Bacteria associated with *Parthenium hysterophorus* root exudate influence olfactory oviposition responses of *Anopheles gambiae*

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Introduction: Previously, we documented that the malaria vector *Anopheles gambiae* responds to volatile emissions from the root exudate water of the invasive plant, *Parthenium hysterophorus*. However, the origin of the volatiles remains to be investigated. Here, we isolated bacteria from the root exudate water of the plant, test the influence of their volatiles in gravid *An. gambiae* oviposition, and examined relationships between volatile profiles and oviposition.

Methods: Bacteria from root exudate water of *P. hysterophorus* were isolated using culture on Luria Bertani medium and identified by sequencing the 16S rRNA gene. Cultures of individual isolates were evaluated for egg laying response by gravid *An. gambiae* and number of eggs laid compared using generalized linear models relative to those in crude bacteria-mixture. Headspace volatile emissions of the bacterial isolates were analyzed by gas chromatography coupled to mass spectrometry (GC-MS) and relationships between volatile organic compound (VOC) profiles and gravid mosquito oviposition examined using Random Forest Analysis. Proximate analysis was performed to assess the difference in volatile chemistry among the different isolates.

Results: Three isolates were identified as Gram-negative bacteria belonging to two families: Enterobacteriaceae (*Enterobacter* sp. and *Enterobacter mori*) and Alcaligenaceae (*Alcaligens aquatilis*). *An. gambiae* laid 3-fold more eggs in cultures of *A. aquatilis* than in those of *Enterobacter* sp. In turn, approx. 4-fold more eggs were laid in cultures of *E. mori* than *A. aquatilis*. Overall, 16 VOCs were identified in the headspace of the isolates belonging to the chemical classes benzenoids, pyrazines, aldehydes, terpenes, alcohols, alkanes, and indoles. Random Forest Analysis identified 10 compounds contributing the most to the attraction of odors of the bacteria isolates to oviposition. Specifically, dodecane and indole were emitted in higher amounts in odors of *Enterobacter sp* than the other two species. Proximate analysis revealed differential attraction of the isolates on the gravid mosquito to be associated with their volatile profiles.

Conclusion: Our results provide first report of *E. mori* or *A. aquatilis* mediating attractive oviposition responses in *An. gambiae* in support of the important role microbes play in insect oviposition. The potential use of the microbes and associated volatiles in malaria vector management needs further investigation.

Introduction

Malaria is a major public health threat in sub-Saharan Africa, where the disease kills thousands of people each year (1). The disease is caused by *Plasmodium* parasites transmitted among people by the bites of infective female *Anopheles* mosquitoes (1). To break the parasite transmission cycle and reduce the disease burden, control efforts have largely focused on interventions that target *Anopheles* vectors. Examples include long lasting insecticide nets (LLINs) and indoor residual spraying (IRS), both targeting mosquitoes that feed and rest indoors (2–4). However, impediments to effective control using these insecticide-based tools include the emergence and spread of insecticide resistance among major malaria vectors (5, 6). As such, it is essential to embrace alternative ways to control vector populations. For example, semiochemical-baited traps, especially those that mediate oviposition represents promising complementary tools for mosquito control (7–10).

To ensure reproductive success, gravid mosquitoes are known to identify and select oviposition sites that can support the growth and survival of their offspring (9, 10). Detection of these preferred oviposition environments by gravid mosquitoes is achieved by integrating a range of cues including olfactory, tactile and visual cues (9). These oviposition cues may be exploited in vector control programmes in several ways. Firstly, olfactory cues can be used to deter mosquitoes from egg laying in a particular environment (8). Secondly, in a "lure and kill" strategy, oviposition attractants can be used as baits to attract gravid mosquitoes in ovitraps laced with larvicidal compounds (11–13). Also, oviposition attractants can be applied as baits in traps for surveillance to track spatial and temporal distribution of mosquitoes (7, 12). Successful deployment of oviposition cues in vector surveillance/control requires an understanding of how gravid females interact with the semiochemicals.

Previous studies have shown that mosquitoes largely rely on olfactory cues to select suitable oviposition substrates (12). Currently, the most documented olfactory cues are those of plant origin because plants constitute most of the biomass in the terrestrial ecosystem. Examples of oviposition attractants that are plant-derived include α-pinene, 6-methyl-5-hepten-2-one (sulcatone), 3-carene, ß-caryophyllene, decanal, nonanal, p-cymene, o-xylene, styrene, 1,8-cineole, N-ethylbenzenamine, undecane and eicosane (14, 15). Some of these compounds elicit antennal responses (14, 16) and in blends attract gravid malaria vector *Anopheles arabiensis* in laboratory behavioral assays (14, 15). Microorganisms including bacteria, fungi and algae have also been linked to emission of volatiles that attract mosquitoes to oviposition sites. For example, the *An. arabiensis* oviposition attractant cedrol is produced by the fungal species, *Fusarium* sp. found on grass rhizomes (11, 17). The algal (*Spirogyra majuscule*) volatiles ethyl acetate, pentacosane, hexacosane, octacosane and docosane have been demonstrated to mediate oviposition behavior of *Anopheles pseudopectipennis* (18). Bacteria isolated from soil and water of oviposition sites were found to elicit oviposition response in *An. gambiae* mosquitoes (13, 19). Similarly, volatiles of bacteria origin (e.g. geosmin) and from plant infusions have been implicated in the attraction of other gravid mosquito species (20–23). However, the volatile profile of a given oviposition substrate may depend on the inhabiting microbial species.

