory, North Carolina State University with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit in an automated ABIPRISM 377DNA Sequencer (Applied Biosystems; Foster City, CA, USA). The amplicons were sequenced using either 518R or 357F primer without GC clamp. Sequences were analyzed with Chromas v. 2.23 software (Technelysium; Tewantin, Australia) and checked for chimeras using the CHECK-

CHIMERA program of the Ribosomal Database Project (http://rdp8.cme.msu.edu/cgis/chimera.cgi?su=SSU). The V3 sequences were compared for homologies to those in the GenBank database with BLAST software (http://www.ncbi.nlm.nih.gov/BLAST).

Analyses of DGGE Gel Images

Gel photographs were analyzed using 1D Analysis software (UVP, Upland, CA, USA) as described by Ponnusamy et al. [32]. Briefly, sample lanes were created manually, and fixed background noise with a radius of 5 pixels was subtracted using the rolling disk algorithm. Band detection was first performed automatically by the software, and then additional bands were assessed and corrected individually by eye. A density profile was created and each OTU was assigned a percentage of the total intensity for each sample lane. Band positions in each lane were converted to R_f values that ranged from 0 to 1, using standard positions on the gel that encompassed the uppermost and lowermost band in each lane.

Phylogenetic Analyses

Partial 16S rRNA gene sequences were aligned using CLUSTAL W [50]. The methods of Jukes and Cantor [18] were used to calculate evolutionary distances between the OTUs, and phylogenetic trees were constructed using the neighbor-joining routine of MEGA version 4 (http://www.megasoftware.net; [49]). A stabile tree topology was achieved by a bootstrapping algorithm after 1,000 iterations.

Origin and Maintenance of Mosquito Colonies

A. aegypti and *A. albopictus* colonies were established from field-collected eggs from New Orleans, LA, USA, in 2003. At least once during each subsequent mosquito season, field-collected eggs from New Orleans were reared, and adults were added to the colonies of each species to maintain genetic diversity. Larvae of both mosquito species were reared and maintained as described by Trexler et al. [51]. Mosquito colonies were maintained in separate insectaries at ~28°C, ~75% RH, and a photoperiod of 14 h light/10 h dark, including dusk and dawn periods (60 min each). Mosquitoes were blood-fed on an arm of the same human host 4 to 5 days prior to set up of each experiment.

Behavioral Assay

The sticky screen olfactory bioassay was performed as described by Ponnusamy et al. [31], except that bioassay cups were painted flat black on the outside to obscure visual cues provided by the dark-colored plant infusions. Visual cues were further masked by a wire mesh screen coated with insect glue that was suspended just above the water in each cup. Briefly, bioassays were conducted in Plexiglas® cages (30×30×30 cm) fitted with stockinet cloth sleeves. In each cage, two 125-mL polypropylene cups, filled with 30 mL of either test or control solution, were placed randomly in diagonal corners of the bioassay cage. Trexler et al. [51] evaluated the oviposition response of gravid *A. albopictus* to 1× 7-day-old WOL infusion that was diluted by 0% to 90% in 10% increments. Maximal responses were reported for infusion diluted by 40% to 60%. Consequently, before behavioral assays were carried out, infusions were diluted by 50% with well water. Each bioassay included six replicate cages, and three separate bioassay trials were completed on different dates for each leaf mass.

Mosquito Response to Native and Sterile Infusions

To verify that attraction of gravid mosquitoes to plant infusions stemmed from microbial metabolites, we tested WOL (0.5×) infusion that was produced using sterile leaves and well water against native infusion (non-sterile leaves and well water) in sticky screen bioassays as described above. To make sterile (microbe-free) infusion, leaves and well water were separately autoclaved for 45 min at 120°C. After cooling, the sterile materials were combined in a sterile glass jar (2 L) fitted with a threaded plastic lid. Similarly, native infusion was produced in sterile jars but with leaves and water that were not sterilized. Jars were held for 14 days at 28°C before infusions were tested in behavioral assays.

Mosquito Response to Cultured Bacteria

Attraction of gravid *A. aegypti* to bacteria cultured from 7-day-old BL infusion was evaluated in sticky screen bioassays. Fourteen bacterial isolates cultured from attractive infusion in R2A media were purified and identified to species as described by Ponnusamy et al. [31]. Bacterial cells (10^4 cells per milliliter) of each of the 14 isolates were mixed and then $100\,\mu L$ of this suspension was inoculated into 100 mL of R2A medium. Bacteria were grown at 28°C with constant shaking, after which bacterial cells from these cultures were used in dose–response sticky screen bioassays [31]. A hemocytometer was used to estimate bacterial cell densities in R2A cultures, which were serially diluted (tenfold) with sterile water to achieve a final cell density of 10^6 to 10^9 cells per milliliter in the 30-mL volumes contained in test bioassay cups. Similarly, R2A media was added to control cups after dilution with sterile water.

Data Analyses

Statistical analyses of infusion parameters were performed using SAS® software (version 9.1, SAS Institute; Cary, NC, USA).

Diversity of OTUs

Band frequency and intensity data were used to calculate Shannon–Weaver diversity (H') and evenness (E) indices as previously described [32, 45]. H' was calculated using the following equation:

$$H' = -\sum P_i \log P_i,$$

where P_i, the proportion of the total diversity represented by the ith species (= OTU), was calculated as

$$P_i = n_i/N,$$

where n_i was the OTU intensity for the ith band and N was the summed intensities of all OTUs in a lane.

Evenness was calculated as $E = H'/\log S$, where S was the number of DGGE OTUs.

Principal Components Analysis

PCA was used to evaluate relationships among the infusion-associated variables. PCA (PROC PRINCOMP) was performed on the composite data set for all infusions defined according to plant species, infusion concentration, and fermentation time combinations. Variables in the analysis included the mean $\log_{10}$-transformed CFUs and DAPI-stained cell counts, mean number of DGGE OTUs, H', and E. The loading scores for the diversity variables for the first two principal components were plotted to visualize the relationship between the infusions in the context of plant species, infusion concentration, and fermentation time.

Effects of Infusion Concentration and Fermentation Time

Effects of infusion concentration (= $conc$) and fermentation time (= day) on bacterial growth (CFU and DAPI) and species diversity (number of DGGE OTUs, H', E) in BL and WOL infusions were determined by fitting a general mixed linear model to observed responses with SAS® procedure PROC MIXED. Prior to statistical analyses, CFU and total bacterial cell counts were subjected to $\log_{10}(x)$ transformations to achieve approximate normality. A visual examination of a scatter plot of predicted values against residuals [8] revealed that the residuals were evenly distributed about a mean of zero, indicating that assumptions about the residual distribution were held and that transformation was adequate to achieve homogeneity of variances and normality.

For WOL CFU and DAPI, the experimental design corresponded to a split-plot design with subsampling and $conc$ as a main unit factor and day as a subunit factor and $trial$ corresponding to the blocking factor. Subsamples were taken from replicated plates within each trial, leaf concentration, and day combination. For BL CFU and DAPI, the experimental design corresponded to a randomized complete block design with subsampling, with day as a treatment factor and $trial$ corresponding to the blocking factor, and $trial \times day$ as a random effect. Subsamples corresponded to replicated plates for each day and $trial$ combination. Separate analyses were carried out by plant species for each response variable. Random-effect covariance estimates were tested for significance (H_o: $\sigma^2_{effect} = 0$) by Wald's asymptotically normal Z test (PROC MIXED, covtest option). Covariance parameters estimated to be zero by PROC MIXED were dropped from the final model or, in the case of $trial$, included in the model as a fixed effect.

For WOL infusion, $conc$, day, and $day \times conc$ were designated as fixed effects, whereas $trial \times conc$ and $trial \times day$ ($conc$) were included as random terms since four CFU or ten DAPI subsamples were available within each replication. For BL infusion, $trial$ and day were considered fixed effects, while $trial \times day$ was included as a random term.

For diversity variables (H', E, and number of bands) in WOL infusion, $conc$, day, and $day \times conc$ were designated as fixed factors, whereas $trial$ and $trial \times conc$ were considered random effects that participated in the construction of the error term used in testing the significance of a given fixed effect. In BL infusion, week was considered a fixed-effect factor, while trial and $trial \times day$ (residual) were considered random effects. The statistical significance of changes in mean cell densities and diversity variables between the levels of infusion concentration (in WOL infusion) or fermentation time points (both plant species) were determined using t tests for pairwise comparison of least-squares means (LSM) at the $P \leq 0.01$ level of significance for control of type I error under the hypothesis of H_o: $LSM_i = LSM_j$. Comparisons between specific infusion concentrations (e.g., 0.5× and 1× compared to 2× and 4× WOL infusion) over fermentation time were carried out with the CONTRAST statement in the SAS® PROC MIXED procedure.

