INVESTIGATING DIFFERENTIAL ATTRACTIVENESS OF HUMAN SKIN MICROBIOTA TO *ANOPHELES GAMBIAE* AS A POTENTIAL COMPONENT OF BAITED SUGAR TRAPS CONTAINING ANTI-*PLASMODIUM* BACTERIA FOR BIOLOGICAL CONTROL

by

Alicia Majeau

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Abstract

Malaria is a pressing global health problem that is difficult to eradicate or even control because of its complex biology. Currently employed control mechanisms are inefficient, and as a result of the need for alternative interventions, some research has focused on investigating the influence of the microbiota on mosquito vector competence. While some bacteria of the mosquito midgut have been shown to confer refractoriness to the *Plasmodium* parasite as well as shorten the mosquito lifespan, we are still far from a real world application of a bacterium as a biological control mechanism for malaria. Spiked sugar feeding stations have been proposed as a mechanism of introducing the bacteria into local vector populations. This project aimed to investigate means of attracting mosquitoes to feed on the malaria parasite-blocking *Enterobacter* (*Esp_Z*) bacteria- spiked sugar, using human skin microbiota isolates as potential attractants. We also investigated the impact of bacterial exposure on mosquito life span and fecundity as general fitness parameters. Minimal fitness costs were observed by sugar-feeding *Plasmodium*-killing bacteria in the lab environment. No foot microbiome isolate was found to attract mosquitoes on its own, although one was found to exert mosquito repelling activity. However when this bacterial isolate was combined with an attractant that zoophilic mosquitoes respond to, an increase in attraction was observed. While much work is still needed, our results provide useful knowledge for the development of this type of biological control.

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Preface

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Introduction

According to the World Health Organization, malaria kills a child under five years of age nearly every minute (1). With 3.4 billion individuals at risk of contracting this disease, it remains one of the most relevant and pressing global public health problems (1). Caused by the *Plasmodium* parasite, the majority of this disease burden lies in sub-Saharan Africa, where more than 90 percent of malaria mortality occurs (1). Those groups most at risk of contracting the disease include children under five and pregnant women. Disease in pregnant women can lead to low birth weights, contributing even further to morbidity and mortality in these regions (1). While the disease has been eradicated in some regions and countries, including the United States, it is still very much a disease of poverty as a result of its complex ecology. Solutions that worked for local elimination in some regions are simply not feasible in many of the affected areas. Considering the great threat to public health, efforts must continue to not only control, but eventually eradicate malaria.

The *Plasmodium* parasite is transmitted by female *Anopheles* mosquitoes, the definitive host of the parasite, when they take a blood meal (1). Male *Anopheles* are uninvolved in transmission of the pathogen because they are not hematophagous, but strict nectar feeders. When a female mosquito takes a blood meal from a human or animal infected with the parasite, it ingests parasite gametocytes, the males of which then exflagellate and the gametocytes fuse to form a zygote (2). Zygotes elongate and become motile, invading the mosquito midgut as ookinetes (2). After crossing the midgut barrier, ookinetes develop into oocysts, which eventually grow as a result of parasite replication, rupture, and release sporozoites that then translocate to the salivary glands (2). When the infected mosquito takes the next blood meal, sporozoites are inoculated into the human or animal on which the mosquito is feeding. This begins the first of two human stages- namely, the liver stage infection. Sporozoites infect the liver and develop into schizonts, which rupture to release merozoites, leading to the second stage

of human infection, the blood stage (2). Merozoies infect erythrocytes and in red blood cells, trophozoites mature into schizonts, which again rupture to release additional merozoites (2). During this blood stage, some parasites will differentiate into gametocytes, which may then be taken up by a mosquito during a blood meal, propagating the cycle (2).

Transmission of malaria may occur at high levels throughout the year, or may also be seasonal, depending on the ecology and climate of a particular location (3). Because of this, there are varying degrees of immunity in differing non-insect populations. However, immunity builds slowly and is not one-hundred percent efficient. Furthermore, it has been demonstrated that immunity in expatriates wanes once the exposure has been removed and upon return to an area of endemnicity, an individual will again be susceptible to contracting the disease.

Historically, research efforts have focused on developing drugs to treat the disease and vaccines to reduce the disease burden. However, drug-resistant strains of *Plasmodium* continue to emerge, and vaccines have been largely unsuccessful as a result of the complex life cycle of the parasite and as hinted at by the lack of true natural immunity. The most successful vaccine to date, the RTS,S vaccine, has only about 30% efficacy, and while this reduction in disease is certainly significant, it is an inefficient solution (4).

When one considers the potential reservoirs for the parasite, it becomes clear that human disease could continue to persist and reemerge, even if successful drugs and/or vaccines were employed. The seven species that make up the *Anopheles gambiae* species complex exhibit varying biting behaviors, with some being generally zoophilic and some generally anthropophilic (5). Some, however, fall in between and take blood meals from animals and from humans at varying rates. While species divisions that feed more on livestock and other mammals may not be ideal vectors of the parasite, it does not mean that their vectorial capacity cannot increase given the right set of circumstances (5).

In theory, a non-human mammal could harbor the parasite and a mosquito could become infected by taking a blood meal. While most *Plasmodium* species that infect humans are rather specific, one need only consider *P. knowelsi* to recognize the potential of one parasite species to spill-over and evolve to a different host. This parasite species naturally infects macaques, but has been found to be an important cause of zoonotic malaria is Southeast Asia, leading to severe disease and even death (6). Other typically non-human *Plasmodium* parasites have also been demonstrated to rarely infect humans (6). No vaccine could possibly provide protection to all *Plasmodium* species.

As a result of the need for alternative interventions, there has been a shift in research with increased focus on transmission blocking methods that target the definitive host. Currently used interventions that target *Anopheline* mosquitoes, however, are similarly inefficient. Some have proposed the use of larvicides to kill off large numbers of mosquitoes before they ever emerge as adults, but breeding sites are often extremely difficult to find in nature and too often very transient. While the use of long-lasting insecticide-treated bed nets and indoor residual spraying has also lessened the disease burden, insecticide resistance is a growing concern in nearly all areas that engage in malaria vector control (Figure 1) (1).

Studies in West Africa found a 40% increase over the course of three years in the prevalence of the Leu1014Phe *kdr* resistance mutation (7). This rapid spread is expected, as the resistance gene confers a large survival advantage where selection pressures are in place and mosquitoes have a relatively short gonotrophic cycle, creating a large evolutionary pressure and selection effect. Insecticide treated nets, in particular, can only be made using pyrethroids at this time, so once resistance enters the mosquito population, these nets become much less efficient, acting primarily as physical barriers (7).

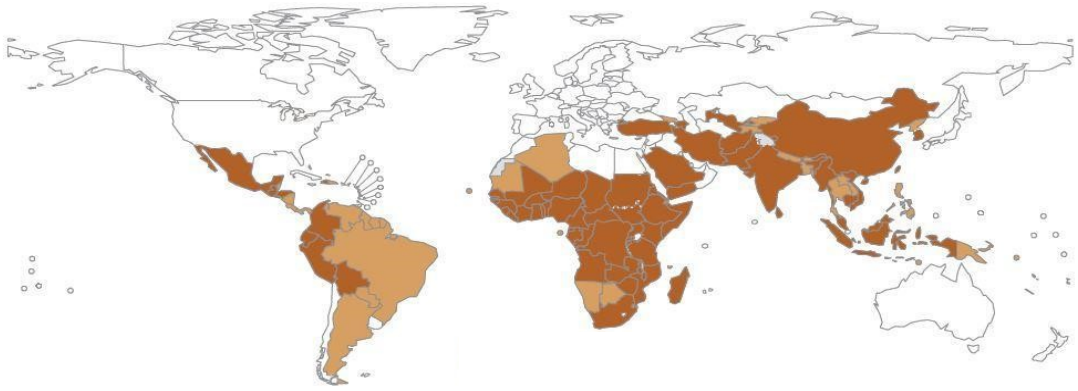


Figure 1. Map indicating countries with ongoing malaria transmission and reports of insecticide resistance. The dark brown color indicates a country where insecticide resistance has been reported (Source: WHO Global Malaria Report, 2013)

Clearly, there is a need for interventions that target either the mosquito or the parasite in an "evolution-proof" manner, allowing for effective control and potentially even eradication. Preventing the vector from becoming infected with the parasite would, in turn, prevent transmission to a new host. The *Anopheles* midgut provides a promising target to prevent such infections (8). This is, in part, due to the bottleneck that occurs at this point of parasite development. While thousands of gametocytes are ingested by the mosquito in an infectious blood meal, forming thousands of ookinetes, very few oocysts ever transverse the midgut in a natural setting (8).

