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Antibiotic-Mediated Microbiota Depletion of *Aedes aegypti* Gut Bacteria Modulates Susceptibility to Entomopathogenic Fungal Infection and Modifies Developmental Factors

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Abstract

Entomopathogenic fungi are promising alternatives to synthetic insecticides for the control of vector species, notably the arbovirus vector, *Aedes aegypti*. The influence of intrinsic mosquito midgut microbiota on host susceptibility to fungal infection and subsequent physiological processes remains poorly understood. Here we treated female *Ae. aegypti* with the broad-spectrum antibiotic carbenicillin to reduce gut bacterial populations, then exposed them to *Metarhizium anisopliae* conidia. Female *Ae. aegypti* offered carbenicillin and then sprayed with fungi had significantly lower survival rates (38.9% ± 1.15) compared to non-antibiotic-treated mosquitoes sprayed with fungus (68.9% ± 0.58). To monitor the kinetics of microbial community recovery, mosquitoes were challenged with conidia at 0, 3, 6, and 9 days following antibiotic removal from the diet. Reduced survival persisted through the 6-day period (survival rates 37.8% to 45.6%), with a significant increase in survival observed 9 days post-antibiotic removal (58.9% vs. control 63.3%), which coincided with recovery of gut bacterial populations. Additionally, antibiotic and fungal treatments reduced egg production, larval eclosion, and pupal formation. These results demonstrate that gut bacteria contribute to mosquito defense against fungal pathogens and support normal reproductive and developmental functions. Understanding the interplay between gut microbiota and entomopathogenic fungi may enhance biological control approaches.

Keywords: entomopathogenic fungi; *Aedes aegypti*; *Metarhizium anisopliae*; gut microbiota; biological control

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1. Introduction

Aedes aegypti (Diptera: Culicidae) L. (1762) is an arbovirus vector responsible for a range of serious human diseases, such as dengue fever, yellow fever, chikungunya, and Zika, and is therefore considered a significant global public health threat [1–4].

The significant increases in *Ae. aegypti* populations worldwide and the resulting arbovirus epidemics are attributed to this insect's anthropophilic behavior, its preference for breeding sites associated with domestic urban environments, the desiccation tolerance of its eggs, and its highly efficient viral transmission [5–9]. In addition to these biological traits,

factors such as urbanization, the expansion of international trade, and increased travel have further contributed to the difficulty of controlling *Ae. aegypti* populations [10,11].

Since the beginning of the 20th century, chemical insecticides have been used to reduce *Ae. aegypti* populations. However, these compounds have toxic effects on humans and select for resistant mosquito populations. The resistance of this species to synthetic organophosphate and pyrethroid insecticides has been shown in several studies [12–15].

In the search for alternatives to the application of synthetic insecticides, the possibility of using biological control strategies should be considered, with the aim of reducing vector insect populations. Biological control strategies are considered safe for human health and the environment [16]. Entomopathogenic viruses, bacteria, and fungi have demonstrated high virulence against disease-vectoring insects [17–20]. Among these, the bacterium *Bacillus thuringiensis* var. *israelensis* (Bti) is widely used to reduce populations of *Ae. aegypti* larvae on a large scale [21]. However, because Bti must be ingested to be effective, its use is limited to larval control [22–25].

Entomopathogenic fungi (EPF) are promising candidates for mosquito control [26–29], with a major advantage being that EPF do not require ingestion to infect their hosts [30]. While some fungi can infect via ingestion [31], they typically penetrate the host through the integument [32,33]. This mode of infection enables fungi to target all stages of insect development, from eggs to adults [32].

Metarhizium anisopliae (Metchnikoff) Sorokin, 1883 (Hypocreales: Clavicipitaceae), and *Beauveria bassiana* (Bals.-Criv.) Vuill., 1912 (Hypocreales: Cordycipitaceae), are highly virulent to *Ae. aegypti* [18,27,29,34–36] and *Anopheles* sp. larvae [37]. Furthermore, a recent study demonstrated the virulence of *M. anisopliae* against *Ae. aegypti* pupae when applied in the form of blastospores, significantly reducing survival compared to conidial applications [38]. Several studies have been carried out on the infection of adult mosquitoes, showing that *M. anisopliae* is also highly virulent against this stage of the insect's life cycle [28,39–41].

Fungal infections are known to alter various physiological aspects of their insect hosts. For instance, infection with *M. anisopliae* reduces both blood-feeding propensity and fecundity in *An. gambiae* [39]. Similarly, *B. bassiana* infection decreases blood-feeding propensity in *Ae. aegypti* [42] and *Anopheles* females, and also impairs the maturation of the protozoan *Plasmodium chaboudi* [43].

In insects, a wide variety of bacterial species are present in the gut [44,45], playing an important role in the physiology of the host—for example, assisting in the process of blood digestion and fecundity [46], in the defense of the insect against natural enemies [47], and during interactions with pathogens such as DENV [48–50].

Studies have shown varying effects of gut microbiota on susceptibility to fungal infection. In female *Anopheles stephensi* (Diptera: Culicidae), removal of the intestinal microbiota with antibiotics made mosquitoes less susceptible to *B. bassiana* infection [51]. In contrast, reducing intestinal bacterial populations in the cockroach *Blattella germanica* (Ectobiidae: Blattodea) increased susceptibility to oral infection by *M. anisopliae* [52]. In oriental fruit flies (*Bactrocera dorsalis*, Tephritidae: Diptera), both axenic and non-axenic individuals were equally susceptible to *M. anisopliae* infection [53].

Little is known about how the intestinal microbiota influences the susceptibility of *Ae. aegypti* females to fungal infection, or about the effects on the development of immature stages following exposure of gravid females to antibiotics and fungi. Therefore, we aimed to test the hypothesis that depletion of the gut microbiota increases the susceptibility of adult *Ae. aegypti* females to infection by the entomopathogenic fungus *M. anisopliae* and negatively impacts reproduction and development.

2. Materials and Methods

2.1. Mosquito Collection and Maintenance

Adult *Ae. aegypti* used in this study were reared from eggs collected directly from the field, as these were considered fitter and more representative of natural populations than more homogeneous laboratory-reared mosquitoes. Eggs were collected using ovitraps, which consisted of black plastic plant pots (12 cm in width × 15 cm in height) with four wooden strips or paddles (3 × 12 cm) placed vertically within the pots, providing highly conducive landing platforms for gravid, ovipositing females. Approximately 300 mL of tap water was added to each ovitrap before placing it outdoors at sites protected from rain and direct sunlight, close to the university insectary (latitude: −21°45′8.17″ S; longitude: −41°19′49.58″ W).

After 5 days, the paddles with eggs were collected and dried at room temperature for 48 h. To initiate egg hatching, the paddles were submerged in water, and the emerging larvae were maintained in plastic trays (approximately 100 larvae per 100 mL) and fed on freshly ground, autoclaved commercial fish food (Nuvilab, São Paulo, Brazil; 0.05 g/L). Pupae were separated into water-filled beakers and transferred to cages before adult emergence. Adults were maintained in cages with 10% sucrose wick-feeders. Recently emerged (2–3-day-old) females that had been maintained in cages with males were initially anesthetized using a stream of CO_2 and then placed on top of a glass plate maintained at a temperature of approximately 6 °C using ice packs underneath the plate. The mosquitoes remained in a dormant state for a maximum of 3 min. This allowed the separation of males and females with the aid of an LED-illuminated magnifying glass.

2.2. Fungal Isolate

Metarhizium anisopliae (isolate ESALQ 818) was obtained from the University of São Paulo Agricultural Faculty (ESALQ) in Piracicaba, São Paulo, Brazil. This isolate has been previously shown to be highly virulent to adult *Ae. aegypti* [54].

2.3. Preparation of Conidia

All of the following methods were carried out using a sterile flow hood and with materials that had been previously autoclaved (20 min at 121 °C). *Metarhizium anisopliae* was initially cultured on Sabouraud dextrose agar (SDA: dextrose 10 g, peptone 2.5 g, yeast extract 2.5 g, agar 20 g in 1 L H_2O). SDA was autoclaved for 20 min at 121 °C before use. Fungi were then cultured for 15 days at 27 °C in Petri dishes (9 cm diameter). Conidia were harvested from the solid media using a spatula and suspended in 5 mL of 0.01% aqueous Tween 80 (TW). This suspension was used to inoculate 25 g of sterile parboiled rice (autoclaved as above) + 10 mL sterile distilled water in a 250 mL conical flask. The flasks were incubated at 27 °C for 15 days before the inoculated rice was transferred to brown paper bags (18 × 9 cm; Fasapel Ltd., São Paulo, Brazil), and humidity was reduced using a forced-air incubator at 34 °C for 24 h before harvesting the conidia using an MR-5 Mycoharvester® (Mycoharvester Company, London, UK). Dry conidia were suspended in 0.01% aqueous Tween 80, and the concentration was estimated using a Neubauer hemocytometer. On average, 0.1 g of dry conidia was equivalent to approximately 5×10^8 conidia mL^{-1}. Viability tests were carried out by plate counting, and only batches with >90% germination were used in experiments.

2.4. Antibiotic Treatments

For experiments where mosquitoes were treated with a broad-spectrum antibiotic, 3-day-old *Ae. aegypti* females were offered carbenicillin disodium salt (C1389 Sigma-Aldrich©, St. 171 Louis, MO, USA), abbreviated as Cb, in wick-feeders at a concentration

of 200 μg mL^{-1} dissolved in a 10% sterile sucrose solution (abbreviated as S) for 3 days before subsequent treatments. Control insects were offered a 10% sterile sucrose solution without Cb for 3 days. Carbenicillin was chosen here because a previous study showed that this antibiotic was efficient in reducing populations of culturable bacteria in *Ae. aegypti* midguts [46].

2.5. Evaluation of Aedes aegypti Mid-Gut Bacterial Populations Following Antibiotic Treatment

All materials and solutions used in these experiments were autoclaved at 121 °C for 20 min or sterilized with 70% ethanol for dissection of *Ae. aegypti* female midguts and cultivation of intestinal bacteria for colony-forming unit (CFU) counts. Before the insects were dissected, their external surfaces were sterilized by submersion in 70% ethanol for 1 min, followed by immersion in 1% sodium hypochlorite solution for 1 min. The mosquitoes were then immediately rinsed in three consecutive phosphate-buffered saline (PBS) solutions. The following methodology was adapted from the protocol developed by Gaio et al. [46].