To date, few data exist on the bacterial diversity associated with plant root exudates as well as their attractive potential to gravid mosquitoes. Malaria vector *Anopheles arabiensis* commonly feeds on plants and selectively on the invasive species *P. hysterophorus* (16). Coincidentally this plant has spread over a large expanse of East Africa, especially western Kenya, which records the highest burden of malaria in Kenya (24). Previously, we documented that the *An. gambiae* preferred host-plant *P. hysterophorus*, releases volatiles in its root exudates that attract gravid female mosquitoes (24). In the current study, we posited that microorganisms, specifically bacteria in the root exudate water emit volatiles that influence gravid mosquito attraction. The purpose of the current study was to (a) isolate and identify the bacteria associated with the root exudate water of *P. hysterophorus*, (b) test the oviposition responses of gravid *An. gambiae* to volatile emissions of the isolated bacteria, and (c) analyze the bacterial volatile emission profiles.

Materials and methods

Plants

Seeds were collected from wild *P. hysterophorus* growing at the garden of the International Centre of Insect Physiology and Ecology (*icipe*), Nairobi, Kenya (1°16′60″S; 36°49′0″E). The seeds were air-dried and planted in plastic pots (12 cm base diameter, 23 cm top diameter and 22 cm depth) with sterilized sand (autoclaved at 121°C for 40 min) and grown using nutrient solution as previously described (25). The plants were watered daily and maintained in a screen house at 22 ± 2 °C and 60–70% relative humidity (RH).

Root exudate water

Flowering *P. hysterophorus* plants (~6 weeks old), were removed from the plastic pots gently avoiding root damage. Thereafter, the roots were wrapped with aluminum foil to maintain the below ground environment. The plants were transported to the laboratory and roots washed gently with distilled water to remove soil. The plant roots were then sterilized using 0.1% sodium hypochlorite and rinsed with sterilized water. Batches of four plants were soaked in 50 ml of distilled water in a glass beaker wrapped with four layers of aluminum foil held tightly to the flask with three tight-fitting rubber bands. After 24 h, the plants were removed and discarded and the water containing the exudate transferred into sterile 50 mL falcon tubes. This experiment was performed in triplicate to obtain root exudate water for bacteria isolation and chemical analysis.

Bacteria

The bacteria used in headspace solid phase micro-extraction (HS-SPME) volatile collection were grown in Luria bertani (LB) agar and incubated overnight at 37°C. Briefly, 100 μL of the root exudate were spread out on the agar using sterile beads. The mixed cultures were then subjected to subsequent sub-culturing to obtain pure cultures. The pure colonies were classified based on macroscopic observation (shape, size and color). The individual colonies were re-inoculated in LB broth to obtain stock bacterial cultures for use in mosquito oviposition bioassays and molecular analysis to identify the isolates.

DNA extraction and identification of bacterial isolates

To obtain bacteria cells, I mL of each pure isolate was aliquoted into 1.5 m L vial and centrifuged for 5 min at 800xg. Thereafter the supernatant was removed, the pellet re-suspended in lysis buffer and DNA was extracted from the isolated bacteria using the Isolate II Genomic DNA extraction Kit (Bioline, London UK) as per the manufacturer's protocol. PCR was used to amplify a portion of the 16S rRNA gene using the V1-V3 primers Forward: 5'-GAGTTTGATCNTGGCTCAG-3' and Reverse: 5'-GTNTTACNGCGGCKGCTG-3' (26). The PCR reaction (20μL) contained 10 μM of each forward and reverse primer, 4x*Taq* Rxn buffer (Bioline, London UK) and 0.4 μL of *Mytaq* DNA polymerase, 11.1 μL of nuclease free water and finally 2μL of DNA sample (~12.5ng). The PCR reaction was carried out in Pro-Flex Thermol Cycler with cycle conditions consisting of initial denaturation at 95°C for 1 min, followed by 35 cycles of 95°C for 15 s, 60°C for 20 s, 72°C for 10 s and a final extension at 72°C for 10 min. Amplicons were resolved in agarose gel (1.5%) electrophoresis after staining with ethidium bromide. The PCR products were purified using QIAquick PCR & Gel cleanup Kit (Qiagen Ltd, North Manchester, UK) following the manufacturer's protocol, and outsourced for Sanger sequencing at Macrogen Europe BV (Amsterdam, the Netherlands) using both primers. The resulting sequences were cleaned and aligned using MEGA v. 11 (27) and queried against the National Center for Biotechnology and Information (NCBI) database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) with the online BLASTn tool (28). Sequence homology greater than >94% to GenBank database reference sequence were used as the criteria for assigning the identity of each isolate (22, 28).

