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Studying the Interaction between *Chromobacterium anophelis* and the Entomopathogenic Fungus *Metarhizium pingshaense* in *Aedes aegypti*, the Dengue Vector in Burkina Faso

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Abstract

Insect-bacteria associations can influence vector competence in multiple ways. *Chromobacterium anophelis*, a bacterium known to be pathogenic to mosquitoes, may also act as an anti-pathogen by inhibiting the development of other pathogens within mosquitoes. The mechanism behind this inhibition remains unclear, with two hypotheses: the bacterium either boosts the mosquito's immunity or directly targets other pathogens within the mosquito. The objective of this study is to elucidate the mechanism behind this pathogen-inhibition effect in mosquitoes. Bioassays (assessing longevity, fecundity, and fertility) were conducted on mosquitoes infected with the bacterium and an entomopathogenic fungus, *Metarhizium pingshaense*, whose hyphae grow on mosquitoes after killing them. The prevalence of hyphal growth was evaluated. The longevity of mosquitoes co-infected with *Chromobacterium anophelis* and *Metarhizium pingshaense* was significantly higher than those infected solely with the more virulent microorganism, which was *Chromobacterium anophelis*. Hyphae were observed on 100% of mosquitoes infected only with the fungus, whereas mosquitoes co-infected with the bacterium exhibited a lower prevalence of fungal hyphal growth. The number of eggs laid by infected mosquitoes was approximately the same, within the typical range (50 - 150). However, the number of larvae observed from co-infected mosquitoes was

significantly higher than those produced by mosquitoes exposed to the microorganism that most reduced egg hatch rates. These results align with the hypothesis that *Chromobacterium anophelis* inhibits the development of other pathogens within mosquitoes by directly targeting them.

Keywords

Biological Control, Vector Control, Anti-Pathogen Activity, *Chromobacterium anophelis*, *Metarhizium pingshaense*, *Aedes aegypti*, Dengue, Burkina Faso

1. Introduction

Epidemic and potentially pandemic diseases remain a significant threat to health security, both regionally and globally. Among these, dengue stands out as the most widespread arboviral disease, as recognized by the World Health Organization (WHO). Each year, dengue affects nearly 390 million individuals, of which 96 million exhibit clinical symptoms. Of the 500,000 severe cases annually, approximately 2.5% result in fatalities [1]. This disease predominantly impacts regions infested by Aedes mosquito vectors, such as *Aedes aegypti* and *Aedes albopictus*, which now inhabit over 100 countries, placing approximately 3.9 billion people more than 55% of the global population at risk [2]. In Burkina Faso, despite significant public health efforts, major dengue outbreaks occurred in 2016, 2017, and 2023 [3]. These Aedes mosquitoes not only transmit dengue but are also vectors for other serious viruses, including chikungunya, yellow fever, and Zika. The severity of these diseases ranges from asymptomatic infections to life-threatening complications affecting vital organs. Alarmingly, global climate change exacerbates this challenge by expanding the geographical range of Aedes mosquitoes and accelerating viral replication cycles, thus increasing the burden of these diseases [4]. Efforts to combat dengue and other arboviral diseases have centered on vaccine development and the widespread use of chemical insecticides for vector control. However, the rise of insecticide resistance among mosquito populations has significantly reduced the effectiveness of chemical approaches [5]. This growing challenge underscores the urgent need to explore alternative control measures, such as biological control using microorganisms. Recent research has highlighted the potential of microorganisms in vector management. For example, in 2014, Ramirez and collaborators demonstrated that a strain of bacterium *Chromobacterium Csp* is not only pathogenic to *Aedes aegypti* but also inhibits dengue virus development within these mosquitoes. Although the exact mechanisms remain unclear, one hypothesis suggests that the bacterium enhances the mosquito's immune response, thereby offering protection against subsequent infections [6]. Similarly, strains of the entomopathogenic fungus *Metarhizium pingshaense* have shown high virulence against mosquitoes, causing a lethal disease known as green

muscardine [7]. Building on these findings, this study seeks to investigate the interactions between *Chromobacterium anophelis* and *Metarhizium pingshaense* in *Aedes aegypti*. Specifically, we aimed to: 1) Assess the impacts of interactions between *Chromobacterium anophelis* and *Metarhizium pingshaense* on *Aedes aegypti*; 2) Evaluate the survival of *Aedes aegypti* following co-infection with these microorganisms; 3) Examine the fecundity and fertility of *Aedes aegypti* after co-infection with *Chromobacterium anophelis* and *Metarhizium pingshaense*.