Mosquitoes were dissected in a glass cavity slide with 100 μL of sterile PBS added to the well. The midgut was removed using fine forceps with the aid of a magnifying glass. Individual dissected midguts were transferred to 1.5 mL Eppendorf tubes containing 100 μL of PBS and macerated with a pestle. Then, 100 μL of liquid BHI medium (brain and heart infusion media, HiMedia, São Paulo, Brazil) was added to each tube. A 100 μL aliquot from each sample was then diluted in 900 μL of liquid BHI before bacterial counts were estimated as follows: To estimate the number of bacterial CFUs in each sample tested here, 100 μL of each homogenate was inoculated onto Petri dishes with solid BHI medium (Kasvi, São Paulo, Brazil). The Petri dishes were incubated at 37 °C for 24 h, after which time the number of CFUs was estimated.

The number of CFUs per midgut was estimated for each of the following treatments:

1. Sucrose (control);
2. Sucrose + antibiotic for 3 days;
3. Sucrose + antibiotic (for 3 days) and then 3 days offering sucrose without antibiotic;
4. Sucrose + antibiotic (for 3 days) and then 6 days offering sucrose without antibiotic;
5. Sucrose + antibiotic (for 3 days) and then 9 days offering sucrose without antibiotic.

Sucrose (control) or sucrose + antibiotic was offered for 3 days in all cases before removal of the antibiotic from the diet. Insects were dissected at "time zero" (3 days after offering diet with sucrose only, or sucrose + antibiotic) and then 3, 6, and 9 days after the removal of antibiotics from the diet.

For each gut sample, three plates were used to estimate bacterial populations, and this experiment was carried out five times, totaling 15 replicates for each treatment group (n = 15; N = 75). The mean bacterial count for each treatment group was calculated for each replicate experiment.

2.6. Blood Feeding

An artificial blood-feeding system was used as follows. Two-day-old adult female *Ae. aegypti* were starved for 24 h prior to offering blood meals. Defibrinated bovine blood was placed in a water-jacketed glass feeder covered with thinly stretched parafilm. The water circulating in the glass feeder was maintained at 37–40 °C using a water bath (Solab Ltd. São Paulo, Brazil) and an aquarium pump. To feed the mosquitoes, the glass feeder was placed in an inverted position in contact with the screen mesh of the holding cages containing the female mosquitoes. The feeding device was maintained in contact with the cages for up to 60 min, and only engorged females were selected for use in experiments.

2.7. Selection of Fungal Concentrations for Use in Subsequent Bioassays

This protocol was developed with the aim of maintaining >50% of the fungus-treated mosquitoes alive following spraying. This was necessary to compare the survival curves of adult mosquitoes from the different treatment groups and to investigate the consequences of the treatments on physiological factors such as blood feeding and egg laying.

Female *Ae. aegypti* were exposed to *M. anisopliae* by spraying them with conidial suspensions using a Potter spray tower (Burkart Ltd., Rickmansworth, UK). Groups of 10–20 mosquitoes were first anesthetized using a stream of CO_2 for 30 s. The anesthetized mosquitoes were then placed on a chilled platform as stated above. Ten females were then quickly transferred, using fine forceps, to a Petri dish lined with a sterile filter paper before being sprayed with 1 mL of *M. anisopliae* conidia at five different concentrations (1×10^4, 1×10^5, 1×10^6, 1×10^7, and 1×10^8 conidia mL^{-1}) in 0.05% (v/v) aqueous Tween 80 (TW). Control insects were sprayed with 1 mL of 0.05% TW only. This methodology has been previously used by our research group [41].

Mosquitoes were then carefully transferred to plastic pots (12 × 7 cm) with mesh netting screen lids. The pots were kept in an incubator (25 °C; 70 ± 10% RH; 12 h light/12 h dark). The mosquitoes were fed daily with 10% sucrose offered on filter paper discs placed on the surface of the netting. Mosquito survival was assessed daily for 7 days. These experiments were performed three times for each conidial concentration, with 30 insects used per treatment/concentration (N = 90 insects per treatment/concentration; controls N = 60). The survival data for replicate experiments was tested for homogeneity using the Log-Rank test. Only homogenous data was used for further data analysis.

2.8. Virulence of Metarhizium anisopliae When Tested Against Female Aedes aegypti Previously Treated with Antibiotics

These experiments were carried out to investigate the possible effects of antibiotic ingestion on mosquito survival following subsequent fungal infection. Two- to three-day-old mosquitoes were offered S or S + Cb for three consecutive days. After this period, 3 groups of 10 females (n = 30) were sprayed with a fungal suspension at 1×10^6 conidia mL^{-1} (conidial concentration determined from the previous experiment). Four treatments were then conducted:

1. Females offered S + Cb and then sprayed with the fungus (F) (S + Cb F);
2. Females offered S and then sprayed with the fungus (S + F);
3. Females offered S + Cb and then sprayed with TW (Tween control = C) (S + Cb C);
4. Females offered S and sprayed with TW (S C).

The fungal application and survival assessment protocols were carried out as described above. Replicate tests were conducted over three different periods using different batches of fungi and mosquitoes (n = 30 insects per treatment group × 3 repetitions per treatment; N = 90 per treatment group (4 treatments). The survival data for replicate experiments was tested for homogeneity using the Log-Rank test.

2.9. Survival of Aedes aegypti Females Exposed to Metarhizium anisopliae at Different Periods After Antibiotic Treatment

Aedes aegypti females were offered either sucrose or sucrose + carbenicillin for 3 days. After this time, the wick feeders with antibiotics were removed from the holding cages, and the females were sprayed with a fungal suspension at a concentration of 1×10^6 conidia mL^{-1}. This treatment group was referred to as "time zero." Subsequently, cohorts of mosquitoes were then sprayed with fungal suspensions at 3, 6, and 9 days after the removal of antibiotics from the diet (see the experimental timeline in Figure 1). For each treatment group, the respective controls were sprayed with 0.05% TW only.

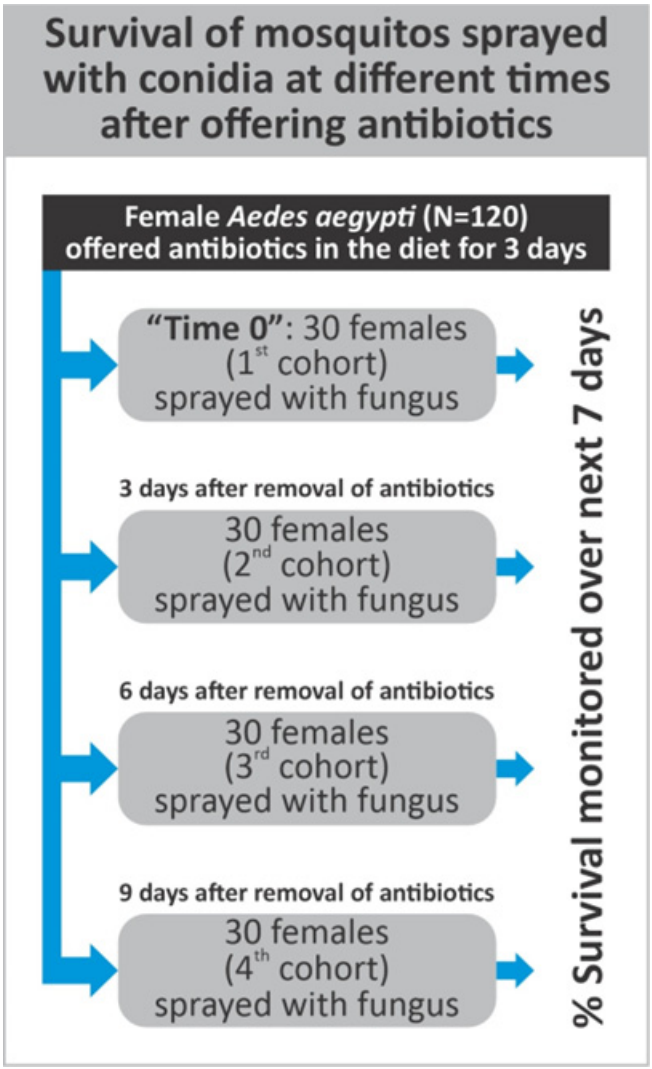

Figure 1. Timeline for experiments carried out to observe mosquito survival following infection of insects previously offered antibiotics in the diet.

The fungal application and survival assessment protocols were carried out as described above. Replicate tests were carried out at three different periods using different batches of fungi and mosquitoes. Thirty mosquitoes were used per time point post-antibiotic treatment (day 0, day 3, day 6, day 9; N = 120), and the experiment was carried out at three different times. The survival data for each replicate experiments was tested for homogeneity using the Log-Rank test. Only homogenous data was used for further data analysis.

2.10. The Effect of Antibiotics on Aedes aegypti Blood-Feeding Propensity

To assess whether the presence of carbenicillin in the diet affected subsequent blood-feeding propensity, two treatments were performed: (1) 3-day-old females were offered sucrose + Cb for 3 days and (2) females were offered sucrose only (controls).

Thirty females were used for each treatment. At the end of the third day, the wick feeders were removed from the cages, and the mosquitoes were deprived of liquids for 24 h. After this period, blood was offered to these insects for 30 min. To determine which females had ingested blood, the mosquitoes were anesthetized with CO_2, placed in Petri dishes, and individually crushed with forceps onto a white sheet of paper. This protocol was used to confirm the presence of blood in each females' alimentary canal. Two cohorts (30 insects each) were used for each treatment, with a total of 120 females per experiment. These

experiments were performed three times. The results were the means of each repetition for the two treatment groups.

2.11. The Effects of Fungal Infection and Antibiotic Treatment on Egg Production

Mosquitoes were first offered a sucrose solution for three days, with or without carbenicillin. At the end of the third day, the mosquitoes underwent a 24 h fasting period and were then offered blood (B) for 30 min as previously described. Two hours later, visibly engorged females were selected for evaluation of egg production. Then, groups of 10 engorged females were sprayed with fungus or 0.05% TW. This experiment was carried out three times (N = 10 × 3). To help clarify this experiment, see the timeline in Figure 2.