Mosquitoes

Anopheles gambiae (Mbita strain) used in this study were from a colony established in 2018 and maintained at *icipe*. The adults were reared at 72% relative humidity (RH) and 28 ± 2°C with a photoperiod of 12 h: 12 h (Light : Dark). For egg development, the mosquitoes were offered human blood through arm feeding, two to three times weekly and had ad libitum access to 6% glucose water solution (wt:vol). Gravid females were selected and used for oviposition experiments. Eggs were laid in oviposition cups (7 cm top diameter, 7 cm depth and 4 cm base diameter) lined with Whatman filter paper (GE Healthcare UK Ltd, Buckinghamshire, London, UK) and transferred to rearing trays (39 × 28 × 4 cm depth). On hatching, larvae were reared at a density of ~500 per tray and the rearing water was changed after every two days. All larval instars were fed daily with Tetramin fish food (Tetra, Germany) until pupation. Pupae were transferred to emergence cups containing 15 ml water and placed in a new cage (15 x 15 x 15 cm). Emerged adults (1 day old) were maintained on 6% glucose water solution as described above.

Oviposition response assays

Three bacterial isolates were assessed for their ability to influence oviposition response of gravid *An. gambiae* in a dual-choice assay. Twelve gravid mosquitoes were presented a choice between the treatment (bacteria suspended in saline water) and normal saline water (0.9% w/v) contained in similar oviposition cups and the number of eggs laid counted using a microscope (Leica M127, Switzerland) after every 24 h for four consecutive days (24). The oviposition cups were placed diagonally in the experimental cages and their positions interchanged every 24 h to avoid

positional bias (22, 24). Each cup was lined with a Whatman filter paper (GE Healthcare UK Ltd, Buckinghamshire, London, UK) and filled with 30 ml of the test solution (saline water with one of the bacterial species) or an equivalent volume of saline water as the control (Saline normally should not contain any bacteria) (13). The bioassays were performed in triplicate and repeated once. The oviposition bioassays were performed with each isolate at three concentrations of different optical densities (OD) i.e. 0.1-0.2 (5.4×10^6 bacteria cells/ml), 0.5-0.6 (1.1×10^7 cells/ml) and 0.8-0.9 (2.2×10^7 cells/ml) (12). Estimate of the concentrations were obtained by counting bacteria cells of known OD (dilution factor of X20) under microscope using a hemecytometer.

Solid phase micro-extraction

Individual bacteria isolates grown in LB agar in a 90 mm Petri dish were placed inside a 250 mL glass beaker and sealed with aluminum foil. The headspace volatiles were collected by solid phase microextraction (SPME) using polydimethylsiloxane/Divinylbenzene (PDMS/DVB) fiber (Supelco, Bellefonte, USA) pre-cleaned via thermal desorption at 250 °C for 30 min. Volatiles were trapped for 24 hr and then analyzed by thermal desorption immediately in a gas chromatography-mass Spectrometry (GC-MS). Flasks with LB agar only served as controls. In preliminary analysis, the GC-MS profile of bacterial isolates in agar and broth were observed to be similar (data not shown), hence the use of agar method was selected because of ease of mobility and low risk of contamination.

Gas chromatography-mass spectrometry analysis

Volatiles were analyzed on a 7890B gas chromatograph (Agilent Technologies, Inc., Santa Clara, CA, USA) coupled to a mass spectrometer (5977A series). The GC was fitted with a HP-5 MS low bleed capillary column (30m x 0.25 mm i.d., 0.25 μm) (J&W, Folsom, CA USA) and helium served as the carrier gas at a flow rate of 1.2 ml/min. The inlet temperature was set at 270 °C whilst the transfer line temperature was 280 °C. The column oven temperature was programmed from 35 to 285 °C, with the initial temperature maintained for 5 min then 10 °C/min to 280 °C for 5.5 min and finally 5 °C/min to 285 °C for 34.9 min. The mass selective detector was maintained at ion source temperature of 230 °C and a quadrupole temperature of 180 °C. Electron impact (EI) mass spectra were obtained at the acceleration energy of 70 eV. SPME-collected volatiles were analyzed by manually inserting the fiber into the GC-MS injector port (250 °C). Fragment ions were analyzed over 38-550 m/z mass range in the full scan mode. The filament delay time was set at 3.0 min. The samples were analyzed in triplicate. The volatile compounds were identified based on the mass spectral library data from Adams and National Institute of Standards and Technology (NIST). Pure standards from sigma analyzed in the same conditions were also used for comparison.