Comparison of 16S rDNA Sequence Libraries

We compared DGGE OTU sequences from BL and WOL infusions using webLIBSHUFF software (http://libshuff.mib.uga.edu) [46]. This software allows comparisons to be made between two 16S rDNA libraries and determines if they are significantly different under the null hypothesis that the two libraries are sampled from the same community. With the CLUSTAL W multiple sequence alignment, a distance matrix was generated with DNADIST, using the

Kimura two-parameter model in the software package BioEdit, v. 7.0.9.0 [11]. The distance matrix was then analyzed with webLIBSHUFF to determine if differences between the sets of BL and WOL infusion OTU sequences were statistically significant ($P \leq 0.05$).

Response of Gravid Mosquitoes

To determine if the response of gravid mosquitoes changed with infusion age and concentration, the proportions of the total number of mosquitoes in each cage that were trapped on the test and control screens were compared using a paired one-tailed *t* test (PROC *TTEST*). Prior to statistical analyses, the proportions were subjected to an arcsine $\sqrt{x}$ transformation to achieve approximate normality. Separate analyses were carried out for each mosquito species and infusion.

To further characterize the response of gravid females of each species to the plant infusions, we compared mean oviposition responses to the corresponding scores for the first two principal components (derived from the PC analyses described above) by regression analysis of variance using a general linear model (PROC GLM) [28]. The arcsine $\sqrt{x}$-transformed proportions of females responding to the infusion were used as dependent variables, and the standardized first two principal component scores for each infusion (*conc* × *day*) were the values of explanatory variables in this analysis.

Nucleotide Sequence Accession Numbers

The partial sequences obtained in this study have been deposited in the GenBank database under accession numbers EU920675–EU920735 (Supplemental Tables 1 and 2).

Results

Abundance of Bacteria in Infusions

For the 1× BL infusions, viable (cultivable) bacterial colony counts (CFUs) were highest in 7-day-old infusions (3.9× 10^6 CFU/mL) and significantly declined over time to the lowest CFU in 28-day-old infusions (0.90×10^6 CFU/mL; Table 1). The respective total bacterial cell counts (cultivable and uncultivable, live and dead), based on DAPI staining, were approximately 50- to 200-fold higher than the cultivable bacterial counts. A curvilinear change in cell densities was observed over the 28-day time course of fermentation with the density of bacterial cells increasing from 7 days (1.84×10^8 cells per milliliter) to 14 days (4.62×10^8 cells per milliliter) and then declining to the end point reading at 28 days (1.10×10^8 cells per milliliter; Table 1). The mean density of total bacterial cells varied significantly over the fermentation period ($F=30.55$; $df=3$, 6; $P=0.0005$; Supplemental Table 3).

For WOL infusions, leaf concentration exerted highly significant effects ($F=24.14$; $df=3$, 24; $P<0.0001$) on bacterial growth irrespective of fermentation time (Tables 2 and 3). Compared to the 0.5× and 1× infusions, CFUs and DAPI-stained cells in 2× and 4× infusions significantly increased ($P<0.05$) by approximately tenfold. There was a highly significant *day* × *conc* interaction ($F=9.98$; $df=9$, 24; $P<0.0001$) in the mixed-model analysis of variance (ANOVA) for viable bacterial cell count (Table 3), indicating that rates of bacterial growth were significantly different between infusion concentrations over the time course of leaf fermentation. Counts of CFUs showed that bacterial growth curves in 0.5× and 1× infusions were curvilinear over the 28-day fermentation period (Fig. 1), and the number of CFUs in the 0.5× infusion was significantly lower ($P<0.0001$) than in the more concentrated infusions. As infusion concentration increased, the functional form of the bacterial CFU growth curve assumed a progressively linear shape with a negative slope over time. The numbers of CFUs for the 1×, 2×, and 4× infusions were significantly ($P<0.01$) separated at the 7-day time point but converged over the 28-day fermentation period with the 0.5× infusions being significantly ($P<0.05$) different from the rest (Fig. 1).

In general, cell densities determined by DAPI staining were 30–50-fold higher than for counts of viable cells at the same leaf biomass and fermentation time points. However, the shape and trajectories of changes in total bacterial cells,

Table 1 Effects of fermentation time on abundance and diversity of bacterial species in 1× bamboo leaf BL infusion

Fermentation time (days)	DAPI[a] (cells × 10^8 mL^{-1})	CFU[a] (× 10^8 mL^{-1})	No. of OTUs[a]	Shannon–Weaver diversity index[a]	Evenness[a]
7	1.84 (0.25)b	0.039 (0.0037)a	49.33 (5.77)a	3.74 (0.17)a	0.86 (0.061)a
14	4.62 (0.32)a	0.020 (0.0031)b	46.67 (4.04)ab	3.67 (0.18)ab	0.85 (0.087)ab
21	4.23 (0.29)a	0.018 (0.0015)b	44.67 (7.23)bc	3.59 (0.23)bc	0.82 (0.076)bc
28	1.10 (0.13)c	0.009 (0.00043)c	42.33 (7.51)c	3.51 (0.25)c	0.81 (0.078)c

1× concentration infusion is made by incubating 33.6 g of senescent leaves in 4 L of well water

[a] Values are means (±SD) for three trials at each fermentation time. Means within each variable followed by the same letter are not significantly different at $P \leq 0.01$ by *t* tests for pairwise comparisons of least-squares means (LSM) under the hypothesis H$_o$: LSM$_{(i)}$=LSM$_{(j)}$

Table 2 Effects of leaf mass on diversity of bacterial species in white oak leaf infusion

Infusion concentration[a]	DAPI[b] (cells × 10⁸ mL⁻¹)	CFU[b] (×10⁸ mL⁻¹)	No. of OTUs[b]	Shannon–Weaver index[b]	Evenness[b]
0.5×	0.43 (0.051)b	0.028 (0.0022)b	47.08 (1.50)a	3.57 (0.048)a	0.76 (0.029)b
1×	0.54 (0.010)b	0.042 (0.00205)b	43.08 (1.20)b	3.53 (0.029)a	0.80 (0.0069)a
2×	6.47 (0.38)a	0.26 (0.023)a	43.32 (0.88)b	3.55 (0.038)a	0.81 (0.014)a
4×	7.22 (0.16)a	0.40 (0.038)a	36.92 (0.88)c	3.31 (0.11)b	0.78 (0.033)b

[a] 1× concentration infusion is made by fermenting 33.6 g of senescent leaves in 4 L of well water

[b] Values are means (±SD) for three trials with each trial averaged over four fermentation times within each leaf mass. Means within each variable followed by the same letter are not significantly different at $P \leq 0.01$ by t tests for pairwise comparisons of least-squares means (LSM) under the hypothesis H_o: $LSM_{(i)} = LSM_{(j)}$

based on DAPI staining, were markedly different. Bacterial cell densities in all infusion concentrations appeared to be relatively constant over time (Fig. 1). Nevertheless, at day 7, cell densities were significantly different ($P < 0.0001$) across all four leaf concentrations of WOL, while at days 14 and 21, they separated in two significantly different groups ($P < 0.0001$), with the lowest two leaf concentrations in one group and the highest two leaf concentrations in the other. Effects of fermentation time were analyzed through a set of contrasts which resulted in a significant linear and quadratic effect ($P < 0.001$) for each of the leaf concentrations and nonsignificant differences between the slopes for the lowest two leaf concentrations ($P = 0.9498$), significant differences between the slopes of the highest two leaf concentrations ($P < 0.0001$), but nonsignificant differences between the average linear slopes of the two lowest concentrations versus the two highest leaf concentrations ($P = 0.9498$).