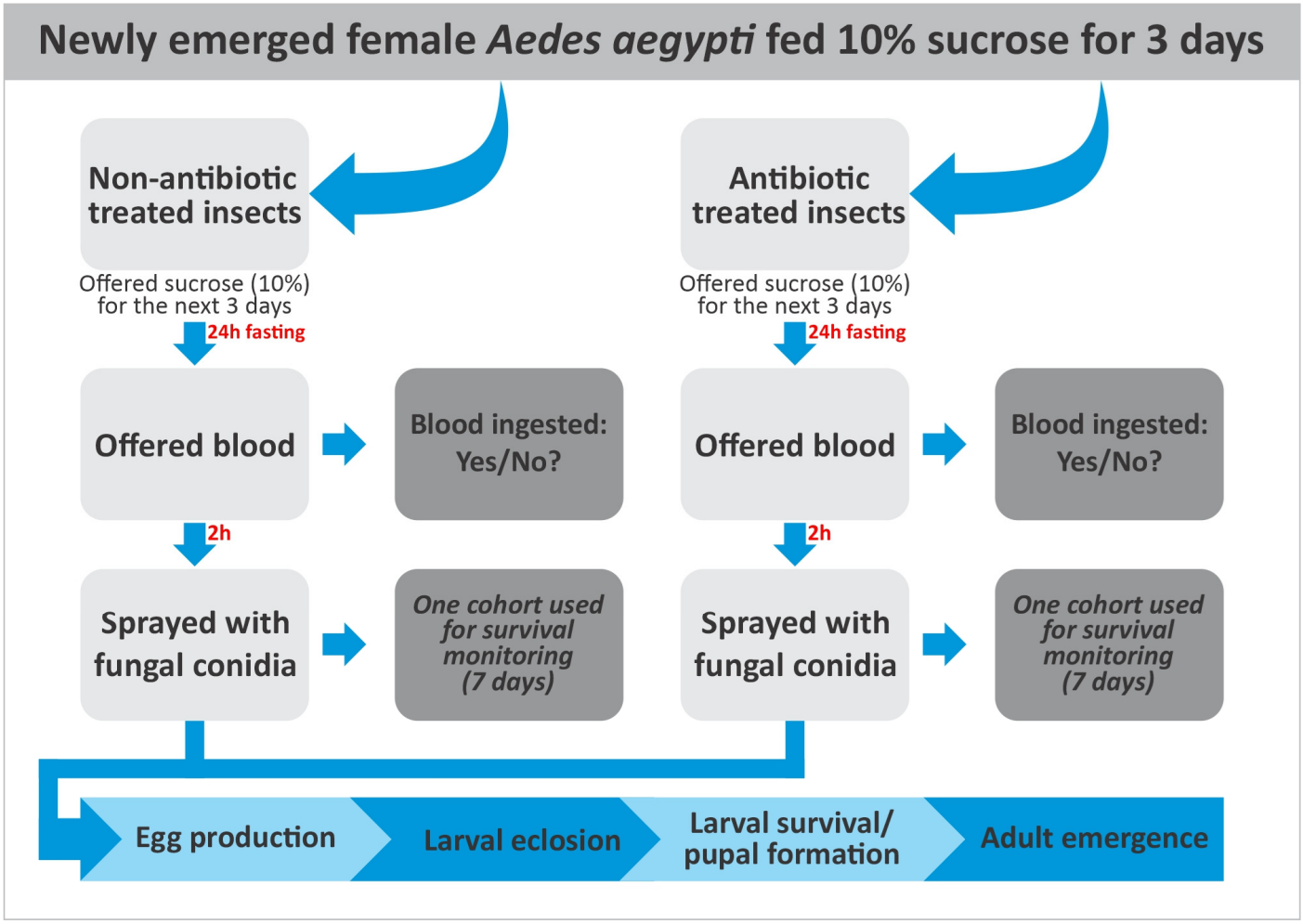

Figure 2. Timeline used for experiments to evaluate the effects of infection and antibiotic treatments on mosquito oviposition and subsequent development of the eggs, larvae, and pupae.

The four treatment groups were:

1. Females offered S + Cb + B and then sprayed with fungus;
2. Females offered S + B and then sprayed with fungus;
3. Females offered S + Cb + B and then sprayed with TW;
4. Females offered S + B and then sprayed with TW.

All of the mosquitoes from each individual treatment were maintained together in the same plastic container for 60 h. After this period, individual females were placed in Petri dishes (9 cm in diameter × 1.5 cm in height). Each Petri dish was inverted, and the bottom of the lid was lined with a filter paper moistened with 700 µL of a solution containing

dechlorinated water mixed with 30% (*v*/*v*) of larval rearing water (water taken from the larval tray to stimulate egg-laying). This procedure was adapted from that described by Valência et al. [55].

One female per group (n = 4; N = 16) was kept individually in a Petri dish in an incubator at 27 °C ± 1 °C; RH > 95%; light/dark photoperiod (12 h light:12 h dark). After 24 h, 300 µL of dechlorinated water was added to each Petri dish to maintain the humidity of the filter paper.

As females oviposit 3 to 5 days after blood feeding [56], the Petri dishes were removed from the BOD on the sixth day after blood feeding. Subsequently, these females were discarded, and the filter paper with the eggs was removed for quantification. The number of eggs was counted using a stereoscopic microscope (Labomed®, São Paulo, Brazil). At the end of the experiment, the mean oviposition rate was calculated considering the total number of eggs divided by the total number of females in each group. Sixteen females were used per repetition, and the tests were conducted at three different times using different batches of fungi and mosquitoes. The filter papers were numbered, and the eggs were dried for 96 h in a climate-controlled chamber (to ensure complete embryogenesis) and stored for use in the next experiment.

2.12. Egg, Larval, and Pupal Viability

Eggs produced by females from each of the above four treatment groups were transferred to Petri dishes containing 10 mL of de-chlorinated water and freshly ground, autoclaved commercial fish food (Nuvilab, São Paulo, Brazil; 0.05 g/L) to stimulate eclosion. The Petri dishes were kept in an incubator at 27 °C ± 1 °C; RH >95%. After 48 h, the number of larvae per Petri dish was counted. Then, the larvae were separated into groups of 20 larvae per Petri dish and were maintained in an incubator. The number of larvae that metamorphosed into pupae were counted, and these pupae were transferred to pots within breeding cages. After 48 h, the number of adults emerging from pupae was recorded for each of the four treatments.

2.13. Statistical Analysis

Survival data analyses (curve comparisons and homogeneity) were performed with GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). The homogeneity of the repetitions was analyzed using the Log-Rank test at a 95% significance level. Homogenous results were then pooled for survival curve comparisons using Log-Rank to contrast treatments. The average survival time (S_{50}) was calculated using the Kaplan–Meier method. Statistical differences between the survival curves of the different treatments were compared using the Log-Rank test (χ^2 significance level 5%). The results for all the controls for each treatment group were pooled, and only one survival curve is shown in the figures.

Comparisons of end-point survival for the different treatments were assessed using a generalized linear model (GLM) using Minitab (version 17). These results are expressed as means and standard deviations where necessary. Differences between groups were considered significant if the *p*-value was $\leq$0.001 using Tukey's post-hoc test. The propensity for blood feeding by females after antibiotic treatments was evaluated for statistical differences between groups by GLM using Minitab (version 17).

Averages for oviposition (number of eggs laid), egg hatching, pupal formation, and adult emergence were also evaluated for statistical differences using GLM. When a significant group effect was observed, data was analyzed using Tukey's post-hoc test, with $p < 0.001$ as the criterion for significance.

3. Results

3.1. The Presence of Antibiotics in the Diet Did Not Affect the Blood-Feeding Propensity of Aedes aegypti Females

There was no significant difference ($p > 0.001$) in blood-feeding propensity when comparing females that had been offered sucrose + antibiotic to females offered sucrose only. Of the total number of females tested here, 86.3% ± 1.52 (mean ± SD) that had been previously offered a diet of sucrose + antibiotics for three days and then offered a blood meal had blood in their digestive tract. For females offered sucrose only and then blood, 88.1% ± 2.08 of the mosquitoes had blood in their digestive tract.

3.2. Antibiotics Reduce Bacterial Populations in the Mosquito Intestine

To evaluate the effects of antibiotic treatments on *Ae. aegypti* intestinal microbiota populations, insects were offered sucrose solutions with and without carbenicillin. Microbiota populations were also monitored over time following the removal of the antibiotic from the diet.

The results for mean numbers of bacterial CFUs are shown in Table 1. Three days after offering a diet with antibiotics, the number of CFUs declined by 99%. Having established the almost complete elimination of the gut microbiota, we then observed whether the bacteria could recolonize the intestine once the antibiotics were removed from the diet. Initially, the bacterial populations remained at a low level three days after the removal of antibiotics. There was a 64% increase in the number of CFUs 6 days after removal of antibiotics. Nine days after the removal of antibiotics, the bacterial population returned to similar levels to that seen in the controls.

Table 1. Bacterial colony counts (mean CFU ± SD) present in *Aedes aegypti* intestines after insects had been offered antibiotics for three days and then the antibiotics were removed from the diets.

Treatment	Bacterial Colonies (CFU)
S	220 ± 2.86 a
S + Cb	2 ± 0.55 b
S + Cb 3D	9 ± 0.84 c
S + Cb 6D	116 ± 2.17 d
S + Cb 9D	218 ± 2 a

Note: Sucrose (S), sucrose + antibiotic (S + Cb), and then sucrose only for 3 (3D), 6 (6D), and 9 (9D) days. The results followed by different letters were significantly different when compared using a generalized linear model and Tukey's post-hoc test ($F_{4,24} = 15{,}930.82$; $p < 0.001$). The results were the means of three experiments per treatment carried out at different time periods. The raw data for this experiment is available in the Supplementary Material.

3.3. Virulence of Metarhizium anisopliae Against Aedes aegypti Females

Different concentrations of conidial suspensions were tested here to define the most appropriate concentration for use in conjunction with antibiotic treatments, with the aim of maintaining approximately 50% of the insects alive over the seven-day period of the experiment. When mosquitoes were sprayed with conidial concentrations of 1×10^8 or 1×10^7 conidia mL^{-1}, none remained alive by the seventh day of evaluation (Figure 3 and Table 2).