Statistical analysis

The oviposition activity index (OAI) was calculated according to the formula described in (23);

$$OAI = (Nt - Nc)/(Nt + Nc)$$

Where Nt refer to the number of eggs laid in the treatment while Nc the number of eggs laid in control (water). OAI ranges from -1 to +1; 0 indicates a neutral response; a positive value indicates an attraction towards the treatment while negative value indicates the converse. The difference in oviposition activity index was compared using one-way ANOVA and Duncan post-hoc test. The number of eggs laid per treatment was analyzed by generalized linear model (GLM) using negative binomial. The model validity was assessed by inspection of residuals (24). The numbers of eggs laid per treatment served as the response variable while the treatments were used as the predictor variable. Mean of peak areas (n=3) of VOCs obtained using GC-MS was also compared using one-way ANOVA and Duncan post-hoc test. Principle component analysis was performed on the VOC profile of the bacteria isolates. All statistical analyses were performed using SPSS 23.0 software (IBM SPSS Statistics) and results considered significant at p ≤ 0.05. Random Forest Analysis (RFA) was performed using the random forest package in R software version 3.4.3 (R core development team). The relative abundance of each identified VOC following GC/MS analysis was used in the RFA analysis executed by running 100,000 iterations (ntree) with 4 volatiles randomly selected at each split (mtry=√q where q is the total number of volatiles) from a total of 16 variables (VOCs). The function 'importance' was used to generate the mean decrease in accuracy (MDA) and volatile with the highest value considered the most important (24).

Results

Identification of bacterial cultures

Using Luria bertani (LB media), three bacterial strains were isolated from *P. hysterophorus* root exudate water. The pure colonies were designated as Isolate A-C based on macroscopic morphological differences (e.g. shape, size, color). For each isolate, a fragment of the 16S rRNA gene was amplified and sequenced followed by query in GenBank database. Sequences (477-485 nt) of two of the isolates were identical to the Gram-negative bacteria species *Enterobacter mori* (95% identity) and *Enterobacter* sp. (99% identity) in the GenBank (Table 1) with both belonging to the family Enterobacteriaceae. The other isolate, also a Gram-negative bacterium showed a 94% similarity to *Alcaligens aquatilis* belonging to the family Alcaligenaceae (Table 1).

Oviposition response of gravid mosquitoes to bacterial isolates

Gravid mosquitoes were presented with a choice between saline water (control, 0.9% w/v) and treatment [either crude bacterial mixture, isolate A (*Enterobacter* sp.), B (*A. aquatilis*) or C

TABLE 1 Phylogenetic affiliation of bacterial isolates from *P. hysterophorus* root exudate water.
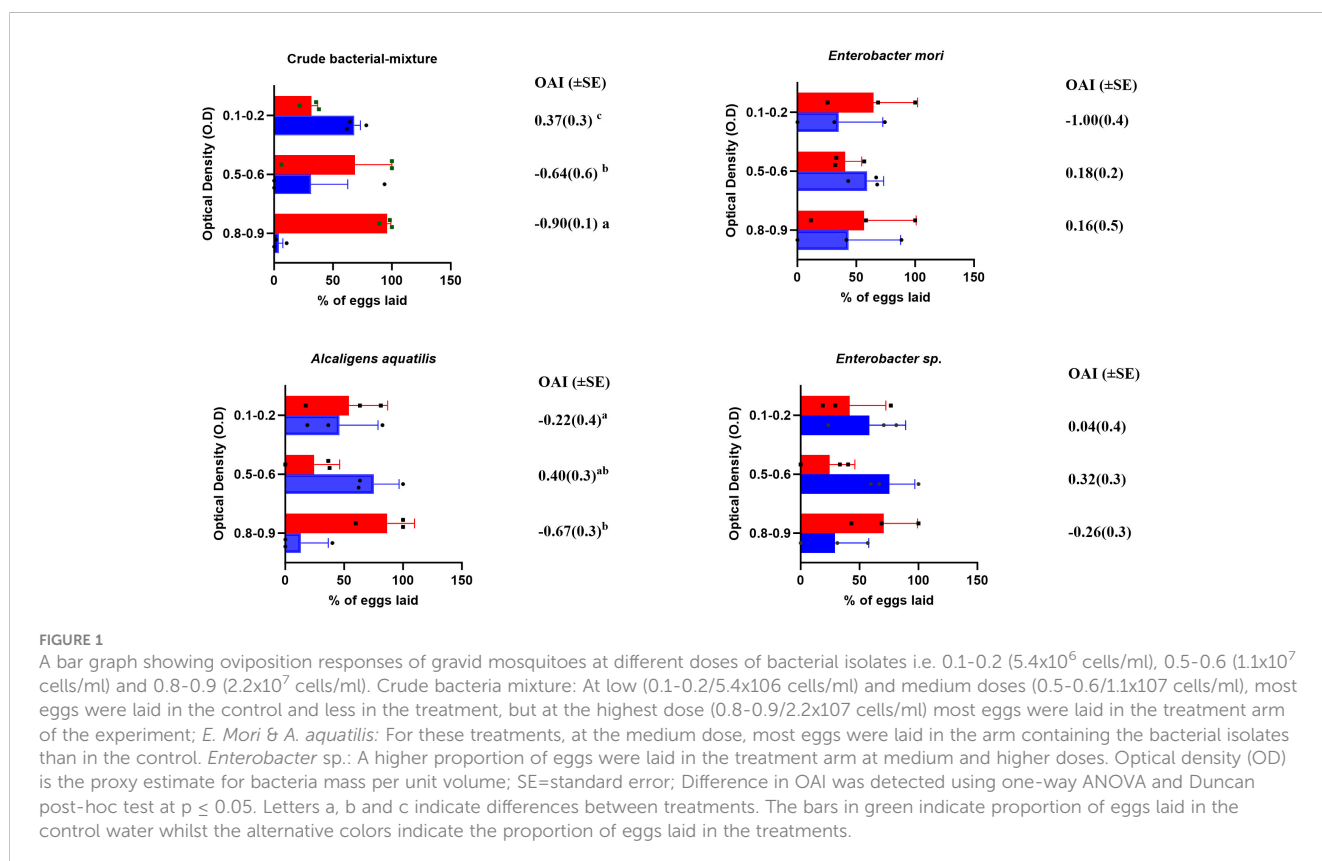
Sample identity	Family	Taxonomic assignment	% identity	GenBank accession no.
Isolate A	Enterobacteriaceae	*Enterobacter* sp.	99	MH790145
Isolate B	Alcaligenaceae	*Alcaligens aquatilis*	94	MT228980
Isolate C	Enterobacteriaceae	*Enterobacter mori*	95	MT239536