Previous studies out of the Dimopoulos group have demonstrated a natural microbe mediated refractoriness to the *Plasmodium* parasite in *Anopheles* (9). *Anopheles arabiensis* (part of the *Anopheles gambiae* species complex) collections were done in Zambia, and various bacterial species were harvested from their midguts. From this group of bacteria, several were identified that conferred refractoriness to the *Plasmodium* parasite when fed back to mosquitoes, including the *Enterobacter Esp_Z*, which will be the focus of the first portion of this investigation (Figure 2) (9). Species in two other genera, namely *Chromobacterium*, and *Serratia* have also been previously shown to reduce infection in the mosquito.

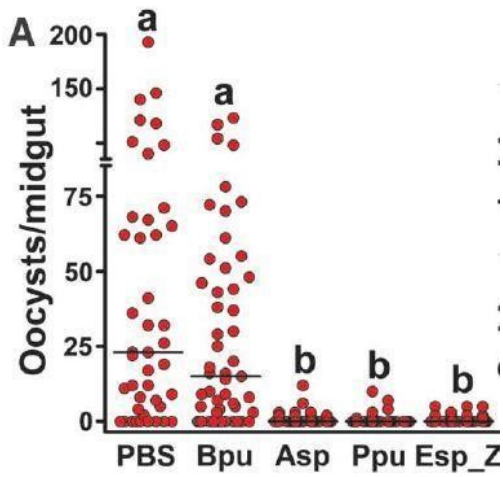


Figure 2. Reduction in median number of oocysts/midgut when *Esp_Z* or other bacteria are fed to *Anopheles* mosquitoes compared to PBS control (Source: Cirimotich et al. 2011)

The anti-*Plasmodium* effects of these bacteria were also demonstrated *in vitro*, indicating that direct killing is occurring and that it is not simply mediated by mosquito immune activation upon bacterial exposure (Figure 3) (9). This distinction is important, as many introduced agents will upregulate innate immune factors, leading to an observed reduction in *Plasmodium* oocysts that is simply an artifact of the system. The refractoriness conferred by feeding these bacteria back to mosquitoes was demonstrated to have a dose-specific response, whereas greater concentrations of *Esp_Z* in the midgut was associated with fewer oocysts up to a cutoff point (9).

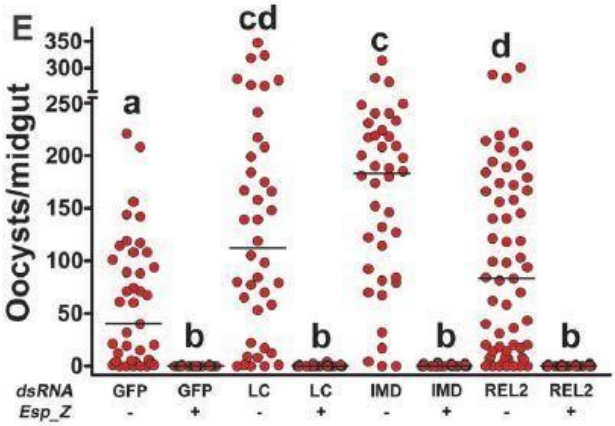


Figure 3. Immune genes knocked down by injecting dsRNA and mosquitoes fed on *Esp_Z* or a control, proving that the observed reduction in *Plasmodium* is not a result of immune activation, but direct killing (Source: Cirimotich et. al 2011).

Since isolation and these initial studies, *Esp_Z* has been adapted in the laboratory by selectively passaging the colonies that demonstrated the greatest reduction in oocysts per midgut. Accordingly, the studies undertaken here used this adapted strain of the bacterium.

In these previous studies, bacteria were fed to mosquitoes through a *Plasmodium*-infected blood meal. Moving forward, practical applications of biological control that employ the use of these microbes would necessitate introduction of the bacterium into the mosquito prior to an infectious blood meal. Stone et.al (2011) has demonstrated that in a sugar-rich environment, mosquitoes will delay their first blood meal, preferentially feeding on nectar in the early stages of life (10). Potentially, then, baited sugar feeding stations could be used to introduce a microbe population that would confer increased refractoriness into the midgut of mosquitoes prior to the first blood meal.

While this design holds a lot of promise, there are several considerations that must be made. First and foremost, the solution would not be sustainable if introducing *Esp_Z*, or any other bacteria with similar properties, to mosquitoes at a high enough concentration to effectively prevent oocyst formation would induce significant fitness costs, unless it would significantly reduce the lifespan of the mosquito to render them incapable of transmission. As only older female *Anopheles* are effective vectors of the parasite due to the weeks-long extrinsic incubation period, killing off any mosquitoes who fed on the bacteria early in life would have the same effect as insecticides, broadly reducing the number of vectors, and could potentially present a resistance-type pressure. If ingesting these bacteria would reduce the fecundity of a mosquito, it is also possible that a mechanism could evolve to prevent the colonization of these species in the mosquito midgut. Next, even if a female mosquito ingests sugar meals throughout her life, there must be a way to preferentially attract the mosquitoes to the baited sugar feeders over the numerous natural nectar sources that could be present in the environment.

Much remains unknown in regards to mosquito olfaction and host-seeking cues, despite many years of focused study. Known attractants do exist, such as carbon dioxide, which has been demonstrated to be greatly important in the attraction of *Anopheles* to human hosts (11, 12). However, carbon dioxide is not necessarily an efficient enough attractant to truly warrant the cost of use as a component of bacterial baited sugar feeding stations in low- and middle-income countries where malaria persists, as the source of the gas in field settings is most often dry ice, which is not easy to transport or store. Some research has also investigated the potential of blocking the host-seeking olfaction pathways, but in the mosquito genome, there is a large amount of redundancy in this pathway, and a number of olfaction receptors can substitute their counterparts.

Some difficulty in the development of olfaction-based malaria control arises in accounting for differences in attraction across *Anopheles* species. Previous studies demonstrated that *An. gambiae* does not respond well to carbon dioxide alone, but responded stronger to a mixture of CO_2 and low concentrations of acetone, and did not respond significantly to CO_2 and 1-octen-3-ol (12). 1-octen-3-ol is a well-established mosquito attractant, derived from bovine ruminants. This same study demonstrated that *A. stephensi* responded very well to the carbon dioxide/1-octen-3-ol mixture, but did not respond at all to the mixture of carbon dioxide and low concentrations of acetone (12). The authors posit that this difference may reflect the inherit difference in natural host preferences, as *An. gambiae* is highly anthropophilic and *An. stephensi* is zoophilic (12). Since similar variation in host preference is observed across many *Anopheline* species complexes responsible for *Plasmodium* transmission, a universal attractant to aid in malaria control may be difficult to find.

Recently, there has been an interest in understanding the role of resident bacterial species on human skin in attracting or repelling a potential vector. Work by the Takken group in the Netherlands has elucidated a number of volatiles produced by resident human skin microbiota

that have attractive properties to female *Anopheles gambiae* (11, 13). Although olfaction work had been undertaken previously in this group, the seeming turning point that led to this interest in the skin microbiota stemmed from a paper published in 2009 demonstrating that cultured skin microbiota attracts mosquitoes (14).

Their experimental design employed blood agar plates, incubated with either skin microbiota or a control bacteria and attractiveness was evaluated in olfactometer bioassays and indoor trapping experiments (14). Thirty unmated female mosquitoes who had not yet taken a blood meal were used in each replicate, alternating the stimuli between olfactometer ports to account for positional differences, and the number attracted to each port was taken as a percentage of the total number released (14). Gas chromatography-Mass spectrophotometery was used to analyze the volatiles being given off by incubated bacteria (13, 15). Importantly, the two most attractive treatments were microbiota incubated for six hours and microbiota incubated for twelve hours, respectively (14).

One critique of the experimental design used in this seminal experiment is the selection of subjects from whom microbiota were isolate. All subjects were Caucasian males between 23 and 31 years of age. Differences in natural microbiota composition may be affected by characteristics such as gender, age, and race. Although there do not seem to be investigations into any skin microbiota differences across groups differing by some of these characteristics, it is certainly plausible to assume that if inherent differences did exist, the natural vectors may be more influenced by the common microbiota compositions of people indigenous to areas of endemic malaria.