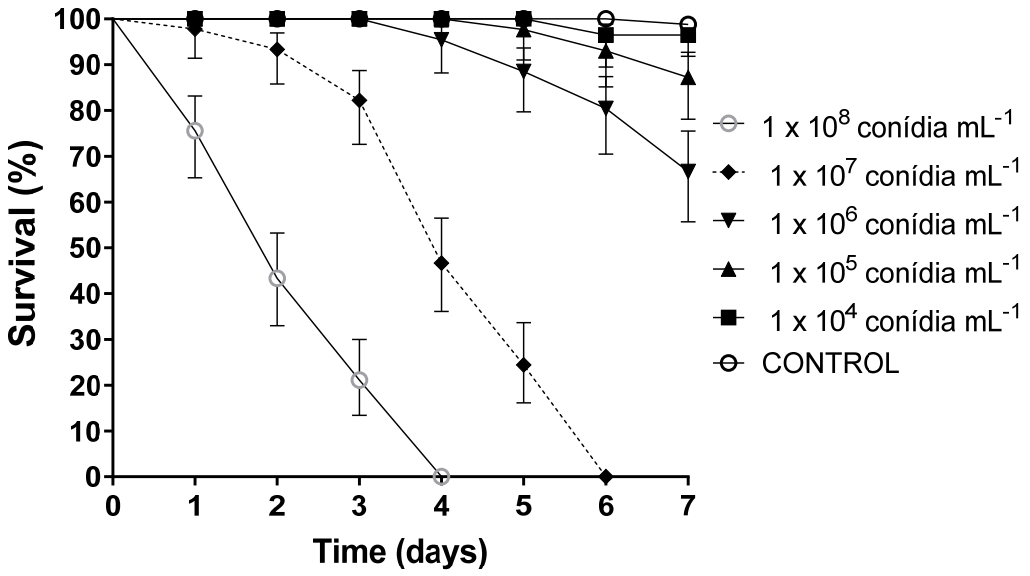

Figure 3. Survival curves of female *Aedes aegypti* sprayed with different concentrations of *Metarhizium anisopliae* conidia and then monitored over 7 days. For each experiment with different fungal concentrations, N = 90 mosquitoes per treatment (n = 30 per repetition). Controls N = 60 (n = 20 per repetition). Bars represent 95% confidence intervals.

However, mosquitoes sprayed with 1×10^6 conidia mL^{-1} had a mean survival rate of 66.7% on the seventh day of evaluation, and 87.8% of the mosquitoes sprayed with 1×10^5 conidia mL^{-1} survived (Table 2). GLM analysis for mean end-point survival data showed significant differences between the treatments ($F_{5,17} = 2110.44$; $p < 0.001$). However, only two of the concentrations tested here (1×10^5 and 1×10^6 conidia mL^{-1}) maintained >50% of the mosquitoes alive, whilst still being significantly different to the control survival curves. The survival curves of mosquitoes when exposed to 1×10^4 conidia mL^{-1} (96.7%), were not significantly different from the control treatment ($\chi^2 = 1.021$, df = 1, $p = 0.3122$). This was also the case for mean endpoint survival when using this concentration of conidia. For all subsequent experiments, a concentration of 1×10^6 conidia mL^{-1} was thus selected to investigate the effects of antibiotics on infection and insect development.

Table 2. Mean survival percentages (± SD) after 7 days and median survival times (S_{50} in days) for female *Aedes aegypti* sprayed with different concentrations of *Metarhizium anisopliae* conidia.

Conidial Concentration	Mean % Survival ± SD	S_{50}
1×10^8 conidia mL^{-1}	0 a	2
1×10^7 conidia mL^{-1}	0 a	4
1×10^6 conidia mL^{-1}	66.7 ± 1 b	NA
1×10^5 conidia mL^{-1}	87.8 ± 0.58 c	NA
1×10^4 conidia mL^{-1}	96.7 ± 0.1 d	NA
Control	98.9 ± 0.58 d	NA

Note: The results followed by different letters were significantly different when compared using a generalized linear model and Tukey's post-hoc test ($F_{5,17} = 2110.44$; $p < 0.001$). The S_{50} values were calculated using Kaplan–Meier analysis. For each experiment with different fungal concentrations, N = 90 mosquitoes were used per treatment (n = 30 per repetition). Control N = 60 (n = 20 per repetition). NA: not applicable. The raw data for this experiment is available in the Supplementary Material.

3.4. Increased Susceptibility of Aedes aegypti to Metarhizium Anisopliae After Females Had Been Offered Antibiotics

In this experiment, it was observed that females offered sucrose + antibiotic and subsequently sprayed with *M. anisopliae* conidia (S + Cb F) had lower survival rates compared

to tests conducted with females offered sucrose only and then sprayed with fungus (S F) (Figure 4).

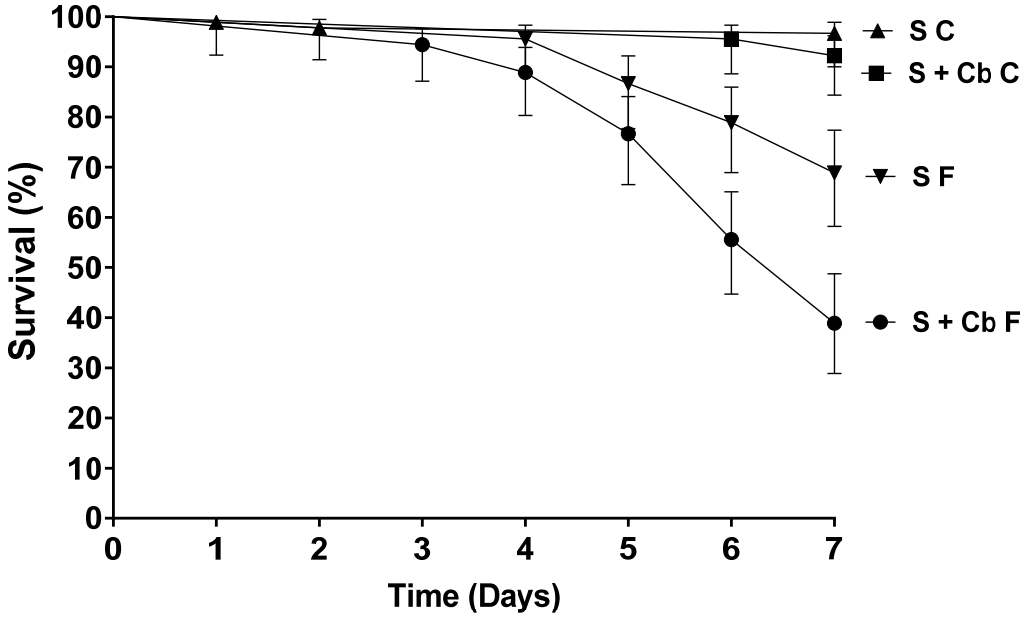

Figure 4. Survival curves of female *Aedes aegypti* sprayed with *Metarhizium anisopliae* conidia (1×10^6 conidia mL^{-1}) after being offered diets with and without antibiotics. Survival was monitored daily over 7 days. N = 90 mosquitoes per treatment group (n = 30 per repetition). Note: S: sucrose; Cb: carbenicillin; F: fungus; C: control. Bars represent 95% confidence intervals.

When comparing the survival curves of females offered carbenicillin and then sprayed with fungus, there was a significant difference ($\chi^2 = 16.07$, df = 1, $p < 0.001$) to the survival curves of mosquitoes sprayed with fungus that had not been offered antibiotics (Figure 4). The mean endpoint survival percentages were 38.9% for antibiotic-treated mosquitoes sprayed with fungal conidia and 68.9% for the sucrose + fungus group (Table 3). GLM analysis confirmed the significant difference between treatments ($F_{3,11} = 317.78$; $p < 0.001$).

Table 3. Mean survival percentages (±SD) after 7 days and median survival times (S_{50} in days) for female *Aedes aegypti* offered different diets and then sprayed with *Metarhizium anisopliae* conidia.

Treatments	Survival ± SD	S_{50}
S + Cb F	38.9 ± 1.15 a	7
S F	68.9 ± 0.58 b	NA
S + Cb C	91.1 ± 1.15 c	NA
S C	96.7 ± 1 c	NA

Note: S: sucrose; Cb: carbenicillin; F: fungus; C: control. The results for survival percentages followed by different letters were significantly different when compared using a generalized linear model and Tukey's post-hoc test ($F_{3,11} = 317.78$; $p < 0.001$). The S_{50} values were calculated using Kaplan–Meier analysis. For each treatment group N = 90 mosquitoes (n = 30 per repetition). NA: not applicable. The raw data for this experiment is available in the Supplementary Material.

3.5. Survival of Aedes aegypti Females Sprayed with Metarhizium anisopliae Conidia at Different Periods After Antibiotic Treatment

This experiment was carried out to investigate the susceptibility of mosquitoes to fungal infection at different times after offering the mosquitoes antibiotics. Survival following fungal application was monitored at different time intervals after the antibiotics had been removed from the diet (see Figure 1 timeline). As in the previous experiment, mosquitoes were offered antibiotics for 3 days, and then they were sprayed with fungus (denominated

here as "time zero"). Additional cohorts were sprayed with fungus 3 days, 6 days, and 9 days after removal of antibiotics from the diet.

Figure 5A shows the survival curves of mosquitoes that had been sprayed with the fungus three days after offering the mosquitoes sucrose + carbenicillin ("time zero"). A comparison of the survival curves of females fed with sucrose + carbenicillin or sucrose only and then sprayed with fungus showed significant differences ($\chi^2 = 13.85$, df = 1, $p < 0.001$). These results were similar to those seen in the previous experiment, with significantly lower endpoint survival ($F_{3,11} = 202.97$; $p < 0.001$) for females offered sucrose + antibiotic compared to those offered sucrose only and then sprayed with the fungus. Table 4 shows the endpoint mean survival analysis.