(*E. mori*)]. As stated earlier, each treatment was tested at three concentrations of different OD (i.e. 0.1-0.2, 0.5-0.6 and 0.8-0.9). Based on the number of eggs laid in each treatment relative to the control, the oviposition activity index (OAI) was estimated as a measure of attractiveness of each isolate to the gravid mosquito (Figure 1). Overall, the crude bacterial mixture was attractive to the mosquitoes at the lowest optical density of 0.1-0.2 (68.7% of eggs laid, OAI=0.37(± 0.3), $\chi^2_{2,57}$=12.2, p<0.001) while the higher bacterial densities were less attractive (4.8%, OAI= -0.9(± 0.1), $\chi^2_{2,57}$=9.7, p<0.001). Similarly, *Enterobacter* sp. and *A. aquatilis* at the highest bacterial density (OD of 0.8-0.9) were less attractive to the mosquitoes; 36.0%, OAI = -0.26 (± 0.3), $\chi^2_{2,142}$=0.4, p=0.51, and 16.7%, OAI= -0.67 (± 0.3), $\chi^2_{2,769}$=30.95, p<0.001 respectively. Likewise, at the lowest bacterial density (OD of 0.1-0.2), gravid mosquitoes avoided odors released from *A. aquatilis* (39.0%, OAI= -0.22 (± 0.4), $\chi^2_{2,148}$=0.3, p=0.58 and *E. mori* (27.1%, OAI= -1.00 (± 0.4), $\chi^2_{2,130}$=1.4, p=0.23, respectively. Notably, for each of the bacterial isolate, the highest OAI was recorded at the OD of 0.5-0.6; this optimum density was selected for further evaluation in binary oviposition assays.

Females laid significantly more eggs in *E. mori* odors than to those released by *A. aquatilis* ($\chi^2_{5,284}$=5.6, p=0.02, Figure 2). Overall, twice as many more eggs were laid in the treatment containing *E. mori* than in *A. aquatilis* (Figure 2). On the other hand, significantly more eggs were laid in the treatment containing *A. aquatilis* than *Enterobacter* sp. ($\chi^2_{5,187}$=3.9, p=0.04, Figure 2). Notably, none of the isolates was as attractive as the crude bacterial mixture (Figure 2).