In addition, while the use of uninfected, never blood-fed mosquitoes is the easiest experimental design, this does not represent a natural model. Microarrays have shown that olfaction genes in *Anopheles* may be up- or down-regulated in response to an aseptic or *Plasmodium*-infected blood meal (16). At the translational point of this research, it will

eventually become important to know how attraction and host-seeking behaviors change in response to a mosquito taking its first blood meal, since multiple blood meals are required to transmit pathogens.

A Takken group publication also demonstrates that human foot odours are more effective attractants than a known attractive blend of ammonia, lactic acid, and carboxylic acids (17). Experiments conducted by this group have also demonstrated that compounds present in the headspace of bacteria, or the airspace immediately around the colony, derived from human skin can have attractive or inhibitory effects (15). Taken together, many of these studies indicate the potential to alter skin microbiota in a way to reduce attractiveness of individuals to malaria vectors.

Recently, it has been demonstrated that the composition of skin microbiota can affect an individual's attractiveness to the *Anopheles gambiae sensu stricto* vector, although not all resident species were included in this analysis (18). This study revealed that individuals who are highly attractive to *An. gambiae* have a much higher abundance of bacteria on their skin, although a lower diversity of bacterial species, compared to individuals who are not as attractive to the vector (18). One common foot microbe that the paper introduced, *Bacillus* spp. was not evaluated in this study, as this genus was not present in the majority of samples (18). This will be discussed further in this thesis, as it could be that the limited samples in which a *Bacillus* species was found may represent an interesting alteration, positively or negatively, in terms of attraction.

Synthetic lures that incorporate human skin microbiota have been tested in both an olfactometer and in semi-field experiments by the Takken group. In the semi-field experiments, carbon dioxide was added to the lure, using either pressurized cylinders or fermenting yeast (17). The use of fermenting yeast is certainly a less expensive source of carbon dioxide, which is important to consider in the low- and middle-income settings malaria interventions will be carried out in. Unpublished work out of the Dimopoulos group also suggests that a certain yeast species

may have some anti-*Plasmodium* effects, which would certainly be beneficial if a field design could incorporate yet another organism to confer refractoriness and simultaneously provide a carbon dioxide source, although further investigation would be needed to first determine if any by-products have attractive or repellent properties and to see if standardization of CO_2 flow would be possible.

Considering our potential field use for sugar traps that incorporate the anti-*Plasmodium* bacteria *Esp_Z, Serratia,* or *Chromobacteria*, we were interested in recreating the results generated by the Takken group to identify human skin bacteria species that attract mosquitoes and that could be incorporated into such lures, enhancing the likelihood of a female *Anopheles* mosquito to take a sugar meal that would confer refractoriness to *Plasmodium*. Here, we hope to demonstrate the real-world feasibility of biological control mechanisms based on naturally-occurring microbes.

Materials and Methods

Investigation of *Esp_Z* introduction on mosquito longevity and fecundity

Survival

In order to determine if feeding *Esp_Z* to *Anopheles* mosquitoes would result in fitness costs, we examined a couple different aspects, including obvious measure of lifespan and fecundity. *An. gambiae* were fed 10% sucrose containing pentamycin, streptomycin, and gentomycin for two days upon emergence to remove any bacteria already in the midgut as a result of rearing. They were then switched to a diet of sterile 10% sugar for another two days to clear the antibiotics. The adapted strain of *Esp_Z* was cultured overnight in 3 mL LB broth, as were 12 bacterial species with no anti-*Plasmodium* effects also isolated from the microbiome of *Anopheles*, as described by previous work in the Dimopoulos group (unpublished data) (Table 1).

ID	Species	Gram
A1	*Exiguobacterium duranticum*	+
A3	*Kocuria sp.*	+
A4	*Pantoea sp.*	-
A5	*Knoellia sp.*	+
A6	*Staphylococcus saprophyticus*	+
A8	*Bacillus cereus*	+
A13	*Pseudomonas sp.*	-
A16	*Bacillus pumilus*	+
A17	*Comamonas sp.*	-
A18	*Arthrobacter sp.*	+
A20	*Acinetobacter sp.*	-
A24	*Pseudomonas putida*	-

Table 1. List of bacterial species isolated from *Anopheles* midgut and used as positive control "cocktail."

The twelve overnight cultures of the microbiota species that do not interact in any way with the parasite were all diluted to an OD_{600} of 1.0 and combined in a 1.5 mL microcentrifuge tube into a "cocktail," at a volume of 10 μL each, to a final volume of 120 μL. The *Esp_Z* overnight culture was also diluted to an OD_{600} of 1.0 and an equal volume (120 μL) transferred to a 1.5 mL microcentrifuge tube. Both the bacterial cocktail (referred to henceforth as CK) and *Esp_Z* were pelleted by centrifugation, re-suspended in 3% sucrose, and diluted to a final bacterial concentration of 10^7 bacteria per mL.

Bacteria were fed to the mosquitoes either via a sugar or a blood meal. Female *Anopheles* were sorted into cups in three groups per replicate, with approximately 75 individuals in each up. One cup was fed on *Esp_Z*, one cup was fed on CK, and the third cup was fed an aseptic meal. For sugar-feeding, 3 mL of the 3% sucrose + bacteria suspension or aseptic 3% sucrose were transferred to a 1.5 mL microcentrifuge tube in 1 mL increments, with a piece of cotton added in between and saturated. These tubes were inserted into the side of the cups to allow the mosquitoes to feed. Sugar-fed mosquitoes were also given a sterile blood-meal at day 4 post-feeding. For blood-feeding, the bacterial suspensions were introduced into a mix of 40% plasma, 60% blood. Using glass membrane feeders and a 37º water bath, mosquitoes that had been starved for several hours were allowed to feed on the blood/plasma mixture, spiked with CK or *Esp_Z*, or alternatively an aseptic blood meal.

For each group, mortality was logged every 24 hours and survival curves were constructed using the Graphpad Prism software.

Fecundity

Female *Anopheles* mosquitoes were sorted into groups of three cups and fed on either *Esp_Z*, CK, or no bacteria, in a blood/plasma mixture or in 3% sucrose. Sugar-fed mosquitoes were given a sterile blood meal at day 4 post- sugar feed to induce a gonotrophic cycle. Mosquitoes were then sorted into individual test tubes with dampened filter paper and cotton in the bottom. The number

of eggs laid by each mosquito was recorded for up to 72 hours post blood-meal. If a mosquito had not laid eggs by this point, the number of eggs was recorded as zero. The eggs that were laid were flooded into larvae trays in deionized water, using fish food as a food source, and allowed to hatch. Larvae were counted in order to determine hatch rates

Midgut Colonization

Esp_Z was sugar-fed to septic mosquitoes in 3% sucrose and midguts were dissected every 24 hours for five days. Trizol was used to extract RNA from the decimated midgut samples and the RNA was DNAse treated. The total RNA concentration was measured and this was used to synthesize cDNA. Using a gradient of concentrations of cDNA as the template, qPCR was run to determine the relative presence of *Esp_Z* compared to the total bacterial load as measured by 16s rRNA-specific primers and *Esp_Z*-specific primers. Three biological replicates were performed for this entire process.

In Vivo Anti-Plasmodium Effects

To investigate if the adapted strain of *Esp_Z* successfully controlled *Plasmodium* infection when fed to the mosquito in sucrose prior to the administration of the infectious blood meal instead of concurrently, female mosquitoes were first fed on antibiotics at day 0, followed by sterile sugar at day 3. At day 5, these mosquitoes were fed on the bacteria cocktail in three percent sucrose, followed by feeding on *Esp_Z* in 3% sucrose at day 7. The bacteria cocktail was fed first to reconstitute a microbiota that the *Esp_Z* would have to compete with to colonize the midgut. Three days later, mosquitoes were given a *P. falciparum* infected blood meal. After one week, midguts were dissected and stained with mercurochrome so that oocysts could be visualized and counted.