The survival curves of females offered sucrose + carbenicillin for 3 days and then sprayed with fungus 3 days after removal of the antibiotics from the diet were significantly different compared to insects offered sucrose only and then sprayed with fungus (Figure 5B; $\chi^2 = 10.55$, df = 1, $p < 0.001$). The mean endpoint survival percentage was 38.8% for the antibiotic-treated insects, while 64.4% of the non-antibiotic-treated insects remained alive on the 7th day of the experiment ($F_{3,11} = 127.48$; $p < 0.001$).

Figure 5C shows the survival curves for tests carried out six days after removal of antibiotics from the diet. *Aedes aegypti* females that had been offered sucrose + carbenicillin during the first three days of the experiment and then exposed to *M. anisopliae* six days later showed significant differences to the other treatments ($\chi^2 = 6.764$; df = 1, $p = 0.0093$). The mean endpoint survival of antibiotic-treated insects was significantly lower than non-antibiotic-treated insects ($F_{3,11} = 182.42$; $p < 0.001$).

When *Ae. aegypti* females were sprayed with the fungus 9 days after removal of carbenicillin from the diet, there was no longer a significant difference observed between the survival curves ($\chi^2 = 0.3876$; df = 1, $p = 0.5336$) compared to the group offered sucrose only and then sprayed with fungus (Figure 5D). There was also no significant difference between endpoint survival (Table 4).

Table 4. Survival (mean % ± SD) and median survival times (S_{50}) of female *Aedes aegypti* sprayed with *Metarhizium anisopliae* at different times ("time zero" up to 9 days) after removing carbenicillin from the diet.

	Time Zero	**3 Days**	**6 Days**	**9 Days**
Treatments	**Survival (%) ±SD**	**Survival (%) ±SD**	**Survival (%) ±SD**	**Survival (%) ±SD**
S + Cb F	37.8 ± 1.15 aA	38.8 ± 1.53 aA	45.6 ± 1.53 aB	58.9 ± 1.53 aC
S F	65.5 ± 1.53 bA	64.4 ± 0.58 bA	63.3 ± 0.58 bA	63.3 ± 1 aA
S + Cb C	90 ± 0.2 cA	91.1 ± 1.15 cA	93.3 ± 0.1 cA	96.7 ± 0.58 bB
S C	98.9 ± 0.58 dA	96.7 ± 1 cA	96.7 ± 0.58 cA	97.8 ± 0.58 bA

S: sucrose; Cb: carbenicillin; F: fungus; C: control. Note: The means followed by different letters were significantly different when compared using a generalized linear model and Tukey's post-hoc test. The uppercase letters indicate statistical differences when comparing individual treatments over time (horizontal lines). The lowercase letters indicate statistical differences when comparing treatments at the same time point (columns). For each different treatment N = 90 mosquitoes (n = 30 per repetition). The raw data for this experiment is available in the Supplementary Material.

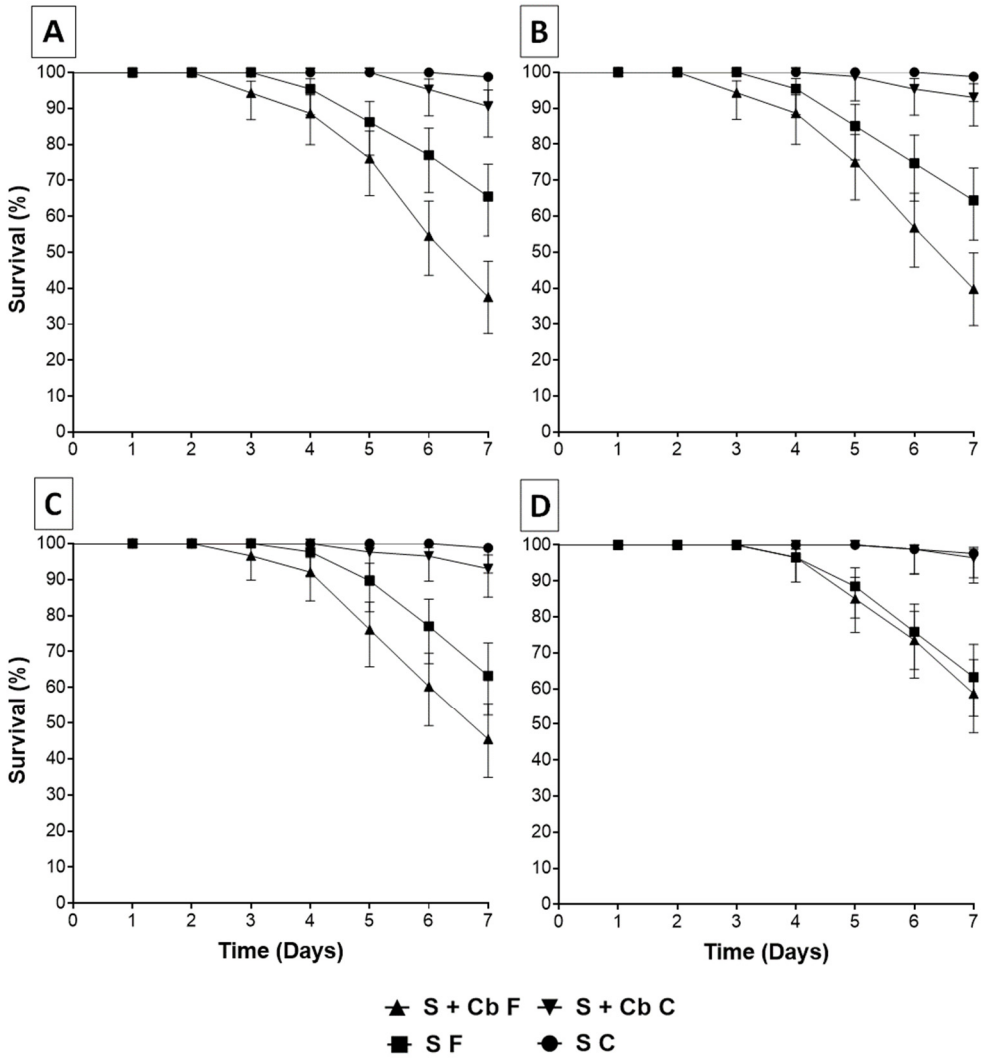

Figure 5. Survival of female *Aedes aegypti* sprayed with *Metarhizium anisopliae* (1×10^6 conidia mL^{-1}) at different times after offering diets: (**A**) = time zero; (**B**) = 3 days; (**C**) = 6 days; (**D**) = 9 days. For each experiment N = 90 mosquitoes per treatment (n = 30 per repetition). S: sucrose; Cb: carbenicillin; F: fungus; C: control. Bars represent 95% confidence intervals.

3.6. Aedes aegypti Oviposition Rates Following Different Treatments

Aedes aegypti females sprayed with *M. anisopliae* after previously being offered diets with or without antibiotics and then allowed to blood feed (S + Cb B F or S B F) showed lower oviposition rates compared to controls (S + Cb B C or S B C). The treatment S + Cb B F showed the lowest average oviposition rate (44.3 eggs/female) compared to the other treatments: S B F (68.7 eggs/female), S + Cb B C (81.7 eggs/female), and S B C (98.8 eggs/female), with significant differences found when comparing treatments ($F_{3,15} = 1483.99$; $p < 0.001$).

3.7. Development from Eggs to Adults Following Different Treatments

Eggs produced by *Ae aegypti* females that had ingested antibiotics and were then subsequently sprayed with *M. anisopliae* (S + Cb B F), or females that had not been offered antibiotics and then were sprayed with fungal conidia (S B F), were evaluated for larval eclosion rates (Table 5). These larvae were then evaluated for pupal formation and adult emergence. The eggs oviposited by females from the S + Cb B F treatment had the lowest viability, with only 47.9% successfully producing larvae. For the S B F treatment, 67.5% of the eggs successfully produced larvae. There was an overall statistical difference between the four treatment groups ($F_{3,15} = 1651.50$; $p < 0.001$).

The larvae that hatched from eggs from the S + Cb B F treatment had the lowest rate of pupal formation (52.5%). Seventy-seven percent of the larvae from the S B F group successfully formed pupae. The respective controls had the highest rates of pupal formation. There were significant differences among the treatments when evaluated using GLM and Tukey's test ($F_{3,15}$ = 1593,43; $p < 0.001$). Interestingly, all of the larvae that pupated subsequently developed into adults.

Table 5. The effects of the different treatments on oviposition (number of eggs) and subsequent development of these eggs > larvae, larvae > pupae, and pupae > adults.

	TREATMENTS			
Stage	**S + Cb B F**	**S B F**	**S + Cb B C**	**S B C**
Eggs	532 ± 2.89 a	824 ± 3.06 b	981 ± 3,61 c	1185 ± 6.24 d
Larvae	255 ± 2.65 a (47.9%)	556 ± 3.79 b (67.5%)	943 ± 404 c (96,1%)	1142 ± 9.61 c (96.4%)
Pupae	134 ± 1.53 a (52.5%)	427 ± 5.13 b (76.8%)	934 ± 4.04 c (99%)	1142 ± 9.61 c (100%)
Adults	134 (100%)	427 (100%)	934 (100%)	1142 (100%)

Mean number of eggs (± SD); mean number of larvae hatching from these eggs (percentage of eggs hatching into larvae); mean number of pupae formed from these larvae (%); number of adults that developed from the pupae (%). S: sucrose; Cb: carbenicillin; F: fungus; B: blood fed; C: control. Lower case letters indicate significant differences ($p < 0.001$) between treatments for individual stages. The raw data for this experiment is available in the Supplementary Material.

4. Discussion

This study explored the role of the gut microbiota in the susceptibility of adult *Ae. aegypti* to EPF infection. This was performed by monitoring the effects of antibiotic treatments on the survival of female *Ae. aegypti*, which were subsequently exposed to *M. anisopliae* conidia. The ingestion of antibiotics significantly increased the susceptibility of mosquitoes to fungal infection. Additionally, this study investigated the effects of fungal infection, with and without antibiotic exposure, on oviposition, larval eclosion, larval to pupal development, and adult emergence. The results showed a range of negative effects on oviposition and subsequent developmental factors.