Analysis of bacterial volatiles

In GC-MS analysis, a total of 16 volatile organic compounds (VOCs) were identified in the headspace of the bacterial isolates belonging to different chemical classes namely benzenoids (styrene), pyrazines (2,6, dimethyl-pyrazine), aldehydes (benzaldehyde, nonanal, decanal), terpenes (1,8 cineole, o-cymene, p-cymene, cedrene), alcohols (phenylethyl alcohol), alkanes (dodecane, tetradecane, pentadecane, hexadecane) and indole. Quantitative analysis revealed a heterogeneous distribution
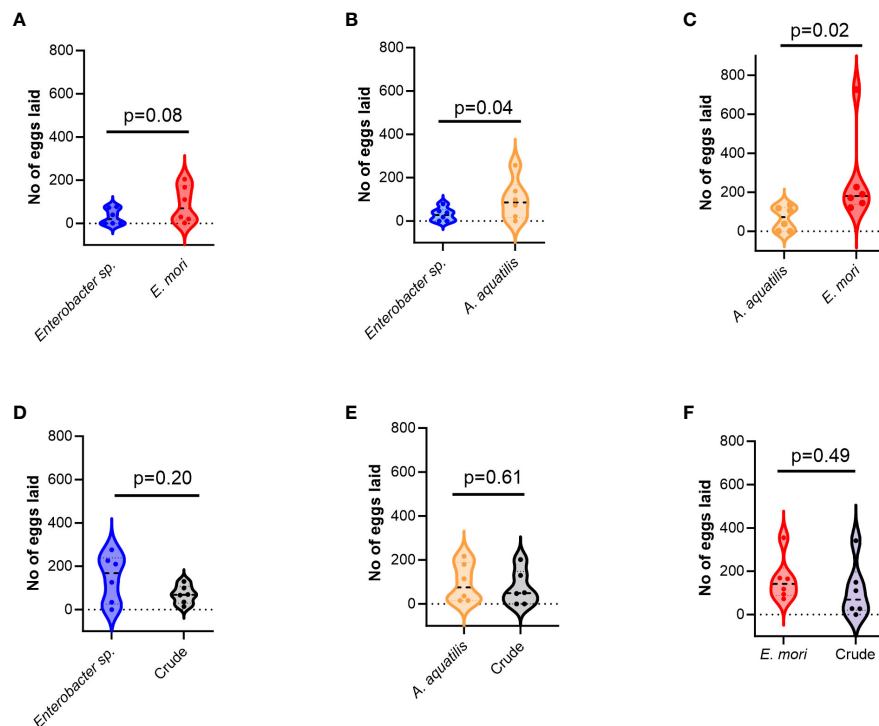

FIGURE 1
A bar graph showing oviposition responses of gravid mosquitoes at different doses of bacterial isolates i.e. 0.1-0.2 (5.4×10^6 cells/ml), 0.5-0.6 (1.1×10^7 cells/ml) and 0.8-0.9 (2.2×10^7 cells/ml). Crude bacteria mixture: At low (0.1-0.2/5.4x106 cells/ml) and medium doses (0.5-0.6/1.1x107 cells/ml), most eggs were laid in the control and less in the treatment, but at the highest dose (0.8-0.9/2.2x107 cells/ml) most eggs were laid in the treatment arm of the experiment; *E. Mori & A. aquatilis:* For these treatments, at the medium dose, most eggs were laid in the arm containing the bacterial isolates than in the control. *Enterobacter* sp.: A higher proportion of eggs were laid in the treatment arm at medium and higher doses. Optical density (OD) is the proxy estimate for bacteria mass per unit volume; SE=standard error; Difference in OAI was detected using one-way ANOVA and Duncan post-hoc test at p ≤ 0.05. Letters a, b and c indicate differences between treatments. The bars in green indicate proportion of eggs laid in the control water whilst the alternative colors indicate the proportion of eggs laid in the treatments.

FIGURE 2
Violin plots depicting number of eggs laid by gravid *An. gambiae* when given a binary choice between different pairs of treatments. **(A)** Eggs laid in response to *Enterobacter* sp and *E. mori* odors was not significantly different **(B)** Females laid significantly more eggs in treatment containing A. aquatilis odors than *Enterobacter* sp. **(C)** Twice as many more eggs were laid in the treatment containing *E. mori* than in *A. aquatilis* **(D−F)** none of the isolates was as attractive as the crude bacterial mixture

of VOCs among the bacteria species (Table 2). For example, whereas benzaldehyde, was 16-fold more abundant in *E. mori* than in the cultures of other bacterial isolates, cedrene and phenyl ethyl alcohol were 6- and 28- fold respectively, more abundant in *A. aquatilis* than in *E. mori*. On the other hand, *Enterobacter* sp. released higher amounts of dodecane, 2,6-dimethyl-pyrazine and indole (Table 2). RFA identified dodecane and indole as the most important compounds (abundant and permanently present) contributing to oviposition responses (Figure 3A). Principle component analysis on the VOC profiles showed separation of the three isolates (Figure 3B).

Discussion

Selection of suitable oviposition sites is a critical determinant of reproductive success in mosquitoes (9). In this study, we evaluated the attractive ability of cultivated bacteria from plant root exudate to the gravid malaria mosquito and analyzed volatiles emitted by these microbes. We show that bacteria associated with *P. hysterophorus* root exudate alter egg-laying decisions by gravid mosquitoes. Furthermore, our analyses reveal VOCs emitted by the associated bacterial species, and variation in the profiles which could account for differences in responses. The data is consistent with previous studies implicating microbial influence of odor-oriented behaviors in mosquito oviposition (11, 13, 17).