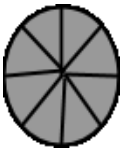
Mosquito Olfaction Investigation

Design of the Olfactometer

As this was the first project in the Dimopoulos lab to investigate mosquito olfaction, it was necessary to design and construct an olfactometer. Traditionally, a "Y-shaped" design has been common in dual-choice assays. While some experts maintain that this design does not work well for an *Anopheline* model, as it does not allow the insect to behave in its usual manner, more complex wind-tunnel box designs require more investment and technology, such as those used to monitor flight paths. Our laboratory-reared mosquitoes have also been reared in small cages for thousands of generations, suggesting that a smaller environment has become more natural to these populations versus mosquitoes in nature. While our design may improve or change moving forward, for the basic needs, a Y-shaped olfactometer would provide the data for proof of principle that would lead to future studies with more complex designs.

The olfactometer was built using two pieces of 6" translucent PVC piping, a Y-connector, two pieces of 4" translucent PVC piping, and four blast gates that can be opened and closed at will. Steel wire was used to hold the various pieces together. The two Y-arm ends were covered with nylon that was tied off in a knot to allow aspirator access to the mosquitoes that had gathered on a given side. The large opening at the opposite end was fitted with mesh that could be removed at will so temporarily knocked-down mosquitoes could be placed into the first olfactometer chamber (Figure 4).

This entire setup was placed into an environment-controlled room to emulate a tropical environment. The room used was part of the school's old insectary, as this area experiences much less foot traffic than the rooms in the current insectary, and we wanted to control for external odors and changes in air flow as much as possible.



(Upstream fan not pictured)

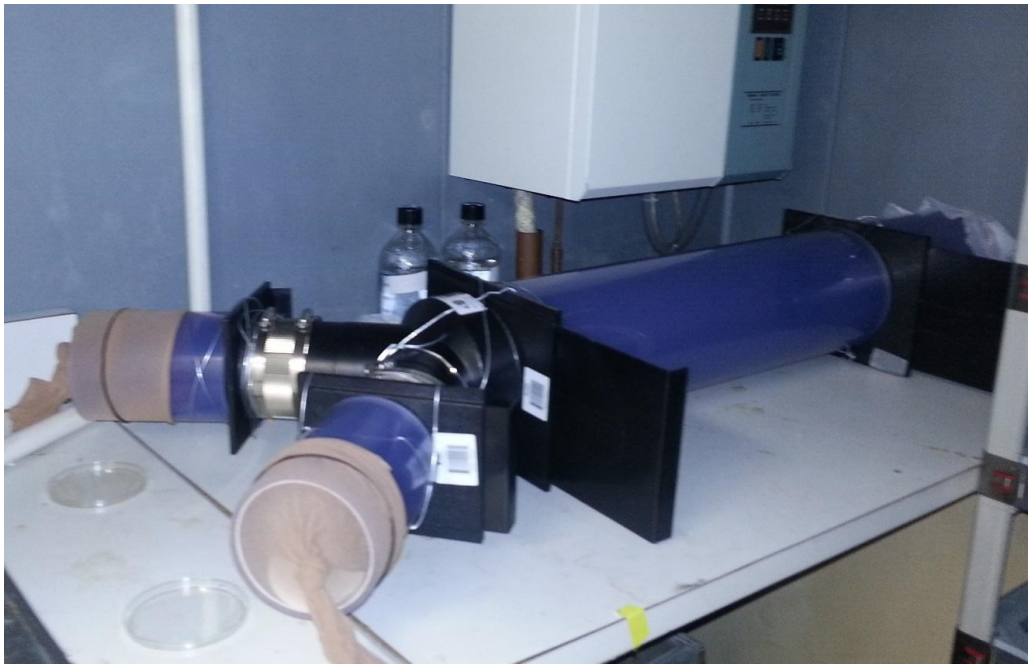


Figure 4. Olfactometer set up

Initial Olfactometer Investigations

To assess whether or not there was an inherent difference between the two arms, female mosquitoes were knocked down on ice and released into the first olfactometer chamber with all blast gates closed. Once they had reanimated, the blast gates were opened and they were allowed to fly throughout the system without bait in either side. After a period of several hours, mosquitoes were aspirated out of either Y-arm and counted. This was repeated in four replicates to help account for potential external variables.

The same process was also done with a fresh blood agar plate in one arm and no bait in the other, to ensure that attraction to a bait could be observed. In the replicates of the assays using a blood agar plate, the plate was swapped between the two arms to help control for any unobserved inherent differences in the system.

Sampling Human Foot Microbiota Isolates

An LB agar plate was pressed against various parts of a person's foot, directly transferring bacteria from the skin. Three lab members were used to sample foot microbiota isolates, attempting to represent both genders and an age range. Similarly to the previous Takken work, however, it was not possible to represent as many different groups as would have been ideal. These plates were incubated at 37º C overnight. Samples were taken over the course of several days. From the plethora of bacteria on the initial LB plates, phenotypically distinct colonies were selected and plated onto individual LB agar plates. This resulted in eight phenotypically distinct bacteria to be used for investigation. While there were likely a much greater number of species present, only those that were very apparently different from others were selected.

Individual bacteria species were grown on blood agar plates overnight at 37º C. This medium was chosen to emulate the Takken group experiments. A control plate was also incubated at the same temperature overnight. Each plate was placed at one end of an arm of the olfactometer and the blast gates #3 and #4 were opened to a width of 2 cm. This was to create a "lobster trap" design, where a small opening allowed inward flight but made exit from the olfactometer chamber more difficult, trapping most of the mosquitoes that entered the specific olfactometer chamber. The two large blast gates were initially set to a closed position. A fan was set up at the Y-end of the olfactometer, to create a current that would carry odors in the direction of the first chamber. Because of the limited space in the room, the distance between the fan and the olfactometer was not able to be manipulated easily, and the current created may not have been ideal.

Mosquitoes were sorted by sex, and 100-300 non-blood-fed female mosquitoes aged three to seven days post-eclosure were released into the first olfactometer chamber and allowed to recover from being knocked down on ice. As olfactory receptor neurons are mainly located in the

antennae and maxillary palps, special care was taken while sorting mosquitoes so that no damage was done to these organs While previous studies on which this investigation was based used far fewer mosquitoes, an increased statistical power may somewhat offset the reduced complexity of our system. Once the majority had reanimated, the two larger blast gates were opened and the room was left undisturbed for a period of three hours. After this time, blast gates were again closed and the number of mosquitoes in each arm was counted. Each isolate was tested in three or four biological replicates, and the arm containing the bacteria plate versus the control plate was alternated to control for any inherent variation in the system.

Inherent attractive properties of the three bacteria shown to have anti-*Plasmodium* effects were also begun employing a similar experimental design, except growing the species on LB agar plates.

1-Octen-3-Ol

1-Octen-3-Ol is a well-known mosquito attractant derived from cattle odours (12). A Takken paper published as early as 1997 elucidated this compound as an attractant for *Anopheles* mosquitoes, although there was differential attraction across species (12). Because of an interesting attractive property found in one of the examined human skin bacteria, the known attractant was placed into the previous set up, such that one arm of the olfactometer contained a control plate, the other a plate with bacteria, and both arms contained a piece of filter paper with 3 µL of 1-octen-3-ol, set just behind the plate. Preliminary experiments with this added component resulted in an interesting result, so it was decided to repeat the assays using the bacteria that had shown no difference in attractiveness with the addition of 1-octen-3-ol.

Although it had been previously demonstrated that *An. gambiae* do not respond well to 1-octen-3-ol alone, it was important to confirm these findings in our own system based on the results observed after undertaking the above experiments. To do so, a piece of filter paper with 1 µL of the alcohol was placed in one arm of the olfactometer, and a piece of filter paper with 1 µL

of water placed in the other. Mosquitoes were allowed to disperse for 24 hours and were then counted in each arm, with the remainder of those not in one of the two end chambers taken to be in the main tube.

Identification of Bacterial Species With Interesting Properties for This Study

Bacterial isolates from the foot that showed any pattern of attraction were identified by sequencing. 16s rRNA was amplified via PCR and the product was purified and cloned into competent *E. coli* cells. A plasmid miniprep was performed and the resulting plasmid was sent to the JHMI sequencing facility. Results were entered into the NCBI BLAST (Basic Local Alignment Search Tool).