It is known that the presence of normal gut microbiota is fundamental to the health of many animals and that alterations/dysbiosis of the microbiota can have significant effects on a range of physiological processes [57]. The current findings confirmed the importance of *Ae. aegypti* gut microbiota for the successful development of eggs through to adulthood. Developmental processes were also modified following fungal infection alone; however, alterations in development were more striking in the absence of the gut microbiota together with exposure to fungi. Although we did not identify the bacteria present in the midguts of the *Ae. aegypti* mosquitoes used here, the *Aedes* mosquito midgut microbiota is made up of predominantly facultative Gram-negative bacteria that belong to four different phyla: Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria [58].

Survival rates were significantly different when comparing non-antibiotic and antibiotic treated groups following exposure to fungal conidia via integumental spraying. This result suggests that the intestinal microbiota may be associated with differences in susceptibility to fungal infections, even though in the experiments here, the pathogen attacks its host via cuticle penetration.

A three-day exposure to carbenicillin reduced the culturable bacterial populations of *Ae. aegypti* intestines by 99% of pre-antibiotic levels. Similar to that seen here, Gaio et al. [46] observed a 97% reduction in bacterial populations of *Ae. aegypti* midguts also when using carbenicillin and a culture-dependent technique. In the case of *An. gambiae*, the ingestion

of an antibiotic cocktail effectively eliminated all culturable bacteria in the midguts of mosquitoes fed on either sugar or human blood with the addition of antibiotics [59].

Culture-dependent methods can significantly underestimate the total microbial diversity of biological samples [60], Therefore, the CFU counts used here may not reflect the complete diversity of bacterial populations. Furthermore, the use of a single antibiotic was a limiting factor in complete elimination of the gut microbiota, although offering carbenicillin alone resulted in significant increases in susceptibility to fungal infections and reductions in egg laying.

When carbenicillin was removed from the diet, the CFU count gradually returned to control levels over a nine-day period. This demonstrated the transient nature of antibiotic treatments, and that bacteria rapidly recolonized the gut. Although other studies have also investigated the elimination of the gut microbiota using antibiotics [46,49,51,59], the current study is the first to observe the recolonization of the gut after the removal of antibiotics from the diet. Fungal virulence was correlated to the numbers of bacteria in the gut. Shortly after treating the mosquitoes with antibiotics ("time zero" to 6 days after removing antibiotics from the diet), the fungus killed significantly higher numbers of insects than 9 days after removing antibiotics from the diet (Table 4 and Figure 5).

In contrast to the current study, Wei et al. [51] found that the gut microbiota of adult *Anopheles stephensi* had a role in increasing the infectivity of the entomopathogenic fungus *B. bassiana*. In their study, the elimination of the intestinal microbiota resulted in lower *B. bassiana* infection rates. This is an unexpected result given that the gut microbiota is known to stimulate immune responses in *An. gambiae* [59].

Entomopathogenic fungi are known to produce low-molecular-mass toxic peptides [61], which inhibit immune responses during colonization of the hemolymph [62]. In the case of *An. stephensi* infection by *B. bassiana*, the fungus secretes oosporein, a peptide toxin that downregulated the insect's immune response, inhibiting antimicrobial peptide secretion and dual oxidase expression in the midgut [51]. This in turn allowed a resident bacterium in the midgut, *Serratia marcescens*, to invade the hemocoel, altering its role as an asymptomatic intestinal symbiont to a hemocoelic pathogen, causing accelerated mosquito death through the fungus–bacterium interaction.

The German cockroach, *B. germanica*, was more susceptible to fungal infection following elimination of the intestinal microbiota [52]. In that case, the microbiota played a crucial role in the protection of this insect against infection by *M. anisopliae* following oral administration of fungal conidia [52].

In mosquitoes, the midgut microbiota influences the IMD pathway by induction of the REL2 "defense gene" in both the midgut and fat body. The elimination of natural microbiota using antibiotics impaired the induction of this immune pathway in defense against pathogens [63]. The induction of immune signaling pathways can lead to the production of antimicrobial peptides (AMPs) that neutralize invasive pathogens. The enhancement of the insect's immune system indirectly increases protection against parasites [59,63,64], and this is correlated to the intestinal microbiota in mosquitoes. The gut microbiota plays a direct role in protecting hematophagous insects against blood-borne parasites [59]. Gram-negative bacteria present in the mosquito midgut are known to significantly reduce *Plasmodium* oocyst numbers in mosquitoes infected with this malaria-causing protozoan [65,66]. In *Anopheles* and *Aedes*, removing the intestinal microbiota with antibiotics increased mosquito susceptibility to *Plasmodium* infection [66] and to the dengue virus [67], respectively.

Although we did not identify the bacteria present in the gut microbiota of the *Ae. aegypti* mosquitoes used here, a previous study of midgut microbiota from a laboratory strain of *Ae. aegypti* (Rockefeller) identified the presence of *Serratia*, *Klebsiella*, *Asaia*, *Bacillus*,

Enterococcus, *Enterobacter*, *Kluyvera*, and *Pantoea*, with *Serratia* representing more than half of the gut microbiota population [68].

The insect gut microbiota also has a role in other aspects of physiology, including facilitating blood digestion [46] and increasing fecundity [46,69,70]. The results of the current study show that elimination of the gut bacterial populations following antibiotic treatment significantly reduced *Ae. aegypti* fecundity.

The intestinal microbiota is crucial to fecundity in *Ae. aegypti*, as demonstrated by a 6–20% decrease in the number of mature oocytes and a 14–22% reduction in egg production when *Ae. aegypti* females were treated with different antibiotics before blood feeding [46]. Here, females offered carbenicillin and then subsequently offered a bloodmeal had a 17% reduction in oviposition rates. Our results confirmed those of previous studies, where the *Ae. aegypti* gut microbiota plays an important role in egg production. In the experiments where mosquitoes were first treated with antibiotics and then exposed to *Metarhizium*, the number of eggs was significantly lower than in all other treatments. During the next step of mosquito development, larval hatching, the interaction between the fungus and the antibiotic treatments also resulted in significant reductions in successful larval eclosion. Larval eclosion rates were also reduced following exposure of females to conidia alone. However, antibiotic treatment alone had no posterior effects on development, as 100% of the eggs hatched into larvae. Furthermore, no negative effects on pupal or adult formation were caused by treating females with antibiotics.

As stated above, antibiotic treatment alone did not negatively affect larval eclosion; however, both fungal treatments (with antibiotics and without antibiotics) resulted in reduced egg viability. For eggs produced by females previously offered antibiotics and then sprayed with fungus, only 47.9% of the eggs developed into larvae. For females offered sucrose and then exposed to fungal conidia, 67.5% of the eggs developed into larvae.

This study showed that exposure to both antibiotics and fungi is associated with reductions in insect developmental parameters. Furthermore, a combination of both agents was highly deleterious for adult mosquitoes. More studies are needed to investigate the role of intestinal bacteria in protecting mosquitoes against pathogens such as EPF.

Entomopathogenic fungi are efficient biological control agents, currently used extensively in crop protection. However, their use against disease-vectoring insects is still incipient. For effective vector control, novel approaches are needed, such as the use of fungus-impregnated traps that attract adult mosquitoes.

A recent study demonstrated that genetically modified *Metarhizium* expressing the volatile organic compound longifolene acted as a mosquito attractant, increasing the fungus's capacity to infect and kill the targeted pests [71].

5. Conclusions

The results presented here confirm the importance of intestinal bacteria in the normal functioning of *Ae. aegypti* defense and developmental processes. The use of a broad range antibiotic increased the susceptibility of female mosquitoes to fungal infection and reduced oviposition rates. The exposure of female *Ae. aegypti* to EPF and antibiotics resulted in cascade effects when considering the development of eggs laid by mosquitoes previously exposed to both agents, reducing egg viability and viability of the larvae that hatched from these eggs.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/parasitologia6010004/s1, Table S1 raw data file: Bacterial colony counts (mean CFU ± SD) present in *Aedes aegypti* intestines after insects had been offered antibiotics for three days and then the antibiotics were removed from the diets. Table S2 raw data file: Mean survival percentages (± SD) after 7 days and median survival times (S_{50} in days) for female *Aedes*

aegypti sprayed with different concentrations of *Metarhizium anisopliae* conidia. Table S3 raw data file: Mean survival percentages (±SD) after 7 days and median survival times (S_{50} in days) for female *Aedes aegypti* offered different diets and then sprayed with *Metarhizium anisopliae* conidia. Table S4 raw data file: Survival (mean % ± SD) and median survival times (S_{50}) of female *Aedes aegypti* sprayed with *Metarhizium anisopliae* at different times ("time zero" up to 9 days) after removing carbenicillin from the diet. Table S5 raw data file: The effects of the different treatments on oviposition (number of eggs) and subsequent development of these eggs > larvae, larvae > pupae, and pupae > adults.

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Abbreviations

The following abbreviations are used in this manuscript:

Bti	*Bacillus thuringiensis israelensis*
EPF	Entomopathogenic fungi
RH	Relative humidity
LED	lllumination light-emitting diode
SDA	Sabouraud dextrose agar
MR-5	Mycoharvester®
S	Sucrose
Cb	Carbenicillin
F	Fungus
TW	Tween 80
C	Control
B	Blood
PBS	Phosphate-buffered saline
BHI	Brain and heart infusion media
CFU	Colony-forming units
BOD	Biochemical oxygen demand
S_{50}	Median survival time in days

glm	Generalized linear model
SD	Standard deviation
NA	Not applicable
REL2	Defense gene
AMP	Antimicrobial peptides