DGGE Analysis of Bacterial Community Structure in Infusions

The melting point profiles revealed by DGGE for DNA OTUs amplified from BL and WOL infusions indicated that temporal changes in the OTU structure of the bacterial community occurred over the 28-day leaf fermentation period (Figs. 2 and 3). Appearance and disappearance of amplicons in DGGE gels and changes in band intensity showed that shifts in species structure of bacterial communities occurred over time. OTU diversity patterns for each infusion at each time point were reproducible in replicate analyses and OTU diversity profiles were highly similar (but not identical) in the three trials for each concentration (data not shown). In 1× BL infusions, the highest mean (± SD) number of OTUs was in the 7-day-old infusion (49.33 ± 5.77) and the lowest was

Table 3 Results of mixed-model ANOVA for effects of trial, infusion concentration (*conc*), and fermentation time (*day*) on bacterial cell counts and diversity in white oak leaf infusions

| Variable	Fixed factor	df	F value	P value	Random factor	Variance component estimate (SE)	P value
DAPI	Trial	(2, 30)	0.31	0.7330	Trial × conc	0	
	Conc	(3, 30)	6336.64	<0.0001	Trial × day (conc)	0.000225 (0.000223)	0.1572
	Day	(3, 30)	323.32	<0.0001	Residual	0.006241 (0.000425)	<0.0001
	Conc × day	(9, 30)	14.45	<0.0001			
CFU	Trial	(2, 6)	1.21	0.3613	Trial × conc	0.01092 (0.01159)	0.1732
	Conc	(3, 6)	53.20	0.0001	Trial × day (conc)	0.03097 (0.01004)	0.0010
	Day	(3, 24)	24.14	<0.0001	Residual	0.01510 (0.001779)	<0.0001
	Conc × day	(9, 24)	9.98	<0.0001			
No. OTUs	Conc	(3, 6)	6.21	0.0286	Trial	52.6545 (54.8148)	0.1684
	Day	(3, 24)	0.56	0.6482	Trial × conc	6.6788 (4.9870)	0.0902
	Conc × day	(9, 24)	0.50	0.8605	Residual	7.6250 (2.2011)	0.0003
H'	Conc	(3, 6)	8.06	0.0159	Trial	0.0175 (0.0190)	0.1779
	Day	(3, 24)	0.71	0.5580	Trial × conc	0.00326 (0.00339)	0.1683
	Conc × day	(9, 24)	1.39	0.2483	Residual	0.00994 (0.00287)	0.0003
E	Conc	(3, 6)	1.60	0.2860	Trial	0.0004 (0.0006)	0.2629
	Day	(3, 24)	0.15	0.9313	Trial × conc	0.0005 (0.0005)	0.1458
	Conc × day	(9, 24)	1.68	0.1501	Residual	0.0013 (0.0004)	0.0003
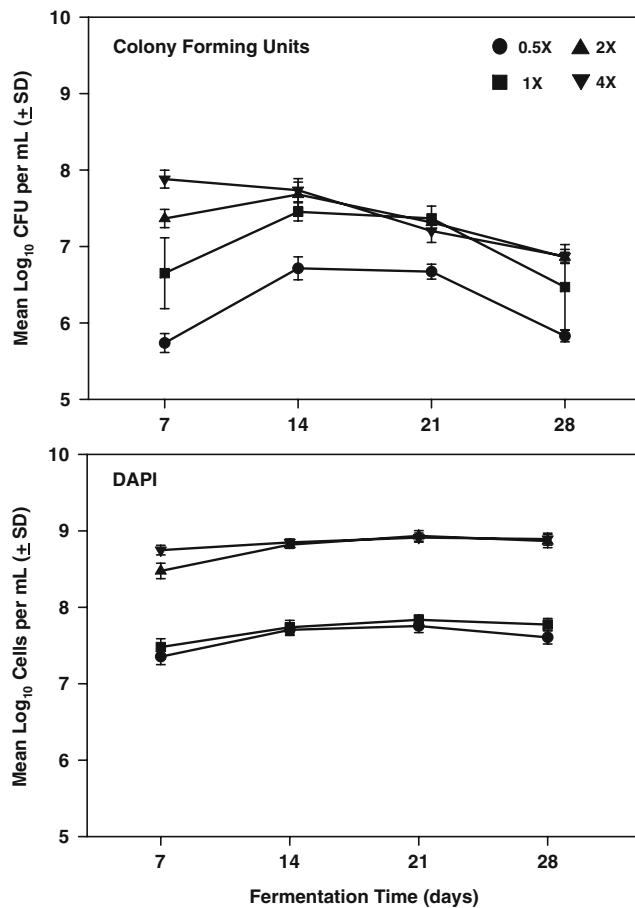
Figure 1 Abundance of bacteria in white oak leaf infusions during fermentation. Total bacterial numbers were determined by DAPI staining and CFUs were obtained by spread plating onto R2A agar. 1× concentration infusion is made by fermenting 33.6 g of senescent leaves in 4 L of well water

obtained after 28 days (42.33±7.51) of leaf fermentation (Table 1). Based on changes in OTU intensity, some bacterial species declined or increased in abundance between days 7 and 28 (Figs. 2 and 3). For example, the intense bands for OTUs BB2 and BB12 on day 7 decreased in intensity over time (Fig. 2). In contrast, OTUs BB24, BB29, and BB35 gradually increased in intensity, indicating that these bacterial species increased in abundance in the infusion.

In WOL infusions, the highest mean number of OTUs occurred in the 0.5× infusion concentration (47.08±1.50) and the smallest mean number was observed in 4× infusions (36.92±0.88; Table 2). Some OTUs (WOB1, WOB6, WOB12, and WOB16) were common in infusions prepared with different leaf masses (Fig. 3). In 0.5× infusions, OTUs WOB1, WOB9, WOB11, and WOB16 were prominent on day 7 but decreased in intensity at the 28-day time point in the fermentation period. Other OTUs, such as WOB17, gradually increased and were the dominant OTU detected at 28 days.
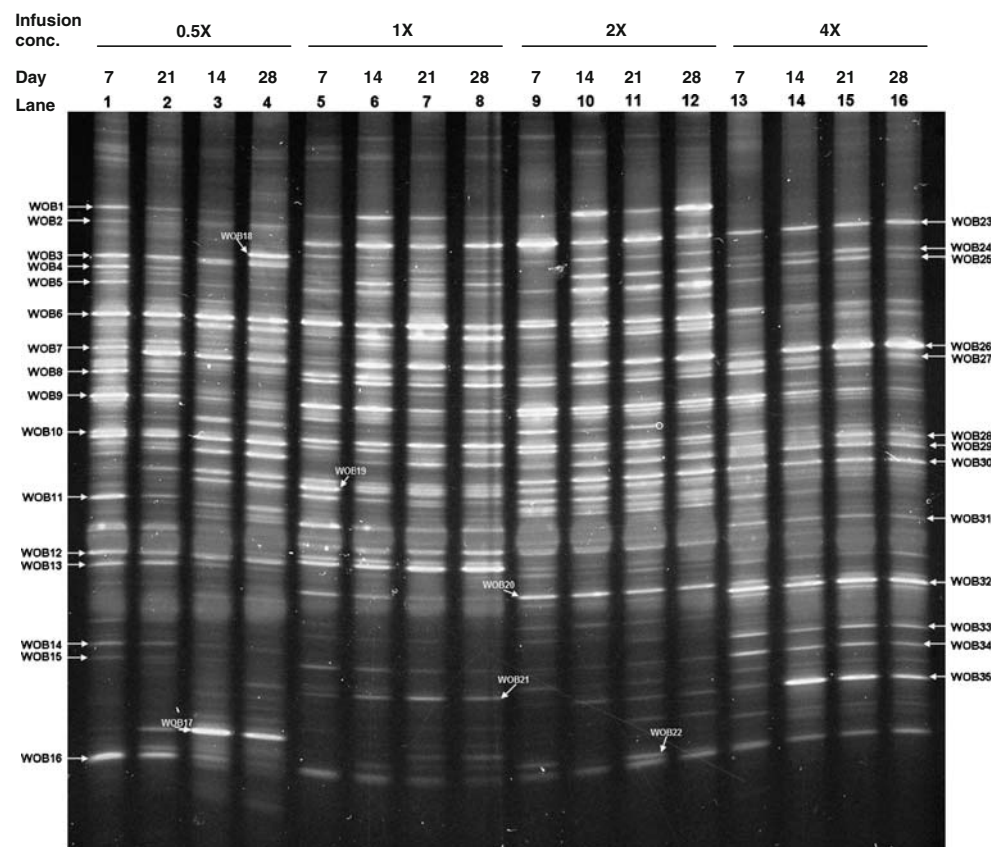
Bacterial Diversity