By employing a culture-based approach, only three bacterial isolates were recovered and identified as *Enterobacter* sp. (Enterobacteriaceae), *A. aquatilis* (Alcaligenaceae) and *E. mori* (Enterobacteriaceae). Odors from these isolates elicited differential oviposition responses in gravid *An. gambiae*. We found that olfactory cues from the bacteria *E. mori* were more attractive to gravid females than those from *A. aquatilis*. In turn, the mosquito was more attracted to *A. aquatilis* than *Enterobacter* sp. This finding is not uncommon as previous studies have reported variation in attractiveness to mosquitoes by different bacterial species (13). Bacteria belonging to the family Enterobacteriaceae have been associated with changes in oviposition behavior of *Anopheles* species (13). Other bacterial families that have been implicated in oviposition response but not identified in the present study include Bacillaceae, Nocardiaceae, Micrococcaceae, Paenibacillaceae and Comamonadaceae (13). The difference could relate to the oviposition substrate type (23). For example, in most previous investigations on mosquito oviposition behavior, the isolated bacteria were from substrates such as soil, lake water, plant infusion and human skins (13, 20, 28, 29). However, influence of culture media type on bacterial composition (29) cannot be ruled out, necessitating use of additional media in future studies. To the best of our knowledge, this is the first report showing the potential effect of bacteria associated with host plant root exudate water on oviposition behavior of gravid *Anopheles* mosquitoes. Moreover, a review of the literature revealed that no previous study had

TABLE 2 Volatiles emitted by different bacterial isolates. .

	Mean of peak areas (± SE)			
	Crude	Enterobacter sp.	A. aquatilis	E. mori
Styrene	19.7(5.2) [a]	30.1(10.7) [a]	28.1(9.6) [a]	1.0(0) [b]
2,6-dimethyl-pyrazine	12.0(1.5) [b]	12.0(1.5) [b]	42.5(21.9) [a]	1.0(0) [c]
Benzaldehyde	8.8(0) [b]	1.0(0) [c]	1.0(0) [c]	16.7(0) [a]
1,8-Cineole	22.5(7.1) [a]	23.2 (13) [a]	10.3(1.2) [a b]	4.7(3.7) [b]
o-Cymene	11.5(2.5) [c]	19.8(0) [b]	31.3(0) [a]	1.0(0) [d]
p-Cymene	13.6(3.7) [b]	1.0(0) [c]	23.3(0) [a]	1.0(0) [c]
n-Nonanal	18.7(1.5) [b]	17.6(3.4) [b]	1.0(0) [c]	42.8(2.0) [a]
Phenyl ethyl alcohol	72.8(20.2) [a]	5.6(0) [c]	26.8(15.4) [b]	1.0(0) [d]
Dodecane	28.4(6.2) [a]	13.8(2.8) [b]	1.0(0) [c]	4.7(3.7) [c]
n-Decanal	23.8(3.2) [b]	31.0(0) [a]	1.0(0) [c]	28.6(2.5) [a]
Indole	283.9(23.9) [a]	13.4(7.7) [b]	1.0(0) [c]	ND
Butyl butanoate	12.8(1.0) [b]	20.7(2.9) [a]	11.6(0.6) [b]	1.0(0) [c]
Tetradecane	23.7(5.0) [a]	29.8(11.4) [a]	23.4(9.9) [a]	1.0(0) [b]
alpha-Cedrene	3.5(0) [b]	4.6(0) [a]	6.3(2.1) [a]	ND
Pentadecane	12.4(3.1) [b]	24.7(0) [a]	11.0(0.3) [b]	1.0(0) [c]
Hexadecane	14.7(1.1) [a]	14.1(0) [a]	11.2(0.9) [b]	1.0(0) [c]

Volatiles in bold indicates those identified in both the headspace of bacterial culture and root exudate water. ND, Not detected; SE, standard error; Difference in mean of peak areas of VOCs obtained using GC-MS was detected using one-way ANOVA and Duncan post-hoc test. Letters a, b and c indicate differences between treatments.

implicated *E. mori* or *A. aquatilis* in the oviposition response of any mosquito vector.

The attraction elicited by odors of different bacterial species to ovipositing females could differ as a function of variations in the composition of VOCs they emit (29). In our study, the abundance of VOCs was observed to vary among the three bacterial isolates. For instance, the aldehydes nonanal and benzaldehyde were abundant in the volatile profiles of *E. mori* while cedrene was abundant in the volatiles of *A. aquatilis*. These two aldehydes have been shown to elicit antennal responses in gravid *Anopheles* mosquitoes (12, 14). Also, nonanal and decanal have been implicated in host seeking behavior of *An. gambiae s.l* (30, 31). and male swarming behavior (32). Additionally, the known *Anopheles* oviposition attractant dodecane and indole were emitted in higher amounts in *Enterobacter* sp. than in the volatile emissions of the other two species. Interestingly, these two compounds were identified by RFA as the most important in contributing to the attraction of the odors of bacterial isolates to oviposition. These and the additional compounds identified may require further investigation to ascertain their roles in mosquito oviposition behavior.