References

1. World Health Organization (WHO). Dengue—Global Situation. Available online: https://www.who.int/emergencies/disease-outbreak-news/item/2024-DON518 (accessed on 27 August 2024).
2. World Health Organization (WHO). Yellow Fever Fact Sheet. Available online: https://www.who.int/news-room/fact-sheets/detail/yellow-fever (accessed on 20 August 2025).
3. World Health Organization (WHO). Chikungunya Fact Sheet. Available online: https://www.who.int/news-room/fact-sheets/detail/chikungunya (accessed on 20 August 2025).
4. World Health Organization (WHO). Zika Virus Fact Sheet. Available online: https://www.who.int/news-room/fact-sheets/detail/zika-virus (accessed on 20 August 2025).
5. Powell, J.R.; Tabachnick, W.J. History of domestication and spread of *Aedes aegypti*: A review. *Mem. Inst. Oswaldo Cruz* **2013**, *108*, 11–17. [CrossRef] [PubMed]
6. Kraemer, M.U.; Sinka, M.E.; Duda, K.A.; Mylne, A.Q.; Shearer, F.M.; Barker, C.M.; Moore, C.G.; Carvalho, R.G.; Coelho, G.E.; Wim Van Bortel, W.G.; et al. The global distribution of the arbovirus vectors *Aedes aegypti* and *Aedes albopictus*. *eLife* **2015**, *4*, e08347. [CrossRef]
7. Lambrechts, L.; Scott, T.W.; Gubler, D.J. Consequences of the expanding global distribution of *Aedes albopictus* for dengue virus transmission. *PLoS Negl. Trop. Dis.* **2010**, *4*, e646. [CrossRef]
8. Sukiato, F.; Shriram, A.N.; Sugumaran, M. Eggs of the mosquito *Aedes aegypti* survive desiccation by rewiring their polyamine and lipid metabolism. *PLoS Pathog.* **2023**, *19*, e1011685. [CrossRef]
9. Sousa-Neto, J.A.; Powell, J.R.; Bonizzoni, M. *Aedes aegypti* vector competence studies: A review. *Infect. Genet. Evol.* **2019**, *67*, 191–209. [CrossRef]
10. Tatem, A.J.; Hay, S.I.; Rogers, D.J. Global traffic and disease vector dispersal. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 6242–6247. [CrossRef] [PubMed]
11. Gubler, D.J. Dengue, urbanization and globalization: The unholy trinity of the 21st century. *Trop. Med. Health* **2011**, *39*, 3–11. [CrossRef]
12. Macoris, M.L.G.; Andrighetti, M.T.M.; Takaku, L.; Glasser, C.M.; Garbeloto, V.C.; Bracco, J.E. Resistance of *Aedes aegypti* from the state of São Paulo, Brazil, to organophosphate insecticides. *Mem. Inst. Oswaldo Cruz* **2003**, *98*, 703–708. [CrossRef]
13. Montella, I.R.; Martins, A.J.; Viana-Medeiros, P.F.; Lima, J.B.P.; Braga, I.A.; Valle, D. Insecticide resistance mechanisms of Brazilian *Aedes aegypti* populations from 2001 to 2004. *Am. J. Trop. Med. Hyg.* **2007**, *77*, 467–477. [CrossRef]
14. Lima, E.P.; Paiva, M.H.; de Araujo, A.P.; Silva, E.V.; Silva, U.M.; Oliveira, L.N.; Santana, A.E.G.; Barbosa, C.N.; Neto, C.C.P.; Goulart, M.O.F.; et al. Insecticide resistance in *Aedes aegypti* populations from Ceará, Brazil. *Parasit. Vectors* **2011**, *4*, 5. [CrossRef]
15. Nakazato, B.M.; Macoris, M.L.G.; Urbinatti, P.R.; Lima-Camara, T.N. Locomotor activity in *Aedes aegypti* with different insecticide resistance profiles. *Rev. Saude Publica* **2021**, *55*, 18. [CrossRef] [PubMed]
16. van Lenteren, J.C.; Bolckmans, K.; Köhl, J.; Ravensberg, W.J.; Urbaneja, A. Biological control using invertebrates and microorganisms: Plenty of new opportunities. *BioControl* **2018**, *63*, 39–59. [CrossRef]
17. Becnel, J.J.; White, S.E.; Moser, B.A.; Fukuda, T.; Rotstein, M.J.; Undeen, A.H.; Cockburn, A. Epizootiology and transmission of a newly discovered baculovirus from the mosquitoes *Culex nigripalpus* and *Culex quinquefasciatus*. *J. Gen. Virol.* **2001**, *82*, 275–282. [CrossRef]
18. Pereira, C.R.; Paula, A.R.; Gomes, S.A.; Pedra, J.P.; Samuels, R.I. The potential of *Metarhizium anisopliae* and *Beauveria bassiana* isolates for the control of *Aedes aegypti* (Diptera: Culicidae) larvae. *Biocontrol Sci. Technol.* **2009**, *19*, 881–886. [CrossRef]
19. Setha, D.; Chantha, N.; Benjamin, S.; Socheat, D. Bacterial larvicide, *Bacillus thuringiensis israelensis* strain AM65-52 water dispersible granule formulation impacts both dengue vector *Aedes aegypti* (L.) population density and disease transmission in Cambodia. *PLoS Negl. Trop. Dis.* **2016**, *10*, e0004973. [CrossRef]
20. Charles, J.; Tangudu, C.S.; Hurt, S.L.; Tumescheit, C.; Firth, A.E.; Garcia-Rejon, J.E.; Machain-Williams, C.; Blitvich, B.J. Discovery of a novel *Tymoviridae*-like virus in mosquitoes from Mexico. *Arch. Virol.* **2019**, *164*, 649–652. [CrossRef]
21. Pruszynski, C.A.; Hribar, L.J.; Mickle, R.; Leal, A.L. A large scale biorational approach using *Bacillus thuringiensis israelensis* (strain AM65-52) for managing *Aedes aegypti* populations to prevent dengue, chikungunya and Zika transmission. *PLoS ONE* **2017**, *12*, e0170079. [CrossRef]
22. Lacey, L.A. *Bacillus thuringiensis* serovariety *israelensis* and *Bacillus sphaericus* for mosquito control. *J. Am. Mosq. Control Assoc.* **2007**, *23*, 133–163. [CrossRef] [PubMed]

23. Pigott, C.R.; Ellar, D.J. Role of receptors in *Bacillus thuringiensis* crystal toxin activity. *Microbiol. Mol. Biol. Rev.* **2007**, *71*, 255–281. [CrossRef] [PubMed]
24. Ben-Dov, E. *Bacillus thuringiensis israelensis* and its mosquitocidal proteins. *Toxins* **2014**, *6*, 1222–1243. [CrossRef] [PubMed] [PubMed Central]
25. Zaki, Z.A.; Dom, N.C.; Alhothily, I.A. Efficacy of *Bacillus thuringiensis* treatment on *Aedes* population using different applications at high-rise buildings. *Trop. Med. Infect. Dis.* **2020**, *5*, 67. [CrossRef]
26. Scholte, E.J.; Knols, B.G.; Takken, W. The potential of entomopathogenic fungi for mosquito control: A review. *J. Insect Sci.* **2004**, *4*, 19. [CrossRef]
27. Gomes, S.A.; Paula, A.R.; Ribeiro, A.; Moraes, C.O.P.; Santos, J.W.A.B.; Silva, C.P.; Samuels, R.I. Neem oil increases the efficiency of the entomopathogenic fungus *Metarhizium anisopliae* for the control of *Aedes aegypti* (Diptera: Culicidae) larvae. *Parasit. Vectors* **2015**, *8*, 1–8. [CrossRef]
28. Imad-Silva, L.E.; Paula, A.R.; Ribeiro, A.; Butt, T.M.; Silva, C.P.; Samuels, R.I. A new method of deploying entomopathogenic fungi to control adult *Aedes aegypti* mosquitoes. *J. Appl. Entomol.* **2017**, *142*, 59–66. [CrossRef]
29. Bitencourt, R.O.B.; Mallet, J.R.S.; Mesquita, E.; Gôlo, P.S.; Fiorotti, J.; Bittencourt, V.R.E.P.; Pontes, E.G.; Angelo, I.C. Larvicidal activity, route of interaction and ultrastructural changes in *Aedes aegypti* exposed to entomopathogenic fungi. *Acta Trop.* **2021**, *213*, 105732. [CrossRef] [PubMed]
30. Samuels, R.I.; Paula, A.R.; Carolino, A.T.; Gomes, S.A.; Morais, C.O.P.; Cypriano, M.B.C. Entomopathogenic organisms: Conceptual advances and real-world applications for mosquito biological control. *Open Access Insect Physiol.* **2016**, *6*, 25–31. [CrossRef]
31. Bitencourt, R.O.B.; de Sousa Queiroz, R.R.; Ribeiro, A.; Ribeiro, Y.R.d.S.; Boechat, M.S.B.; Carolino, A.T.; Santa-Catarina, C.; Samuels, R.I. Encapsulation of *Beauveria bassiana* conidia as a new strategy for the biological control of *Aedes aegypti* larvae. *Sci Rep.* **2024**, *14*, 31894. [CrossRef]
32. Charnley, A.K. Fungal pathogens of insects: Cuticle degrading enzymes and toxins. *Adv. Bot. Res.* **2003**, *40*, 241–321. [CrossRef]
33. Butt, T.M.; Coates, C.J.; Dubovskiy, I.M.; Ratcliffe, N.A. Entomopathogenic fungi: New insights into host-pathogen interactions. *Adv. Genet.* **2016**, *94*, 307–364. [CrossRef] [PubMed]
34. Silva, R.O.; Silva, H.H.G.; Luz, C. Effect of *Metarhizium anisopliae* isolated from soil samples of the central Brazilian Cerrado against *Aedes aegypti* larvae under laboratory conditions. *Rev. Patol. Trop.* **2004**, *33*, 207–216. [CrossRef]
35. Bitencourt, R.O.B.; Farias, F.S.; Freitas, M.C.; Balduino, C.J.R.; Mesquita, E.S.; Corval, A.R.C.; Golo, P.S.; Pontes, E.G.; Bittencourt, V.R.E.P.; Angelo, I.C. In vitro control of *Aedes aegypti* larvae using *Beauveria bassiana*. *Int. J. Biol. Sci.* **2018**, *12*, 400–404. [CrossRef]
36. Carolino, A.T.; Teodoro, T.B.P.; Gomes, S.A.; Silva, C.P.; Samuels, R.I. Production of conidia using different culture media modifies the virulence of the entomopathogenic fungus *Metarhizium* against *Aedes aegypti* larvae. *J. Vector Borne Dis.* **2021**, *58*, 346–351. [CrossRef]
37. Bukhari, T.; Middelman, A.; Koenraadt, C.J.M.; Takken, W.; Knols, B.G.L. Factors affecting fungus-induced larval mortality in *Anopheles gambiae* and *Anopheles stephensi*. *Malar. J.* **2010**, *9*, 22. [CrossRef] [PubMed]
38. Carolino, A.T.; Gomes, S.A.; Teodoro, T.B.P.; Mattoso, T.C.; Samuels, R.I. *Aedes aegypti* pupae are highly susceptible to infection by *Metarhizium anisopliae* blastospores. *J. Pure Appl. Microbiol.* **2019**, *13*, 1629–1634. [CrossRef]
39. Scholte, E.; Takken, W.; Knols, B.G. Infection of the malaria mosquito *Anopheles gambiae* with the entomopathogenic fungus *Metarhizium anisopliae* reduces blood feeding and fecundity. *J. Invertebr. Pathol.* **2006**, *91*, 43–49. [CrossRef] [PubMed]
40. Mnyone, L.L.; Kirby, M.J.; Mpingwa, M.W.; Lwetoijera, D.W.; Knols, B.G.; Takken, W.; Koenraadt, C.J.M.; Russell, T.L. Infection of *Anopheles gambiae* mosquitoes with entomopathogenic fungi: Effect of host age and blood-feeding status. *Parasitol. Res.* **2011**, *108*, 317–322. [CrossRef]
41. Paula, A.R.; Imad-Silva, L.E.; Ribeiro, A.; Silva, G.A.; Silva, C.P.; Butt, T.M. *Metarhizium anisopliae* blastospores are highly virulent to adult *Aedes aegypti*, an important arbovirus vector. *Parasit. Vectors* **2021**, *14*, 555. [CrossRef]
42. Darbro, J.M.; Johnson, P.H.; Thomas, M.B.; Ritchie, S.A.; Kay, B.H.; Ryan, P.A. Effects of *Beauveria bassiana* on survival, blood-feeding success, and fecundity of *Aedes aegypti* in laboratory and semi-field conditions. *Am. J. Trop. Med. Hyg.* **2012**, *86*, 656–664. [CrossRef]
43. Blanford, S.; Chan, B.H.K.; Jenkins, N.; Sim, D.; Turner, R.J.; Read, A.F.; Thomas, M.B. Fungal pathogen reduces potential for malaria transmission. *Science* **2005**, *308*, 1638–1641. [CrossRef]
44. Dillon, R.J.; Dillon, V.M. The gut bacteria of insects: Nonpathogenic interactions. *Annu. Rev. Entomol.* **2004**, *49*, 71–92. [CrossRef]
45. Engel, P.; Moran, N.A. The gut microbiota of insects: Diversity in structure and function. *FEMS Microbiol. Rev.* **2013**, *37*, 699–735. [CrossRef]
46. Gaio, A.O.; Gusmão, D.S.; Santos, A.V.; Berbert-Molina, M.A.; Pimenta, P.F.P.; Lemos, F.J. Contribution of midgut bacteria to blood digestion and egg production in *Aedes aegypti* (Diptera: Culicidae) (L.). *Parasit. Vectors* **2011**, *4*, 105. [CrossRef]
47. Dale, C.; Moran, N.A. Molecular interactions between bacterial symbionts and their hosts. *Cell* **2006**, *126*, 453–465. [CrossRef]
48. Jayakrishnan, L.; Sudhikumar, A.V.; Aneesh, E.M. Role of gut inhabitants on vectorial capacity of mosquitoes. *J. Vector Borne Dis.* **2018**, *55*, 69–78. [CrossRef]