Changes in bacterial species diversity were reflected in the DGGE OTU profiles for each infusion over time. For bamboo leaf infusions, the *day* variable was significant ($P \leq 0.0288$; Supplemental Table 3), indicating that diversity of bacterial species changed over the fermentation period. Mean diversity of OTUs declined significantly for all three metrics (number of OTUs, H', E) over time (Table 1). In comparison, in WOL infusions, *day* and *day* × *conc* were
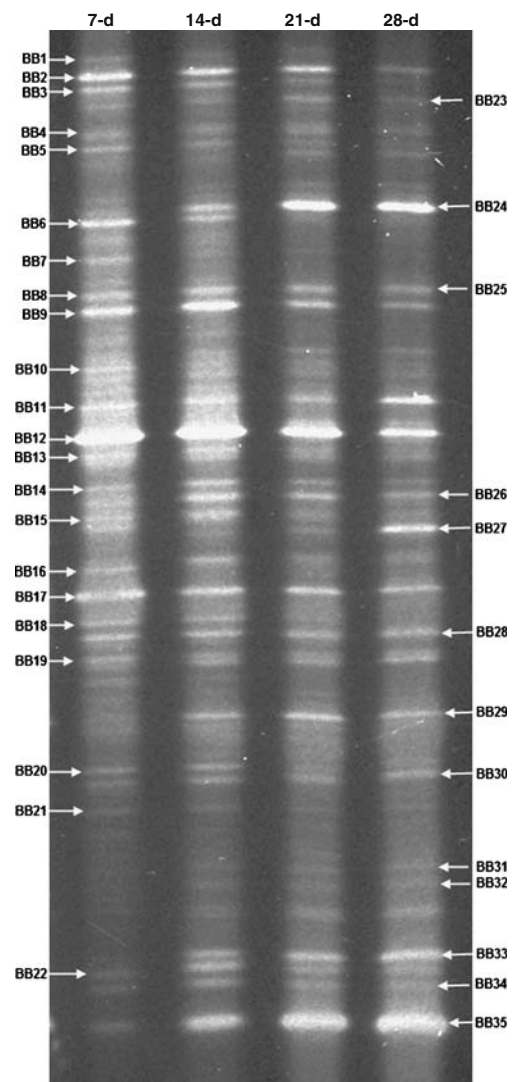
Figure 2 DGGE analysis, in a 40–65% denaturing gradient, of PCR products amplified from 1× bamboo leaf infusion using universal primers for eubacterial 16S rRNA gene (518R and 341F; see "Materials and Methods"). Each *lane* represents a sample taken from 7-, 14-, 21-, or 28-day-old infusions of a single trial. The *numbered arrows* (*BB* = bamboo bacteria) indicate bands that were excised for sequencing. 1× concentration infusion is made by fermenting 33.6 g of senescent leaves in 4 L of well water

Figure 3 DGGE analysis, in a 40–65% denaturing gradient, of PCR products amplified from white oak leaf infusion using universal primers for eubacterial 16S rRNA gene (518R and 341F; see "Materials and Methods"). Each *lane* represents a sample of infusion taken from a different leaf concentration and fermentation time from a single trial. The *numbered arrows* (*WOB* = white oak bacteria) indicate bands that were excised for sequencing. 1× concentration infusion is made by fermenting 33.6 g of senescent leaves in 4 L of well water

not significant ($P > 0.05$, Table 3), and there was no specific pattern of change in diversity over time for the four different infusion concentrations (Supplemental Table 4); however, there was a significant ($P < 0.05$) difference in bacterial diversity for the number of OTUs and H' at the low and high infusion concentrations (Table 2).

Principal Components Analysis

The number of OTUs and H' were highly correlated and both diversity indices were negatively correlated with CFU and DAPI (Supplemental Table 5). Principal components analysis (Fig. 4) resulted in the distribution of points in a plane formed by two principal axes, showing higher infusion concentration on the left side of PC 1 and lower concentration on the right side of PC 1. The first two principal components (PC 1 and PC 2) accounted for 53.1% and 29.6% of the variance, respectively, or 82.7% of the total variance. PC 1 was a measure of diversity with high positive loading scores for the number of OTUs and H' and negative loading scores for bacterial abundance estimated by CFU and DAPI staining. PC 2 primarily reflected high OTU evenness in the structure of bacterial communities in infusions. PC 2 also contained moderately high positive loading scores for CFU and DAPI.
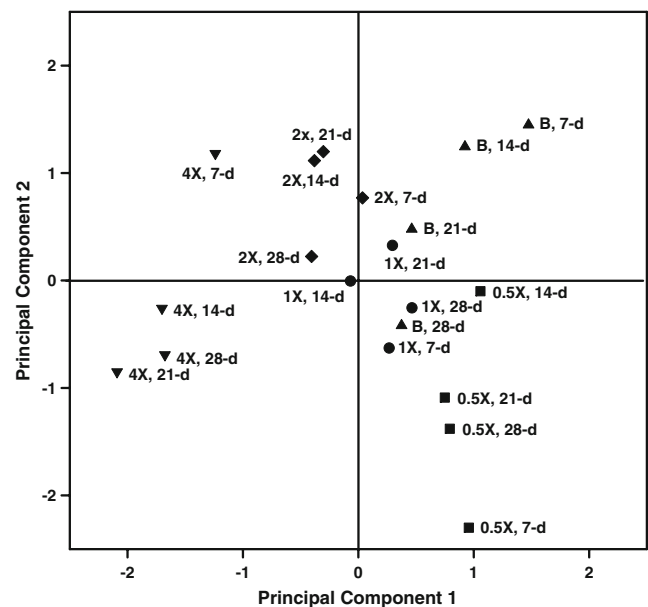
Figure 4 Principal components analysis of CFU- and DAPI-stained bacteria, and the number of bacterial phylotypes H' and E data for white oak and bamboo leaf infusion samples. *Symbols* correspond to specific 1× bamboo (B) and white oak leaf (unlabeled) infusions that vary by infusion concentration (0.5×, 1×, 2×, and 4×) and age (7, 14, 21, and 28 days). 1× concentration infusion is made by fermenting 33.6 g of senescent leaves in 4 L of well water

Identification of OTUs

To identify the species of bacteria in the infusions, DGGE gel bands were excised and the DNA was extracted and sequenced from BL and WOL infusions from the first trial (see Figs. 2 and 3). We attempted to elute DNA from 35 excised gel bands each from BL and WOL infusions for reamplification by PCR. Amplicons were not detected for two (5.7%) of 35 BL infusion bands, but the V3 DNA fragments were successfully reamplified and sequenced for the remaining 33 OTUs. However, four (12.1%) of the 33 BL infusion sequences and three (8.6%) of the 35 WOL infusion sequences had too many ambiguous nucleotide positions, which prevented these bacteria from being identified. For the sequenced bands, 23 BL and 31 WOL infusion OTUs were more than 98% identical to sequences in the GenBank database and seven (30.4%) and 13 (41.9%) of these sequences, respectively, were closest matches to undescribed species. For BL infusions, six (20.7%) of the 29 sequences exhibited 91–97% identity with sequences in the GenBank database (Supplemental Table 1), while for WOL infusions one (3.1%) of the 32 sequences exhibited 96% identity with sequences in the GenBank database (Supplemental Table 2).

Phylogenetic Affiliation of Sequenced Bacteria

A phylogenetic tree was constructed by the neighbor-joining method to characterize the relationship between the 61 OTUs from BL and WOL infusions that were sequenced (Fig. 5). Based on 16S rDNA amplicon sequences, the OTUs were classified as Proteobacteria (65.5%), Firmicutes (9.8%), Bacteroidetes (9.8%), and a group (14.7%) that could not be classified. The Proteobacteria were the dominant heterotropic bacteria of those detected and sequenced in infusions, comprising 19 different phylotypes with representatives from four sub-classes (Alpha-, Beta-, Delta-, and Gammaproteobacteria). Nine sequences from BL infusions had relatively low similarity (<97%) to sequences previously deposited in GenBank databases and could not be associated with any known bacterial division. Notably, OTUs from WOL and BL infusions were generally segregated, indicating that the species structure of the bacterial communities were different (Fig. 5). In addition to differences in the clustering patterns of OTUs in the phylogenetic tree, the DGGE sequence library for OTUs from BL infusions was significantly different ($P=0.030$) from the library derived for WOL infusion. Accordingly, we rejected the hypothesis that the two 16S libraries were sampled from the same community.

Responses of Gravid Mosquitoes to Native and Sterilized WOL Infusions

Native WOL infusion was culture positive for a diverse array of bacterial species. Failed attempts to culture bacteria from sterilized WOL infusion verified that it was bacteria free. Native WOL infusion was significantly more attractive to both *A. aegypti* ($t=6.34$; $df=17$; $P<0.0001$) and *A. albopictus* ($t=6.39$; $df=17$; $P<0.0001$) than was infusion made using leaves and water sterilized by autoclaving. A mean (± SD) of 71.9% (±10.8) of gravid *A. aegypti* and 77.8% (±14.04%) of gravid *A. albopictus* were trapped during 24-h assays in test cups containing native infusion, reflecting the high level of attraction of gravid mosquitoes to WOL infusion that contained microbes.