The bacteria sp. that influences mosquito oviposition behavior may be exploited for mosquito control. For instance, the attractive bacteria sp. can be utilized in the development of microbial based oviposition traps to target gravid mosquitoes. Alternatively, the less attractive bacteria species can be used to manipulate breeding sites

to produce unattractive volatiles and thus prevent colonization of habitats by mosquitoes. Overall, the positive influence of microbes on the oviposition behavior of gravid mosquitoes agrees with previous findings (13, 33), although, our approach is unique and opens up a novel avenue for identifying lures that can compete with natural breeding sites.

As a limitation, we assayed bacteria singly and using concentration ranges that could differ from the natural root exudate. Generally, it is possible for a combination of bacteria to elicit a stronger oviposition response. Bacterial populations in the natural substrate could be more diverse than just a single species (13, 23, 29), necessitating evaluation of bacteria also in blends. In addition, the current study did not investigate the impact of the bacteria on mosquito life history traits but focused only on oviposition behavior. With respect to VOCs detection, some of the detected VOCs (e.g., 2,6, dimethyl-pyrazine, 1,8 cineole, phenylethyl alcohol, dodecane, hexadecane) did not overlap in the headspace of the bacteria and root exudate water. Perhaps in the root exudate water, only trace amounts of these volatiles were present beyond the detection limit of the GC-MS. Also, the difference can be attributed to the adsorbents employed in volatile trapping. Whereas plant root exudate volatiles were trapped using Super Q (24), SPME was utilized for bacteria headspace samples. Nevertheless, the study provides relevant baseline investigating the role of bacteria associated with host plant root exudate water in mediating mosquito oviposition behavior.
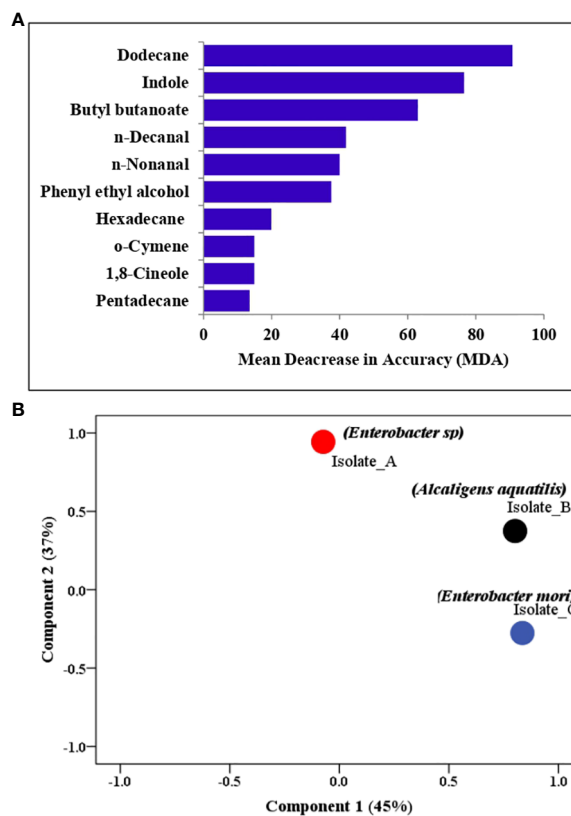
FIGURE 3
(A) Bar graph depicting the most important compounds (abundant and permanently present) contributing to oviposition responses **(B)** Principal component analysis (PCA) plots showing the variation of the isolates based on the VOCs profile. Component 1 explained 37% of the total variation while component 2 explained 45% of the total variation.

Conclusion

Our study provides a first report demonstrating the ability of culturable bacteria associated with *P. hysterophorus* root exudate to attract gravid *An. gambiae* mosquitoes. The bacterial isolates elicited differential attraction to gravid *Ae. gambiae* mosquito and varied in their emitted VOCs. The attractive isolates (*E. mori* and *A. aquatilis*) were associated with abundance of compounds known to attract gravid malaria vectors including nonanal, benzaldehyde and decanal. These VOCs can be exploited in semiochemical-based mosquito control strategies. Collectively, the current findings improve our knowledge of *P. hysterophorus* root exudate-mosquito-microbe interaction.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

Ethics statement

The manuscript presents research on insects that do not require ethical approval for their study.

Author contributions

TM: Conceptualization, Formal Analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. BT: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing. DT: Conceptualization, Data curation, Formal Analysis, Software, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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