49. Zhang, L.; Wang, D.; Shi, P.; Li, J.; Niu, J.; Chen, J.; Wang, G.; Wu, L.; Chen, L.; Yang, Z.; et al. A naturally isolated symbiotic bacterium suppresses flavivirus transmission by *Aedes* mosquitoes. *Science* **2024**, *384*, eadn9524. [CrossRef]
50. Cai, J.A.; Christophides, G.K. Immune interactions between mosquitoes and microbes during midgut colonization. *Curr. Opin. Insect Sci.* **2024**, *63*, 101195. [CrossRef] [PubMed]
51. Wei, G.; Lai, Y.; Wang, G.; Chen, H.; Li, F.; Wang, S. Insect pathogenic fungus interacts with the gut microbiota to accelerate mosquito mortality. *Proc. Natl. Acad. Sci. USA* **2017**, *114*, 5994–5999. [CrossRef] [PubMed]
52. Zhang, F.; Sun, X.X.; Zhang, X.C.; Zhang, S.; Lu, J.; Xia, Y.M.; Huang, Y.H.; Wang, X.J. The interactions between gut microbiota and entomopathogenic fungi: A potential approach for biological control of *Blattella germanica* (L.). *Pest Manag. Sci.* **2018**, *74*, 438–444. [CrossRef] [PubMed]
53. Gichuhi, J.; Khamis, F.; Van den Berg, J.; Mohamed, S.; Ekesi, D.; Herren, J.K. Influence of inoculated gut bacteria on the development of *Bactrocera dorsalis* and on its susceptibility to the entomopathogenic fungus *Metarhizium anisopliae*. *BMC Microbiol.* **2020**, *20*, 321. [CrossRef]
54. Paula, A.R.; Brito, E.S.; Pereira, C.R.; Carrera, M.P.; Samuels, R.I. Susceptibility of adult *Aedes aegypti* (Diptera: Culicidae) to infection by *Metarhizium anisopliae* and *Beauveria bassiana*: Prospects for dengue vector control. *Biocontrol Sci. Technol.* **2008**, *18*, 1017–1025. [CrossRef]
55. Valência, M.D.; Miller, L.H.; Mazur, P. Permeability of intact and dechorionated eggs of the *Anopheles* mosquito to water vapor and liquid water: A comparison with *Drosophila*. *Cryobiology* **1996**, *33*, 142–148. [CrossRef]
56. Forattini, O.P. *Culicidologia Médica. 1º Edição*; Edusp: São Paulo, Brazil, 2002; Volume 2, p. 864, ISBN 13: 9788531406997.
57. Hou, K.; Wu, Z.X.; Chen, X.Y.; Wang, J.Q.; Zhang, D.; Xiao, C.; Zhu, D.; Koya, J.B.; Wei, L.; Li, J.; et al. Microbiota in health and diseases. *Signal Transduct. Target. Ther.* **2022**, *7*, 135. [CrossRef] [PubMed]
58. Zouache, K.; Raharimalala, F.N.; Raquin, V.; Tran-Van, V.; Raveloson, L.H.; Ravelonandro, P.; Maving, P. Bacterial diversity of field-caught mosquitoes, *Aedes albopictus* and *Aedes aegypti*, from different geographic regions of Madagascar. *FEMS Microbiol. Ecol.* **2011**, *75*, 377–389. [CrossRef] [PubMed]
59. Dong, Y.; Manfredini, F.; Dimopoulos, G. Implication of the mosquito midgut microbiota in the defense against malaria parasites. *PLoS Pathog.* **2009**, *5*, e1000423. [CrossRef]
60. Demko, A.M.; Patin, N.V.; Jensen, P.R. Microbial diversity in tropical marine sediments assessed using culture-dependent and culture-independent techniques. *Environ. Microbiol.* **2021**, *23*, 6859–6875. [CrossRef] [PubMed] [PubMed Central]
61. Samuels, R.I.; Charnley, A.K.; Reynolds, S.E. The role of destruxins in the pathogenicity of three strains of *Metarhizium anisopliae* for the tobacco hornworm *Manduca sexta*. *Mycopathologia* **1998**, *104*, 51–58. [CrossRef]
62. Vilcinskas, A.; Götz, P. Parasitic fungi and their interactions with the insect immune system. *Adv. Parasitol.* **1999**, *43*, 267–313. [CrossRef]
63. Barletta, A.B.; Nascimento-Silva, M.C.L.; Talyuli, O.A.C.; Oliveira, J.H.M.; Pereira, L.O.R.; Oliveira, P.L.; Sorgine, M.H.F. Microbiota activates IMD pathway and limits Sindbis infection in *Aedes aegypti*. *Parasit. Vectors* **2017**, *10*, 103. [CrossRef]
64. Rodrigues, J.; Brayner, A.B.; Alves, L.C.; Dixit, R.; Barillas-Mury, C. Hemocyte differentiation mediates innate immune memory in *Anopheles gambiae* mosquitoes. *Science* **2010**, *329*, 1353–1355. [CrossRef]
65. Seitz, H.M.; Maier, W.A.; Rottok, M.; Becker-Feldmann, H. Concomitant infections of *Anopheles stephensi* with *Plasmodium berghei* and *Serratia marcescens*: Additive detrimental effects. *Zentralbl. Bakteriol. Mikrobiol. Hyg. A* **1987**, *266*, 155–166. [CrossRef]
66. Beier, M.S.; Pumpuni, C.B.; Beier, J.C.; Davis, J.R. Effects of para-aminobenzoic acid, insulin, and gentamicin on *Plasmodium falciparum* development in anopheline mosquitoes (Diptera: Culicidae). *J. Med. Entomol.* **1994**, *31*, 561–565. [CrossRef] [PubMed]
67. Xi, Z.; Ramirez, J.L.; Dimopoulos, G. The *Aedes aegypti* Toll pathway controls dengue virus infection. *PLoS Pathog.* **2008**, *4*, e1000098. [CrossRef] [PubMed]
68. Gusmão, D.S.; Santos, A.V.; Marini, D.C.; Bacci, M.; Berbert-Molina, M.A.; Lemos, F.J.A. Culture-dependent and culture-independent characterization of microorganisms associated with *Aedes aegypti* (Diptera: Culicidae) (L.) and dynamics of bacterial colonization in the midgut. *Acta Trop.* **2010**, *115*, 275–281. [CrossRef] [PubMed]
69. Hill, P.D.S.; Campbell, J.A. The production of symbiont-free *Glossina morsitans* and an associated loss of female fertility. *Trans. R. Soc. Trop. Med. Hyg.* **1973**, *67*, 727–728. [CrossRef]
70. Nogge, G. Sterility in tsetse flies (*Glossina morsitans* Westwood) caused by loss of symbionts. *Experientia* **1976**, *32*, 995–996. [CrossRef]
71. Tang, D.; Chen, J.; Zhang, Y.; Tang, X.; Wang, X.; Yu, C.; Cheng, X.; Zhang, J.; Shi, W.; Zhen, Q.; et al. Engineered *Metarhizium* fungi produce longifolene to attract and kill mosquitoes. *Nat. Microbiol.* **2025**, *10*, 3075–3093. [CrossRef]