Effects of Bacterial Diversity on the Response of Gravid Mosquitoes to Plant Infusions

Regression ANOVA revealed that attraction of gravid *A. aegypti* was significantly associated with PC 1 ($F=90.53$; $df=1$, 17; $P<0.0001$) but not PC 2 ($F=0.68$; $df=1$, 17; $P=0.4222$) and that 83.7% of the variation in response of gravid females could be explained by PC 1. Similarly, the response of gravid *A. albopictus* to plant infusions was significantly related to PC 1 ($F=35.59$; $df=1$, 17; $P<0.0001$) but not to PC 2 ($F=0.17$; $df=1$, 17; $P=0.6895$); PC 1 explained 67.5% of the total variation in the response of gravid females to the plant infusions. As stated above, PC 1 reflected bacterial community diversity with high positive loading scores for the number of OTUs and H'.

Responses of Gravid Mosquitoes to Bamboo Leaf Infusions

Bioassays of *A. aegypti* and *A. albopictus* females with 1× BL infusions (33.6 g of senescent leaves in 4 L water) that were fermented for various periods showed that both mosquito species exhibited the strongest attraction to infusions that had been fermented for 7 days (Fig. 6). Attraction of gravid *A. aegypti* was highly significant to 7-day-old ($t=6.15$; $df=23$; $P<0.0001$) and 14-day-old ($t=3.59$; $df=20$; $P=0.0018$) infusions, but there were no significant differences between either 21-day-old ($t=-1.72$; $df=21$; $P=0.1003$) or 28-day-old ($t=1.90$; $df=20$; $P=0.0716$) infusions and well water (Fig. 6a). *A. albopictus* females were significantly attracted to 1× concentration infusion at each 7-day interval in the 28-day time course of fermentation ($t=8.36$; $df=23$; $P<0.0001$; Fig. 6b). In general, a higher percentage of *A. albopictus* than *A. aegypti* females was trapped on sticky screens above BL infusions.

Figure 5 Phylogenetic tree
constructed by the neighbor-
joining method using the
bacterial V3 region 16S rRNA
gene sequences of DGGE bands
of white oak and bamboo leaf
infusion samples. Bootstrap
values for a total of 1,000
iterations are shown at the nodes
of the tree and only bootstrap
values higher than 50% are
shown. The bar indicates a
Jukes–Cantor
distance of 0.05
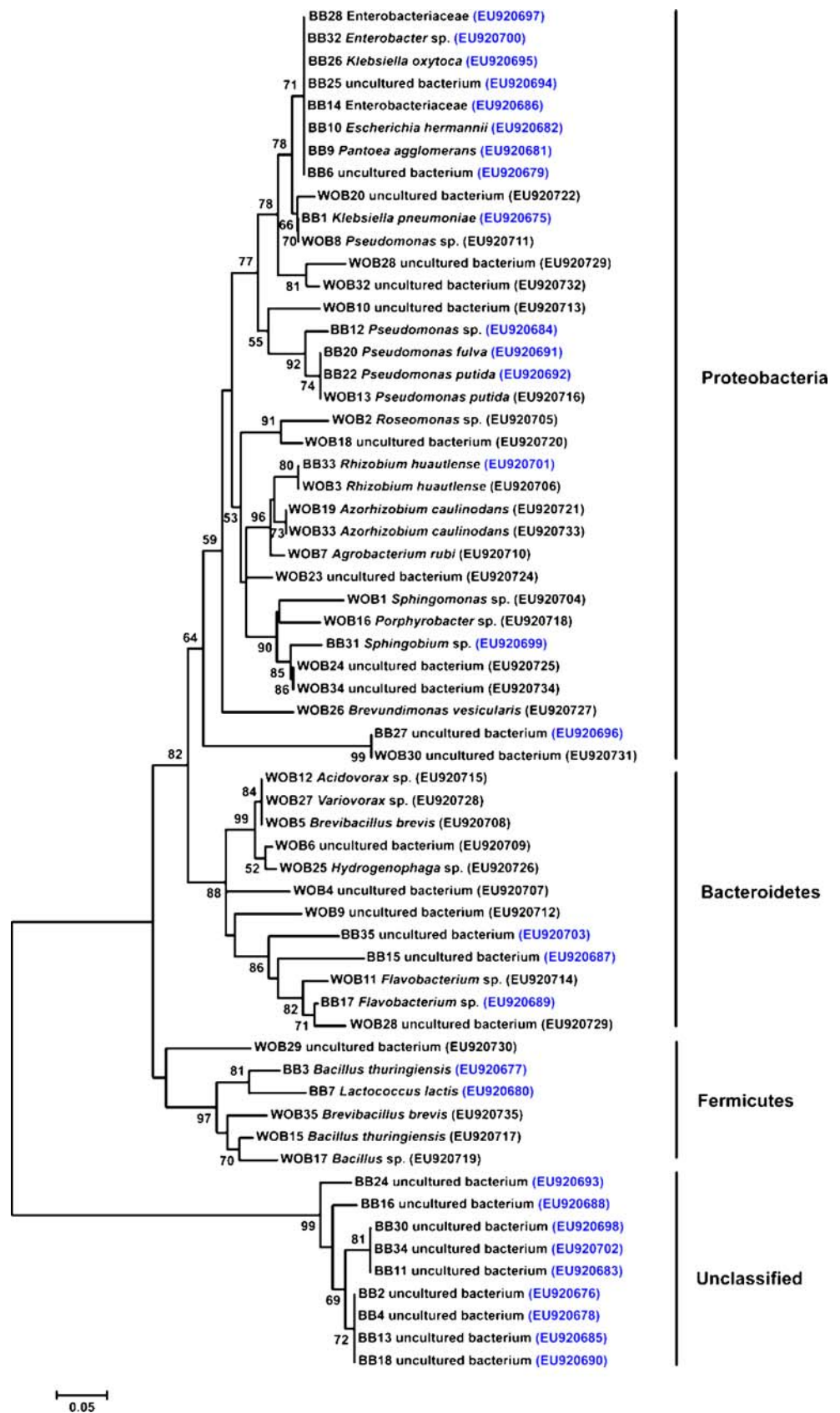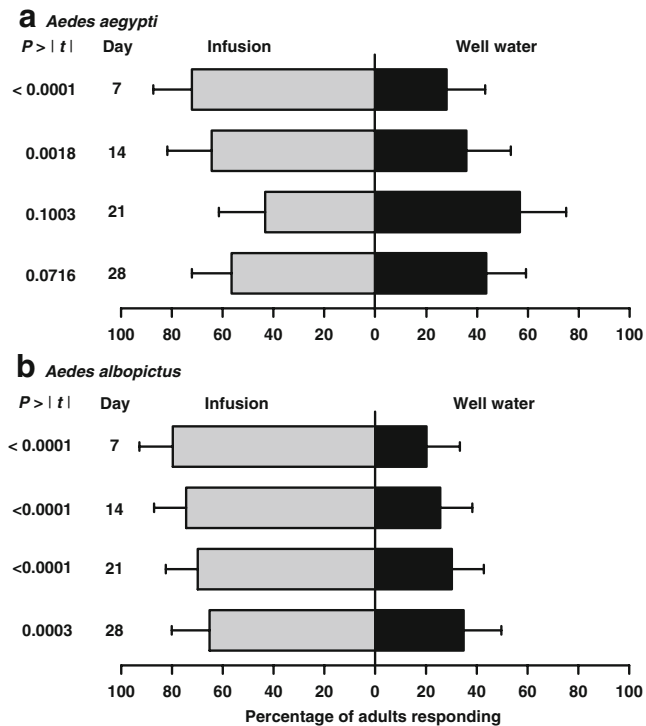
Figure 6 Effects of fermentation time on attraction of gravid *Aedes* mosquitoes to bamboo leaf infusion (1× infusion concentration). The percentages of mosquitoes trapped during a 24-h assay on sticky screens over the infusion and control (well water) cups were subjected to an arcsine $\sqrt{x}$ transformation before they were compared using a paired *t* test. 1× concentration infusion is made by fermenting 33.6 g of senescent leaves in 4 L of well water

Responses of Gravid Mosquitoes to WOL Infusions

We examined the attraction of both mosquito species to four concentrations of WOL infusions, each fermented over a 28-day period and sampled at 7-day intervals. Across the entire 28-day fermentation period, both species were significantly attracted to infusions of low leaf biomass (0.5× infusion) at each of the weekly sampling intervals (Fig. 7). In general, relatively fewer gravid *A. aegypti* were attracted to the more concentrated WOL infusions. For 1× infusions, *A. aegypti* females were significantly attracted only to 21-day-old infusions, infusions made using twice the leaf mass failed to attract females, and infusions made using 4× the leaf mass became significantly repellent after 14, 21, and 28 days of fermentation (Fig. 7a). In contrast, *A. albopictus* was, in general, attracted to oak leaf infusions over a broader range of concentrations and leaf fermentation times. Nevertheless, similar to *A. aegypti*, attraction of gravid *A. albopictus* to WOL infusions declined—although at a lower rate—as more leaves were added to the infusion. The 4× infusion was neither attractive nor repellent to *A. albopictus* at any of the four time points in the 28-day fermentation period (Fig. 7b).