Results

Investigation of *Esp_Z* colonization on mosquito longevity and fecundity

There was no statistically significant difference in the survival rates between the aseptic group and the group fed *Esp_Z* via a sugar meal. There was, however, a statistically significant difference between the CK and the *Esp_Z* fed mosquito groups, as well as between the aspectic and CK groups, with the lifespan of the CK-fed group below both of the other two groups (Figure 5). Interestingly, a different pattern of survival was observed when the bacteria were introduced into the mosquito midgut via a blood meal. At this condition, the *Esp_Z* group had a statistically significantly reduced lifespan compared to the CK and aseptic groups, which were not significantly different from one another (Figure 6).

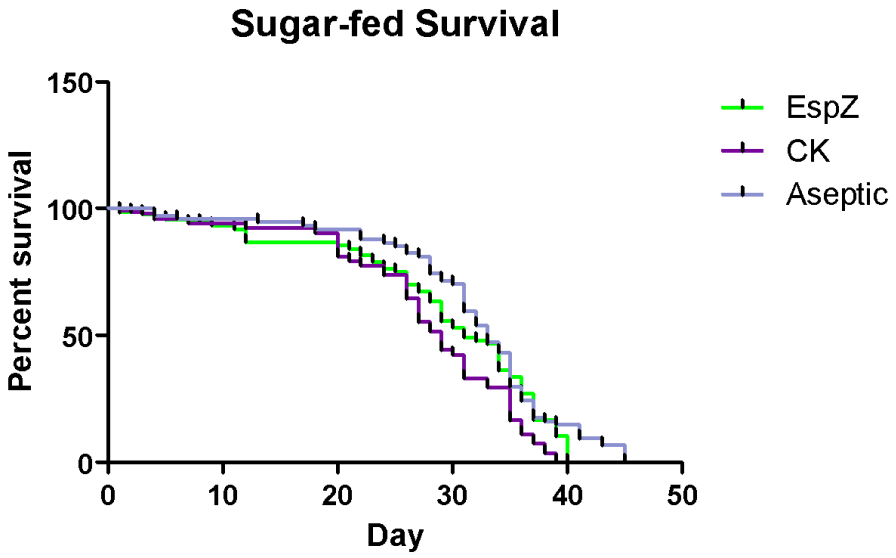


Figure 5. Survival analysis of *An. gambiae* fed on either sterile or bacteria-spiked 10% sucrose

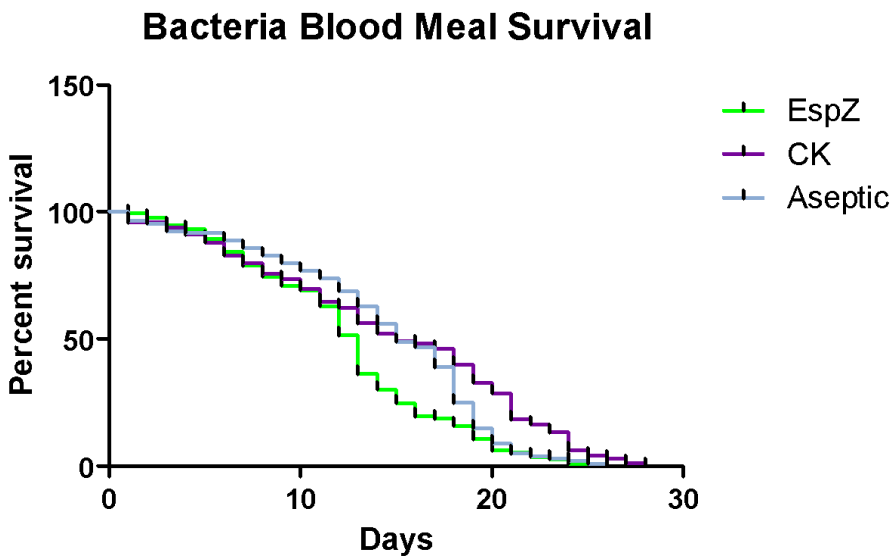


Figure 6. Survival analysis for female *An. gambiae* that had fed on aseptic or bacteria-spiked blood and then maintained on 10% sucrose.

For the fecundity assays, when the bacteria were introduced to female mosquitoes via a sugar meal, there were no statistically significant differences in egg deposition between any of the three groups (Figure 7).

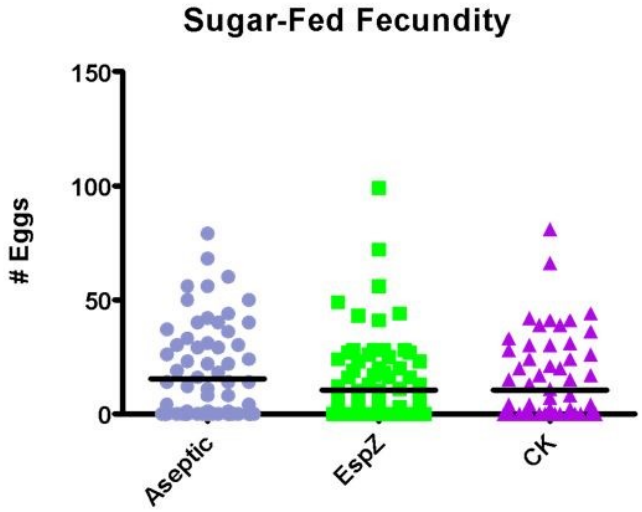


Figure 7. Mean number of eggs laid by female *An. gambiae* fed on aseptic 10% sucrose or 10% sucrose + bacteria, followed by a sterile blood meal.

When bacteria were introduced via a blood meal, there was a significant difference in the number of eggs laid between the aseptic group and CK group and an even more significant difference between the aseptic group and *Esp_Z* group. There was not any significant difference between the CK group and *Esp_Z* group. Mosquitoes that had fed on bacteria-supplemented blood displayed a greater fecundity compared to the control group that had fen on sterile blood. (Figure 8).

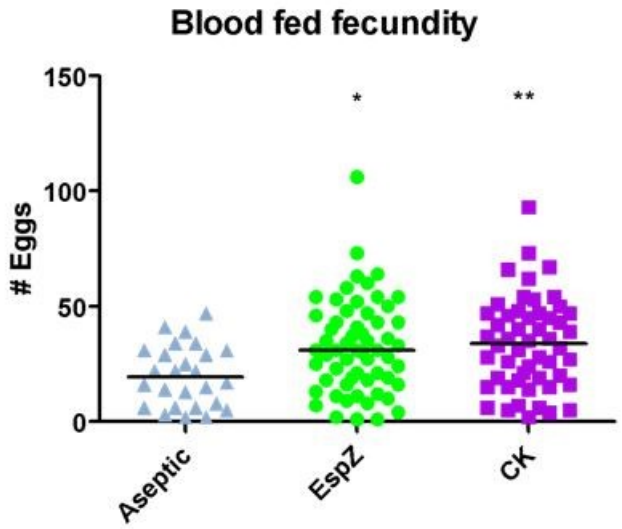


Figure 8. Mean number of eggs laid by female *An. gambaie* fed an aseptic blood meal or a blood meal spiked with bacteria, then maintained on 10% sucrose.

Hatch rates are also an important indicator of fecundity, as laying unviable eggs is also a reduction in the fitness of an individual. The hatch rates tended to follow the same pattern as the fecundity assays, with the blood-feeding-mediated bacterial introduction groups showing higher hatch rates overall compared to the sugar-feeding-mediated bacterial introduction groups. The *Esp_Z* group had a slightly higher hatch rate than the aseptic group in both the sugar-feeding-mediated and the blood-feeding-mediated bacterial introduction groups, while the CK group had a higher hatch rate than the aseptic group when introduced by sugar-feeding, but a comparable hatch rate when introduced by blood-feeding (Data not shown).

Midgut Colonization

Although the results of each replicate differed slightly, it was found that *Esp_Z* fed to female mosquitoes persisted in the midgut up to 96 hours after the bacteria was ingested. At this time point, the percent of *Esp_Z* relative to the total bacteria load was approximately one percent. The highest documented percentage, at 24 hours post-ingestion, was about ten percent (Table 2). In the representative table, there is no detected *Esp_Z* at 72 hours after ingestion although there is at 96 hours post-ingestion. This is likely due to an error in the RNA extraction or subsequent process instead of truly reflecting the absence of *Esp_Z*. All percentages shown represent the mean of three technical replicates.

Time Point	***Esp_Z*: Total bacteria**
24 h	9.974%
48 h	7.878%
72 h	N/A
96 h	1.198%
120 h	N/A
168 h	N/A

Table 2. Representative qPCR results indicating the ratio of *Esp_Z* to total bacteria load in the mosquito midgut after it was fed to *An. gambiae*. N/A indicates that there was no detected *Esp_Z* at that time point.