Response to Odorants from a Mixture of Bacterial Isolates

Gravid *A. aegypti* exhibited a differential response to a mix of bacterial species cultured from BL infusion, depending on the bacterial cell density to which females were exposed to (Fig. 8a). Significant attraction was found to 10^8 cells per milliliter ($t=4.84$; $df=17$; $P=0.0002$) and 10^7 cells per milliliter ($t=3.66$; $df=17$; $P=0.0019$). Notably, females were significantly repelled by 10^9 cells per milliliter ($t=-2.45$; $df=17$; $P=0.0253$). Similarly, significantly more gravid *A. albopictus* were trapped in bioassay cups containing 10^8 cells per milliliter ($t=5.35$; $df=17$; $P<0.0001$) and 10^7 cells per milliliter ($t=3.39$; $df=17$; $P=0.0012$) than in cups containing control media (Fig 8b). Responses to other bacterial cell densities were not significantly higher ($P>0.05$) than to the control media.

Discussion

Senescent plant leaves commonly accumulate in water-holding containers in residential landscapes. Tree leaves are known to contain bacterial populations composed of diverse species [55], and these bacteria and other microbes catabolize and mineralize organic carbon in leaves and other detritus [47], producing metabolites that attract and stimulate gravid mosquitoes to lay eggs [31]. Although the activity of organic infusions [2, 36, 42, 51] and bacteria [5, 12, 13, 36, 48, 52] in mediating oviposition responses of gravid mosquitoes has been previously investigated, our study is the first to characterize how changes in bacterial diversity, in response to fermentation time and leaf concentration, affect attraction of gravid *Aedes* (*Stegomyia*) mosquitoes to plant infusions. Results of our present study provide compelling evidence of the keystone role of microbes in guiding gravid mosquitoes to their container habitats. When presented with native and sterilized infusions, gravid *A. aegypti* and *A. albopictus* were significantly more attracted to infusions containing microbes. Similarly, the mosquito *Culex tarsalis* exhibited a greatly diminished egg-laying response to Bermuda grass infusion that had been filtered to remove microorganisms [17]. *Aedes* (*Stegomyia*) mosquitoes laid more eggs in infusions of *Panicum maximum* leaves than in tap water but equivalent numbers of eggs in containers holding 7-day-old sterilized *P. maximum* infusion versus tap water [42]. Bacterially derived semiochemicals have been postulated to provide gravid females with cues to the quality of food resources available in egg-laying sites [31, 42]. The dual roles of bacteria in semiochemical production and as a principal food resource for mosquito larvae [24] support this biological construct.

Observed significant increases in the abundance of bacteria in response to increases in WOL concentration,

Figure 7 Effects of fermentation time and infusion concentration on attraction of gravid *Aedes* mosquitoes to white oak leaf infusion. The percentages of mosquitoes trapped during a 24-h assay on sticky screens over the infusion and control (well water) cups were subjected to an arcsine $\sqrt{x}$ transformation before they were compared using a paired *t* test. Values are means±SD. 1× concentration infusion is made by fermenting 33.6 g of senescent leaves in 4 L of well water
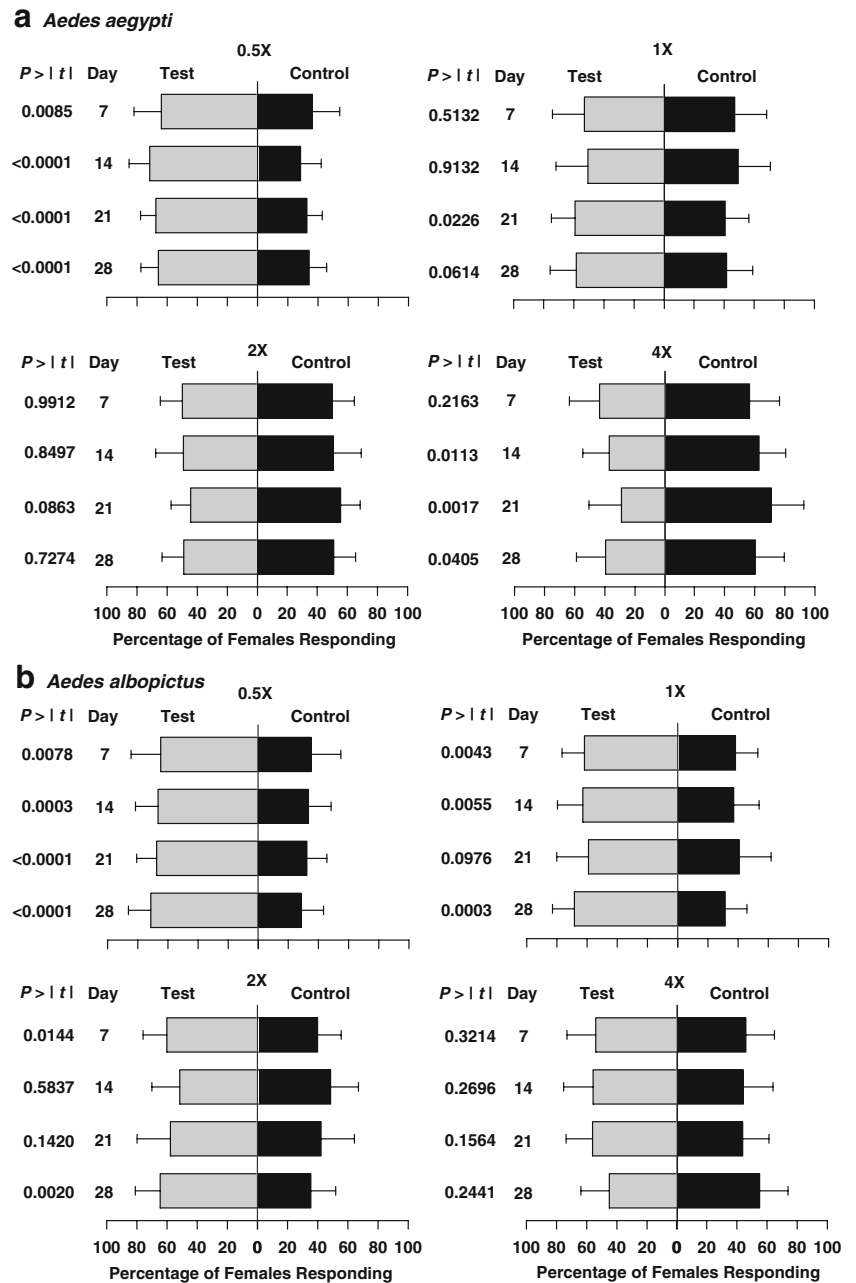
determined by two enumeration techniques (colony counts and DAPI), suggested that infusions made with the lowest leaf mass were nutrient-limited. Nutrient composition and availability have been shown to affect the structural organization of bacterial communities in leaf biofilms [54]. We can only speculate why total cell counts in WOL infusions were not more responsive to increases in leaf biomass. Oak leaves contain tannins, which have been reported to be toxic to microorganisms [26, 43]. Consequently, in our experiments, it was likely that growth of bacteria was inhibited by high levels of tannins in 2× and 4× WOL infusions. Additionally, the more concentrated leaf infusions were darkly colored, which may have had a masking effect, preventing some

stained bacteria from being visualized. Similar problems in visualizing stained bacteria in some sediment types has been previously encountered [44].

Phylogenetic analysis revealed that white oak and bamboo leaves were inhabited by bacteria representing the phyla Proteobacteria, Firmicutes, and Bacteroidetes (formerly known as the Cytophaga–Bacteroides–Flavobacteria group). Major bacterial taxa found in our study are typical of freshwater habitats that are often dominated by Proteobacteria [3, 20, 21]. Members of the Bacteroidetes group are found in nearly every habitat of the biosphere and are known to play an important role in the degradation of complex biopolymers in marine and freshwater environ-
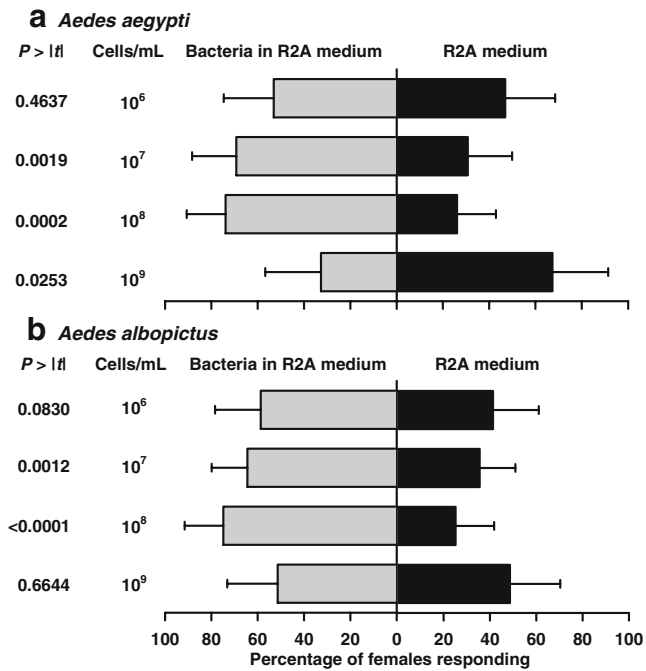
Figure 8 The percentages of gravid *Aedes* mosquitoes trapped during a 24-h assay on sticky screens over the 14 mix bacterial isolates and control (R2A medium) cups were subjected to an arcsine $\sqrt{x}$ transformation before they were compared using a paired *t* test

ments [21]. In our study, 9.8% of the phylotypes sequenced from BL and WOL infusions were classified in this phylum. Overall, 14.7% of the sequences were classified to the level of Eubacteria. These sequences clustered in a separate phylum level lineage, indicating that there are unique bacterial species present in BL infusions. Notably, the library of 16S rDNA sequences from DGGE amplicons from BL infusion was significantly different from the library generated for WOL infusion.

Although 16S rDNA sequence libraries for both plant infusions were significantly different, there is some overlap in the bacterial species cultured from these plant infusions [31]. Notably, *Bacillus thuringiensis*, *Enterobacter asburiae*, *Klebsiella granulomatis*, *Brevundimonas vesicularis*, *Rhizobium huautlense*, and some *Pseudomonas* spp. have been cultured from both infusions [31]. In an earlier investigation, *Aerobacter aerogenes* was the principal bacterial species cultured from the hay infusion that elicited oviposition responses from *Culex quinquefasciatus* and *A. aegypti* [13]. In subsequent research, Millar and coworkers [25] isolated and identified the chemical structures of five bacterial metabolites from fermented Bermuda hay that attracted and stimulated gravid *C. quinquefasciatus* to lay eggs. The principal compound, 3-methylindole, was active in concentrations that spanned five orders of magnitude. A metabolite (7,11-dimethyloctadecane) of *Pseudomonas aeruginosa* grown on capric acid substrates was attractive

to gravid *A. aegypti* [15]. Similarly, in another study, *A. aegypti* exhibited significant oviposition responses to *P. aeruginosa* [12]. *Acinetobacter calcoaceticus*, isolated from water holding late-instar *A. aegypti*, elicited significant oviposition responses from gravid female mosquitoes [5]. Behavioral bioassays showed that both olfaction and contact stimulation were involved. *Sphingobacterium multivorum* from soil-contaminated towels, *Psychrobacter immobilis* from larval rearing water, and an undetermined *Bacillus* species from WOL infusion elicited oviposition responses from gravid *A. albopictus* that were significantly higher than to water without the bacteria [52]. However, only volatiles collected from larval rearing water elicited significant electroantennogram responses in females, suggesting that a mixture of bacteria was responsible for production of attractive odorants. Alternatively, uncultured bacterial species in the infusions could also have been responsible for production of bioactive odorants. In our present investigation, population levels of bacteria estimated by culture-independent DAPI staining were 30–50-fold higher than estimates derived from culturing-based CFUs. Sequencing of phylotypes revealed that the majority of bacteria in BL and WOL infusions were uncultured. Although the bacterial communities in these infusions varied considerably in species composition, infusions made from both plant species produced odorants that were attractive to gravid mosquitoes. Therefore, the shared bacterial species in the two infusion types play key roles in attracting mosquitoes, or different bacterial species or assemblages of bacteria in the two infusions might be functionally equivalent in mediating mosquito behavior. Recently, we have evaluated single bacterial species isolates separately with gravid *A. aegypti*. Bacterial isolates varied in activity, with gravid mosquitoes exhibiting significant attraction to some isolates while other isolates were significantly repellent, suggesting that the net bioactivity of an infusion represents the interplay of its constituent microbes (Ponnusamy et al., unpublished data).

Our ongoing investigation with experimental infusions extend our previous studies of species diversity of microbial communities in water-filled human-made and natural containers in suburban landscapes of the city of New Orleans, LA, USA [32]. Highly diverse DGGE OTU patterns were found that could not be related to the kinds of containers or their local spatial distribution. The high diversity of bacterial species in field containers was understandable because these containers were of different sizes and shapes and held varying amounts of water and detritus from a variety of plant species. These variables are known to affect the species composition of bacterial communities [4, 41, 55]. Our results indicate that detrital biomass would also be an important determinant of bacterial diversity in containers used as oviposition sites by mosquitoes.

Plant infusions contain a mix of bacterial species, some of which release odorants that attract gravid *Aedes* (*Stegomyia*) females from a distance, while others stimulate gravid females to oviposit [31]; yet other bacterial species produce potent oviposition repellents (Ponnusamy et al., unpublished data). Results of our present investigation provide insight into the dynamics and molecular ecology of bacterial communities in plant infusions. The diversity of bacterial species and abundance of bacteria in infusions and correspondingly the blend of oviposition attractants, stimulants, and repellents changes with plant species and the amount of leaf and other organic biomass and fermentation time. Our findings are highly relevant to the operational use of plant infusions by public health agencies in traps used in surveillance and monitoring programs for these mosquito species. Deployment of traps baited with plant infusions containing high leaf biomass or infusions that have been aged excessively would not be optimally effective in attracting gravid females and, indeed, may repel females. Responses of mosquitoes to plant infusions that would be used in mosquito monitoring programs have been reported to vary significantly from batch to batch and with infusion age [16, 42]. Observed differences in mosquito response were thought to result from changes in bacterial activity in plant infusions. Our present investigation provides an experimental basis for these observations.

Our present research involves the isolation, culture, and bioassay of single as well as a mix of bacterial species from plant infusions that are highly attractive to *Aedes* (*Stegomyia*) mosquitoes. A sustained release formulation of these bacteria may provide a practical tool for enhancing the efficacy of oviposition traps used for monitoring and surveillance of *A. aegypti* and *A. albopictus*.

References

1. Allan SA, Bernier UR, Kline DL (2005) Evaluation of oviposition substrates and organic infusions on collection of *Culex* in Florida. J Am Mosq Control Assoc 21:268–273
2. Allan SA, Kline DL (1995) Evaluation of organic infusions and synthetic compounds mediating oviposition in *Aedes albopictus* and *Aedes aegypti* (Diptera: Culicidae). J Chem Ecol 21:1847–1860
3. Amann R, Ludwig W (2000) Ribosomal RNA-targeted nucleic acid probes for studies in microbial ecology. FEMS Microbiol Rev 24:555–565
4. Bell T, Ager D, Song J-I, Newman JA, Thompson IP, Lilley AK, van der Gast CJ (2007) Larger islands house more bacterial taxa. Science 308:1884