Anti-Plasmodium Effect of Adapted Esp_Z Strain

Although only one replicate was performed before this aspect of the project was taken over by someone else, the results are still worth presenting and discussing. There was no statistically significant difference between the average number of oocysts per midgut between the adapted *Esp_Z* colonized and control groups. However, there does appear to be a slight reduction in the adapted *Esp_Z*-fed group (Figure 9). Important to note is the difference in the number of "zeroes," or midguts without any oocysts. In the aseptic group, only two midguts appeared to have an absence of oocysts after the infectious blood meal. Comparatively, oocsysts were not found in nine midguts of the *Esp_Z*-fed group. If several seeming outliers in the *Esp_Z*-fed group are removed, the difference in means becomes much more evident. These potential outliers will be addressed further in the discussion.

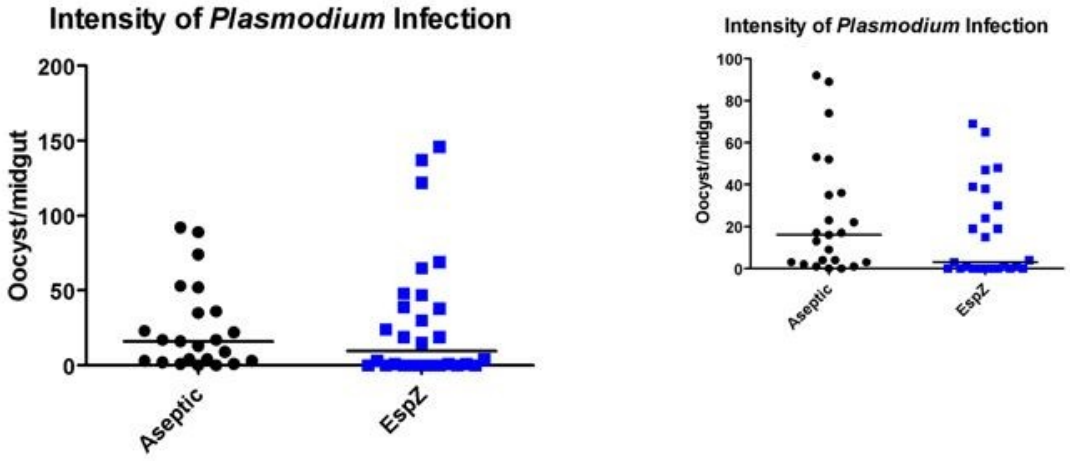


Figure 9. Number of oocysts per midgut in female *An. gambaie* that had been colonized with *Esp_Z* via sugar feeding bacteria, and then blood fed *Plasmodium* parasites, compared to a control group given a sterile sugar meal followed by infectious blood meal. The smaller inset graph demonstrates the same results, with the three potential outliers removed.

Mosquito Olfaction Investigation

Initial studies to investigate the olfactometer set-up employed demonstrated no significant or noticeable differences between the two end olfactometer chambers without a bait, but a noticeable attraction when a bait of a blood agar plate was placed in one olfactometer chamber (data not shown), indicating that the system's basic function is acceptable for our purposes.

Most of the foot bacterial isolates that were examined did not show any significant difference in attraction compared to the control (Figure 10). These results are represented using a low-high graph, with the bottom line of the box showing the lowest replicate percentage, the top line of the box showing the highest replicate percentage, and the middle line representing the overall mean. The variation tended to be greater in the bacterial groups, but the means were nearly identical. One may notice that the percentage means for the various control groups ranges from about ten percent to about thirty percent. This may indicate differences such as length of starvation prior to experimental set up, which although always within a few hours range, sometimes varied slightly as a result of experiment scheduling. Resulting discrepancies may have occurred in dispersal, or even host-seeking behavior, and changes in olfactometer chamber air flow or other fluctuating conditions may have also had some influence.

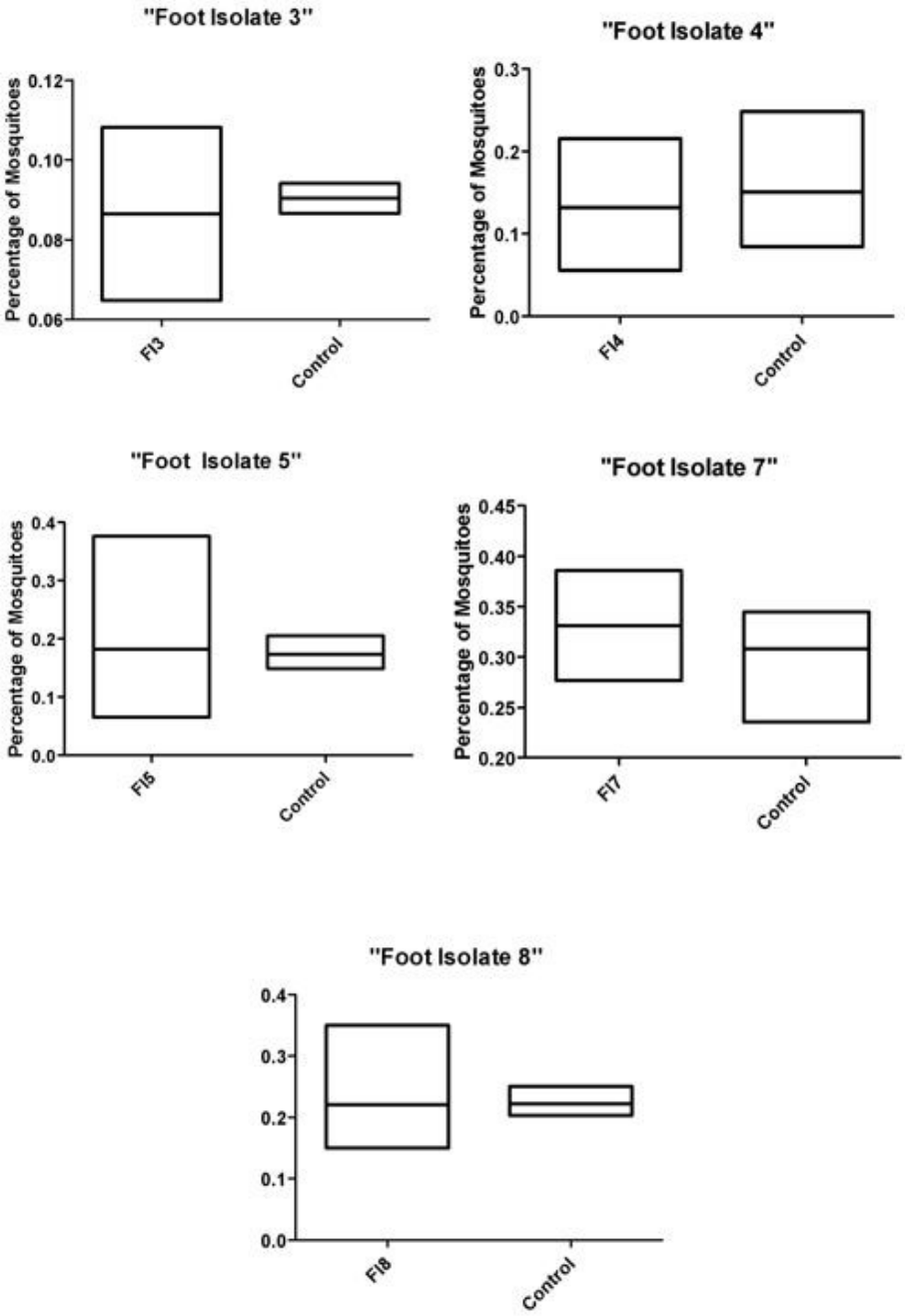


Figure 10. Five foot isolates that demonstrated no mean difference in attraction compared to a control. Graphs are low-high graphs, with the upper arm of the box indicating the highest replicate, the lower arm of the box indicating the lowest replicate, and the middle arm representing the mean.

One particular foot isolate did show a repellent effect (Figure 11), with fewer mosquitoes attracted to the bacteria olfactometer chamber versus the control olfactometer chamber in all replicates. Although the difference in means was not statistically significant using a t-test for analysis, at a p-value of 0.07, it is near-significant and represents an approximate ten percent reduction in attractiveness from the control group. Keeping in mind that statistical significance does not always correlate with biological significance, this result was taken to be meaningful. Because of this interesting difference in behavior, Foot Isolate 1 was sequenced as described in the methods section. BLAST analysis of the 16s rRNA sequence of Foot Isolate 1 demonstrated that it was a *Bacillus* species.

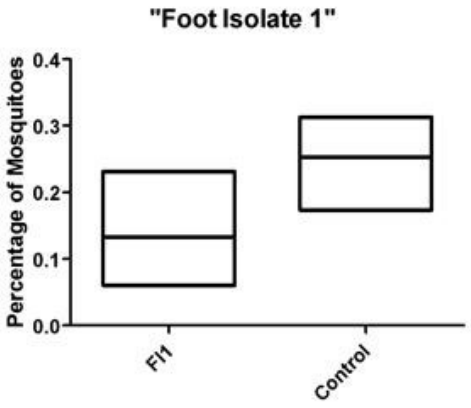


Figure 11. Arbitrarily named, "foot isolate 1," demonstrated a repelling effect, representing an approximate 10% reduction in attraction compared to the control (p-value= 0.07). This microbe was determined to be a *Bacillus* species.

"Foot isolate 1," determined to be a *Bacillus*, was the first tested in the olfactometer in conjunction with 1-octen-3-ol to determine if the inhibitory effects observed could offset the attractive effects of the organic attractant. Since the species used was *A. gambiae,* the attraction to 1-octen-3-ol was known to be rather mild, so we hypothesized that a 10% repellent effect should be sufficient to offset the level of attraction to the organic attractant bait. When these two cues were placed together, there was an interesting reversal of the previously observed trend.

Namely, there was a large increase in attraction to the bacteria/1-octen-3-ol olfactometer chamber compared to the control/1-octen-3-ol olfactometer chamber (Figure 12).

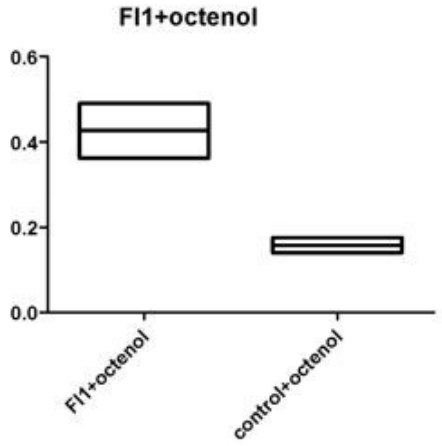


Figure 12. Comparison in attraction between Foot Isolate 1 on blood agar + 1-octen-3-ol and a control blood agar plate + 1-octen-3-ol. A marked synergistic increase in attraction is observed between the microbe and the alcohol.

When 1-octen-3-ol was similarly added to an olfactometer chamber containing a blood-agar plate growing "Foot Isolate 4," and to an olfactometer chamber containing a sterile control plate, there was, again, an observed increase in attraction to the olfactometer chamber containing the bacteria versus the olfactometer chamber containing the control (Figure 13). The control olfactometer chamber also corroborated the previous finding that the attraction to 1-octen-3-ol by *An. gambiae* is relatively weak (12), especially considering that some attraction does occur to the blood agar plate itself, which may account for a good portion of the 10-18% of mosquitoes attracted to the control olfactometer chamber, in conjunction with random dispersal.

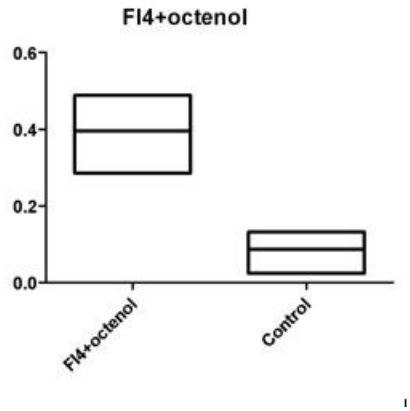


Figure 13. Comparison in attraction between Foot Isolate 4 on blood agar + 1-octen-3-ol and a control blood agar plate + 1-octen-3-ol. A marked synergistic increase in attraction is, again, observed between the microbe and the alcohol.

Experiments to confirm a lack of inherent attraction to 1-octen-3-ol compared to a control of deionized water showed no significant difference between the two groups, as expected given the published findings from the Takken group (12) (data not shown).

Discussion

Investigation of *Esp_Z* influence on mosquito longevity and fecundity

Both the survival assays and the fecundity investigations demonstrated no apparent negative effects of sugar-feeding-introduced *Esp_Z* to female mosquitoes. This supports the potential use of such a bacterium as biological control, in the absence of negative selection. Some fitness costs were observed when bacteria were introduced via a blood meal, but this does not reflect the imagined realistic field application, as bacteria would be introduced via artificial nectar. Before any use in field studies, the amount of bacteria that would be fed in sugar-feeding stations should be optimized.

It is worth considering that the bacteria may have been back to the mosquitoes at a concentration that overwhelmed the innate immune system. In the previous studies that demonstrated a dose-specific response, a concentration of 10^4 CFU/midgut was enough to confer refractoriness (9). While the aim was to feed the bacteria at a higher concentration in the hopes that it would better colonize the midgut, there is a cost-benefit ratio that must be carefully considered. Costs may be more evident in the blood-fed groups since taking a blood meal initiates a gonotrophic cycle, potentially diverting some of the mosquito's resources away from the immune system, allowing the massive influx of bacteria to go partially unchecked.

Interesting as well is the increased fecundity in the two bacteria fed groups and the reduced hatch rate in the sugar-feeding- versus blood-feeding-mediated bacteria introduction groups. This suggests an influence of the natural microbiota of the mosquito on the gonotrophic cycle. While this difference in fecundity was not observed in the group that had been exposed to bacteria via sugar-feeding followed by an aseptic blood meal, it is of note that many more "zeroes" existed in this experimental set-up, versus mosquitoes fed bacteria directly through a blood meal. These observations taken together may suggest that colonization of the midgut is

inefficient at the several days post bacteria spiked sugar meal to properly play its role in reproduction, or that the presence of blood helps to more efficiently colonize the midgut.

Although the one replicate of the anti-*Plasmodium* investigations did not demonstrate a significant reduction, there are a number of possible explanations why may have happened other than the adapted strain not having the same observed effects as the parental strain. First of all, the observed outliers may be explained by differential sugar-feeding propensity. While blood-fed mosquitoes were sorted from those who did not feed, there was no confirmation of amount of bacteria actually consumed through the sugar-feeding prior to the blood meal. This variance would be addressed with further replications, which were not undertaken here.

While our investigations focused on *Esp_Z*, an ideal baited feeding station may employ a different microbe, or a combination of microbes for a number of reasons. The aforementioned *Serratia* can be vertically transmitted, as well as horizontally transmitted from male to female during mating (Jacobs-Lorena Lab, unpublished data). This may make the spread of the bacterium, and therefore refractoriness to the *Plasmodium* parasite, into the mosquito population occur at a much greater rate than using *Esp_Z* or *Chromobacterium*. *Chromobacterium* has also been shown to have an entomopathogenic potential, which could further enhance its transmission-blocking activity (Dimopoulos Lab, unpublished data). There may also be some inherently attractive or inhibitory properties of these three bacteria that would encourage or discourage feeding from spiked sugar stations, which is the focus of currently ongoing work.

Mosquito Olfaction Assays

A possible design flaw with our olfactometer is the inability to accurately control air flow and recreate identical conditions from one assay to the next. While the rearing room that was used was much less trafficked than the currently in-use insectary, several other lab members conduct experiments in the same room and could have influenced assays to some degree. The fan employed may have helped to direct air flow, but the room itself was also much less closely monitored. Considering this, it is possible that slight variations in the external environment may have had some impact on the consistency of the olfactometer results.

One consideration to make when evaluating the results of the microbiota attraction assays is the lack of standardization in bacterial concentration. Because these experiments were designed to be crude initial investigations, bacteria was streaked out to cover the plate as best as possible, but optical density or CFU normalization was not undertaken for the first set of investigations. Takken et. al (2011) reported that, the concentration of bacteria may have an effect on observed attractiveness to *A. gambiae*, so it is possible that our findings may over-or under-estimate attractiveness. Regardless, these experiments provide a preliminary proof of principle for future investigations.