5. Benzon GL, Apperson CS (1988) Reexamination of chemically mediated oviposition behavior in *Aedes aegypti* (L.) (Diptera: Culicidae). J Med Entomol 25:158–164
6. Chadee DD, Mendis C, Beier JC (1993) Diel oviposition periodicity of Anopheline mosquitoes (Diptera: Culicidae) from the Americas: *Anopheles albimanus* Wiedemann and *Anopheles freeborni* Aitken. Ann Trop Med Parasitol 87:501–507
7. Colton YM, Chadee DD, Severson DW (2003) Natural skip oviposition of the mosquito *Aedes aegypti* indicated by codominant genetic markers. Med Vet Entomol 17:195–204
8. Draper NR, Smith H (1981) Applied regression analysis. Wiley, New York
9. Duineveld BM, Kowalchuk GA, Keijzer A, van Elsas JD, van Veen JA (2001) Analysis of bacterial communities in the rhizosphere of chrysanthemum via denaturing gradient gel electrophoresis of PCR-amplified 16S rRNA as well as DNA fragments coding for 16S rRNA. Appl Environ Microbiol 67:172–178
10. Ellis RJ, Morgan P, Weightman AJ, Fry JC (2003) Cultivation-dependent and -independent approaches for determining bacterial diversity in heavy-metal-contaminated soil. Appl Environ Microbiol 69:3223–3230
11. Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 41:95–98
12. Hasselschwert D, Rockett CL (1988) Bacteria as oviposational attractants for *Aedes aegypti* (Diptera: Culicidae). Great Lakes Entomol 21:163–168
13. Hazard EI, Mayer MS, Savage KE (1967) Attraction and oviposition stimulation of gravid female mosquitoes by bacteria isolated from hay infusions. Mosq News 27:133–136
14. Hoefel D, Monis PT, Grooby WL, Andrews S, Saint CP (2005) Culture-independent techniques for rapid detection of bacteria associated with loss of chloramine residual in a drinking water system. Appl Environ Microbiol 71:6479–6488
15. Ikeshoji T, Ichimoto I, Konish J, Naoshima Y, Ueda H (1979) 7, 11-Dimethyl octadecane—an oviposition attractant for *Aedes aegypti* produced by *Pseudomonas aeruginosa* on capric acid substrate. J Pestic Sci 4(187):194
16. Isoe J, Beehler JW, Millar JG, Mulla MS (1995) Oviposition responses of *Culex tarsalis* and *Culex quinquefasciatus* to aged Bermuda grass infusions. J Am Mosq Control Assoc 11:39–44
17. Isoe J, Millar JG (1995) Characterization of factors mediating oviposition site choice by *Culex tarsalis*. J Am Mosq Control Assoc 11:21–28
18. Jukes TH, Cantor CR (1969) Evolution of protein molecules. In: Munro HN (ed) Mammalian protein metabolism. Academic, New York, pp 21–132
19. Kadlec RH, Knight RL (1996) Treatment wetlands. CRC/Lewis, Boca Raton 893 pp
20. Kaufman MG, Walker ED, Smith TW, Merritt RW, Klug MJ (1999) Effects of larval mosquitoes (*Aedes triseriatus*) and stemflow on microbial community dynamics in container habitats. Appl Environ Microbiol 65:2661–2673
21. Kirchman DL (2002) The ecology of *Cytophaga–Flavobacteria* in aquatic environments. FEMS Microbiol Ecol 39:91–100
22. Maw MG (1970) Capric acid as a larvicide and an oviposition stimulant for mosquitoes. Nature 227:1154–1155
23. Mboera LEG, Knols BGJ, Braks MAH, Takken W (2000) Comparison of carbon dioxide-baited trapping systems for sampling outdoor mosquito populations in Tanzania. Med Vet Entomol 14:257–263
24. Merritt RW, Dadd RH, Walker ED (1992) Feeding behavior, natural food, and nutritional relationships of larval mosquitoes. Annu Rev Entomol 37:349–376
25. Millar JG, Chaney JD, Mulla MS (1992) Identification of oviposition attractants for *Culex quinquefasciatus* from fermented Bermuda grass infusions. J Am Mosq Control Assoc 8:11–17

26. Min BR, Pinchak WE, Merkel R, Walker S, Tomita G, Anderson RC (2008) Comparative antimicrobial activity of tannin extracts from perennial plants on mastitis pathogens. Sci Res Essays 3:66–73

27. Muyzer G, de Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59:695–700

28. Neter T, Kutner MH, Nachtsheim CJ, Wasserman W (1996) Applied linear statistical models, 4th edn. McGraw-Hill, Boston

29. Nguyen TTH, Su TY, Mulla MS (1999) Bacteria and mosquito abundance in microcosms enriched with organic matter and treated with a *Bacillus thuringiensis* subsp. *israelensis* formulation. J Vector Ecol 24:191–201

30. Polz MF, Cavanaugh CM (1998) Bias in template-to-product ratios in multitemplate PCR. Appl Environ Microbiol 64:3724–3730

31. Ponnusamy L, Xu N, Nojima S, Wesson DM, Schal C, Apperson CS (2008) Identification of bacteria and bacteria-associated chemical cues that mediate oviposition site preferences by *Aedes aegypti*. Proc Natl Acad Sci U S A 105:9262–9267

32. Ponnusamy L, Xu N, Stav G, Wesson DM, Schal C, Apperson CS (2008) Diversity of bacterial communities in container habitats of mosquitoes. Microb Ecol 56:593–603

33. Porter KG, Feig YS (1980) The use of DAPI for identifying and counting aquatic microflora. Limnol Oceanogr 25:943–948

34. Rawlins SC, Martinez R, Wiltshire S, Legall G (1998) A comparison of surveillance systems for the dengue vector *Aedes aegypti* in Port of Spain, Trinidad. J Am Mosq Control Assoc 14:131–136

35. Reasoner DJ, Geldreicht EE (1985) A new medium for the enumeration and subculture of bacteria from potable water. Appl Environ Microbiol 49:1–7

36. Reiter P, Amador MA, Colon N (1991) Enhancement of the CDC ovitrap with hay infusions for daily monitoring of *Aedes aegypti* populations. J Am Mosq Control Assoc 7:52–55

37. Rejmankova E, Higashi R, Grieco JJ, Achee NN, Roberts D (2005) Volatile substances from larval habitats mediate species-specific oviposition in *Anopheles* mosquitoes. J Med Entomol 42:95–103

38. Rejmankova E, Roberts DR, Manguin S, Pope KO, Komárek J, Post RA (1996) *Anopheles albimanus* (Diptera: Culicidae) and cyanobacteria: an example of larval habitat selection. Environ Entomol 25:1058–1067

39. Ritchie SA (1984) Hay infusion and isopropyl alcohol-baited CDC light trap—a simple effective trap for gravid *Culex* mosquitoes. Mosq News 44:404–407

40. Rivera ING, Lipp EK, Gil A, Choopun N, Huq A, Colwell RR (2003) Method of DNA extraction and application of multiplex polymerase chain reaction to detect toxigenic *Vibrio cholerae* O1 and O139 from aquatic ecosystems. Environ Microbiol 5:599–606

41. Sanford A, Morgan J, Evans D, Ducklow H (2001) Bacterioplankton dynamics in estuarine mesocosms: effects of tank shape and size. Microbiol Ecol 41:45–55

42. Sant'Ana AL, Rogue RA, Eiras AE (2006) Characteristics of grass infusions as oviposition attractants to *Aedes* (*Stegomyia*) (Diptera: Culicidae). J Med Entomol 43:214–220

43. Scalbert A (1991) Antimicrobial properties of tannins. Phytochemistry 30:3875–3883

44. Schallenberg M, Kalff J, Rasmussen JB (1989) Solutions to problems involving enumeration of sediment bacteria by direct counts. Appl Environ Microbiol 55:1214–1219

45. Shannon C, Weaver W (1963) The mathematical theory of communication. University of Illinois Press, Urbana

46. Singleton DR, Furlong MA, Rathbun SL, Whitman WB (2001) Quantitative comparisons of 16S rRNA gene sequence libraries from environmental samples. Appl Environ Microbiol 67:4374–4376

47. Sinsabaugh RL, Linkins AE (1990) Enzymatic and chemical analysis of particulate organic matter from a boreal river. Freshwater Biol 23:301–309

48. Sumba LA, Guda TO, Deng AL, Hassanali A, Beier JC, Knols BGJ (2004) Mediation of oviposition site selection in the African malaria mosquito *Anopheles gambiae* (Diptera: Culicidae) by semiochemicals of microbial origin. Int J Trop Insect Sci 24:260–265

49. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599

50. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 24:4876–4882

51. Trexler JD, Apperson CS, Schal C (1998) Laboratory and field evaluations of oviposition responses of *Aedes albopictus* and *Aedes triseriatus* (Diptera: Culicidae) to oak leaf infusions. J Med Entomol 35:967–976

52. Trexler JD, Apperson CS, Zurek L, Gemeno C, Schal C, Kaufman M, Walker E, Watson DW, Wallace L (2003) Role of bacteria in mediating oviposition responses of *Aedes albopictus* (Diptera: Culicidae). J Med Entomol 40:841–848

53. Vázquez-Martínez MG, Rodríguez MH, Arredondo-Jiménez JI, Méndez-Sánchez JD, Bond-Compeán JG, Gold-Morgan M (2002) Cyanobacteria associated with *Anopheles albimanus* (Diptera: Culicidae) larval habitats in Southern Mexico. J Med Entomol 39:825–832

54. Wolfaardt GM, Lawrence JR, Robarts RD, Caldwell SJ, Caldwell DE (1994) Multicellular organization in a degradative biofilm community. Appl Environ Microbiol 60:434–446

55. Yang CH, Crowley DE, Borneman J, Keen NT (2001) Microbial phyllosphere populations are more complex than previously realized. Proc Natl Acad Sci U S A 98:3889–3894