Two of the initially isolated skin microbiota were not easily grown on blood agar plates and were, thus, eventually excluded from investigation because of time constraints, which accounts for this paper only reporting results for six of the aforementioned isolates. The previous studies by Takken's group found several more genera with attractive properties, and this may be a result of a more detailed and thorough study using a much greater sample size (15). Another consideration in this vein is the period of incubation for the bacterial plates. Because of various constraints, our bacterial plates were generally incubated for 18-20 hours before being introduced into the olfactometer. At this point, active growth may have slowed to the point where volatiles are not being as readily produced and attraction/repellence is not as apparent. The experimental

design of Takken et al. (2009) elucidated the identity of bacterial species before undertaking investigations. In contrast, our approach was to first evaluate for attractiveness and then identify bacterial species that induce vector behavior.

Although somewhat surprising that no bacterial isolate tested on its own demonstrated attractive properties, the response to "foot isolate 1," subsequently determined to be a *Bacillus* agrees with earlier findings from the Takken group that some skin-isolated bacteria has inhibitory effects in mosquito olfaction/attraction (15). Noting that the studies out of this group did not investigate the effects on attractiveness relative to abundance for any *Bacillus* species, this inhibitory effect may be worth pursuing further, as one can imagine a lotion or cream that alters microbiota as a natural repellant. The *Bacillus* that we isolated is not necessarily the same as that discussed in the Takken group paper, but further investigation may be warranted regardless.

The DNA sequence analysis using BLAST showed that there was no perfect match to a known, common *Bacillus* species, yet enough sequence similarity was present to definitively categorize "Foot Isolate 1" into this genus. Future directions include analyzing volatiles and compounds produced by this isolate in order to better understand exactly what is producing the observed inhibitory effect. Many common *Bacillus* species produce biologically important compounds and volatiles, including some with mosquito control applications (19). While likely not directly related, *Bacillus*-derived toxins have been employed as larvicides, and there may be an evolutionary reason for the aversion on the part of *Anopheles* to bacteria of this genus in general (19). Considering the lack of previous studies on this genus' role in mosquito attraction to humans, further investigation is warranted.

The observed synergistic effect noted between foot microbiota and 1-octen-3-ol is as yet unreported. Since these experiments utilized *A. gambiae* it is initially somewhat surprising that there would be such a response to this attractant. The control olfactometer chamber certainly demonstrated a slight attractiveness, but this paled in comparison to the bacterial olfactometer

chamber. Mosquito olfaction systems are complex pathways comprising tens of thousands of receptors and various signal transduction cascades. Because of this, it is possible for two odorants to show no attraction independently, yet have a synergistic attractive effect, and it is well documented that a mixture of two or more odorants can produce a non-additive receptor code.

Although much more investigation would be necessary to make this claim, it is certainly possible that due to the close relationship between humans and livestock and the often close quarters in which they are housed in low and middle income countries, large mammal volatiles could potentially act as secondary cues for host-seeking behavior of *An. gambiae*. Future directions also include elucidating attractive versus repellent effects for the other isolates from the foot in conjunction with the octenol to investigate whether this synergistic result is observed across all isolates tested.

Five to ten mosquito generations may be sufficient to shift host preferences, indicating that mosquito bait systems could impose a selective pressure if not late-acting. Baits that would employ the use of a cocktail of microbiota and other attractants along with a microbe to confer *Plasmodium* refractoriness should, then, be thought of and designed more as feeding stations that do not harm the mosquito in any way to ensure that the attraction would not change over time. This ease in change of feeding preference also points to the issue of the fact that the mosquitoes used for all experiments were lab-reared, and derive from a long lineage of lab-reared mosquitoes. Mosquitoes are maintained with 10% sucrose in their cage and never have to forage for a blood meal. The evolutionary pressures have, thus, changed, and with each subsequent generation, they increasingly diverge genetically from those mosquitoes first caught and brought to establish the colony. Particularly with something as malleable as olfaction and host-seeking behavior, one must consider that these findings are not yet ready for field application, and multi-

generation lab-based experiments or small-scale field-based experiments would be necessary to confirm the findings and feasibility before any kind of broad implementation.

A further consideration is that our experiments only used *Anopheles* mosquitoes that had not yet been blood-fed. In the field, a female mosquito may take sugar meals throughout its life when a blood meal is not available. The attraction to various nectar sources, however, may change after the first blood meal. As stated before, previous research has demonstrated a differential up-regulation or down-regulation of olfaction genes in response to blood feeding. Changes may also take place in response to infection with the parasite. Future directions may include elucidating changes in response to human skin microbiota following the first blood meal and post *Plasmodium* infection.

Before any field implementation, refractoriness to other strains and even species of human *Plasmodium* parasites should also be evaluated. While *P. falciparum* is responsible for most of the morbidity and mortality in sub-Saharan Africa, eliminating only this species may simply open up a niche for another species of parasite to become more widespread.

Overall, these studies have further affirmed the potential for such a field-employed design. Studies looking into combinations of attractants should continue, as well as investigations into the attractive or inhibitory properties of *Chromobacterium, Serratia,* and *Esp_Z*, which are currently ongoing, as this may also play a role in the decision of the best bacterium or bacteria to use as the biological control agent.

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Alicia C. Majeau

2315 Saint Paul Street. Apt. 102. Baltimore, MD 21218

(508) 340-1403 · alicia.majeau@gmail.com

EDUCATION

Johns Hopkins Bloomberg School of Public Health, *Baltimore, MD*

September 2012- Present

Masters of Science, ScM. Molecular Microbiology and Immunology

Hofstra University, *Hempstead, NY*

August 2008- May 2011

Bachelor of Science, B.S. with High Honors in Biology, Specialization

In Cell and Molecular Biology; Minor in Biochemistry; Summa cum laude

RESEARCH EXPERIENCE

Dimopoulus Laboratory Research Assistant, , *Baltimore, MD*

November 2012-Present

Department of MMI, JHU Bloomberg School of Public Health

- Rear and care for *Anopheles* and *Aedes* mosquitoes
- Conduct both sterile and *Plasmodium*-infected blood feeds
- Double-stranded RNA injections and dissections of mosquitoes
- Isolate various bacteria from the human skin and in conjunction with bacteria known to have anti-*Plasmodium* effects, test level of mosquito attraction or repulsion
- Weekly laboratory meetings and presentations

Microbiology Laboratory Research Assistant, *Hempstead, NY*

September 2010-May 2011

Department of Biology, Hofstra University

- Create gene-deletion mutants in the bacterium *Streptomyces coelicolor,* attempting to identify the unknown protease responsible for cleaving *SapB* from its hyphae to its aerial form
- Design and create necessary plasmids and competent cell lines
- Conduct PCR and verify phenotypes
- Routine laboratory supply maintenance of glassware, media, pipette tips, etc.

Summer Research Intern, *Boston, MA*

June 2010- August 2010

Department of Pathology, Boston University Medical Center,

- Conduct ELISA assays
- Interpret immunofluorescence results using analytical software

VOLUNTEER EXPERIENCE

Uganda Village Project Healthy Villages Intern June 2011- August 2011
Healthy Villages Team Leader June 2012- August 2012

Uganda Village Project *Iganga Distract, Uganda*

- Conduct health needs assessment in rural, underserved villages
- Design and conduct health care initiatives and education sessions
- Partner with sub-county and district officials and various other NGOs to perform public health interventions
- Observe local practices at health centers and the district hospital

LABORATORY SKILLS

Mosquito Rearing, Dissection, Blood Feeding, dsRNA injections, *Plasmodium* Handling, PCR, Production of Competent Cells, Septic and Aseptic Techniques, Broth Culture, Plate Streaking, Nucleic Acid Extraction (DNA/RNA), Western Blotting, Agarose Gel Electrophoreses, Rodent Handling, Buffer and Media Preparation, pH Measurement, Molecular Subcloning

PROFESSIONAL DEVELOPMENT

Computer Skills: Microsoft Office Suite, Prism

Languages: English (Fluent), French (Proficient)

HONORS AND AWARDS/ CERTIFICATES

Member Phi Beta Kappa, Golden Key International Honor Society

Provost's List Scholar & Dean's List Scholar

Distinguished Academic Scholarship full tuition scholarship recipient, Hofstra University

High School Valedictorian, Shepherd Hill Regional High School 2008

Recipient of Bausch and Lomb Honorary Science Award

National Honor Society