2. Materials and Methods

2.1. Rearing and Maintenance of Adult Female *Aedes aegypti* in the Insectarium

To obtain adult female *Ae. aegypti*, we hatched eggs collected from oviposition substrates. These eggs were originally laid by mosquitoes captured in the field. The hatching process involved immersing the oviposition substrate in water. Submerged eggs underwent embryonic development, culminating in hatching, which could take from one day to a week. Daily monitoring of the container ensured detection of hatching, evidenced by the presence of larvae. Fragile larvae were carefully transferred to water-filled containers and fed twice daily with TetraMin. The quality and quantity of the larval diet were critical for survival and subsequent development. Overfeeding led to high larval mortality, while underfeeding resulted in smaller-than-average adults. Once larvae developed into pupae, they were sorted, isolated in cups, and transferred to mosquito-rearing cages (30 cm × 30 cm × 30 cm or 15 cm × 15 cm × 15 cm) for adult emergence. Adults were provided with a 5% glucose solution absorbed in cotton placed on top of the cages for daily feeding. After four days of cohabitation to promote mating, males were separated from females using a mouth aspirator, a process known as sexing based on morphological differences. Males continued receiving the glucose solution, while females were fed either glucose solution or chicken blood, depending on the study requirements. The insectarium conditions, including a thermostat, humidifier, and neon lighting, were standardized to maintain temperature at 27˚C ± 2˚C, humidity at 80% ± 10%, and a photoperiod from 7:30 AM to 6:30 PM. These environmental controls ensured the reliability and repeatability of the experiments by replicating natural mosquito habitats.

2.2. Mosquito Infection Method with *Metarhizium* Strain Met_S26 and *Chromobacterium anophelis*

2.2.1. Infection with *Chromobacterium anophelis*

For mosquitoes, the midgut is a critical site for the propagation of pathogens such as bacteria, viruses, and certain eukaryotic parasites [8]. Mosquitoes were infected with *C. anophelis* via ingestion by offering a sugar meal (5% glucose solution absorbed in cotton) supplemented with bacterial cells at a concentration of 1×10^7 cells/mL. This concentration, as per Gnambani *et al.*, is effective for controlling mosquito populations.

2.2.2. Infection with Met_S26

Unlike bacteria and viruses, microscopic fungi such as Metarhizium spp. infect mosquitoes through direct contact with their cuticle [9]. To infect mosquitoes with Metarhizium strain Met_S26, mosquitoes were immobilized using a freezer for 30 seconds and subsequently sprayed with fungal spores at varying concentrations (1×10^8, 1×10^7, and 1×10^6 spores/mL) using a sprayer.

2.3. Assessment of the Virulence of *C. anophelis* and *Metarhizium pingshaense* Co-Infection in *Aedes aegypti*

This experiment assessed the longevity of *A. aegypti* after co-infection with Met_S26 and *C. anophelis*. Fifteen groups of 50 mosquitoes were used in cups (800 cm^3), divided as follows:

Nine groups: Doubly infected with Met_S26 and *C. anophelis* at three fungal spore concentrations (1×10^8, 1×10^7, and 1×10^6 spores/mL), each with three replicates. Six groups: Control and single-infection replicates:

Two uninfected control replicates.

Two replicates infected with Met_S26 (1×10^7 spores/mL).

Two replicates infected with *C. anophelis* (1×10^7 bacterial cells/mL).

Post-infection, mosquitoes were maintained under standard insectarium conditions, fed a 5% glucose solution, and monitored for mortality over 14 days [10]. Dead mosquitoes were collected twice daily, disinfected with 2% bleach for 20 seconds to remove residual Met_S26 spores, rinsed with sterile distilled water for 40 seconds, and incubated on 1% agar at 25˚C ± 1˚C. Five days post-incubation, the cause of death was determined using a stereomicroscope to observe fungal hyphae growth. *Metarhizium* spp. fungi are saprophytic and grow for 3 - 5 days postmortem, forming white mycelia and green spores. Additionally, mosquitoes exposed to *C. anophelis*-contaminated sugar meals were homogenized with beads and cultured on BCP agar to confirm bacterial presence. The control groups played a crucial role in this study by serving as a baseline for comparing the effects of infections. By maintaining uninfected and single-infection groups, we ensured that observed differences in mortality and other outcomes were specifically attributable to the co-infection treatment.

2.4. Evaluation of the Impact of *C. anophelis-M. pingshaense* Co-Infection on Fertility and Fecundity of *A. aegypti*

We evaluated the impact of co-infection on fertility and fecundity using four groups of 60 inseminated females:

Control group: Uninfected mosquitoes.

Single-infection groups: Mosquitoes infected with either Met_S26 (1×10^7 spores/mL) or *C. anophelis* (1×10^7 bacterial cells/mL).

Co-infection group: Mosquitoes infected with both Met_S26 and *C. anophelis* (respective concentrations: 1×10^7 spores/mL and 1×10^7 cells/mL).

Females were placed in individual oviposition cups (20 cL, filled ¼ with water and containing an oviposition substrate) after a 30-minute chicken blood meal.

Eggs were collected and counted daily for three days using a manual counter and stereomicroscope. Eggs were subsequently hatched, and L1 larvae were counted 2 - 5 days post-hatching. Females that did not lay eggs within a week were excluded from the analysis.

2.5. Data Processing and Analysis

Data were entered and verified using Microsoft Excel 2013. Statistical analysis was performed using R Studio version 1.1.453 (2018). All relevant R codes for the analysis are available on the GitHub repository: GitHub Repository. Environmental controls and inclusion of control groups enhanced the robustness of the experimental design, minimizing external variability and ensuring reproducibility.

3. Results

3.1. Virulence of Co-Infection with *C. anophelis* and *M. pingshaense* in *Aedes aegypti*

Mortality monitoring of treated mosquitoes and controls over 14 days, as shown in Figure 1, revealed significant differences in survival across treatments (Kaplan-Meier survival analysis, log-rank test, $p < 0.001$).

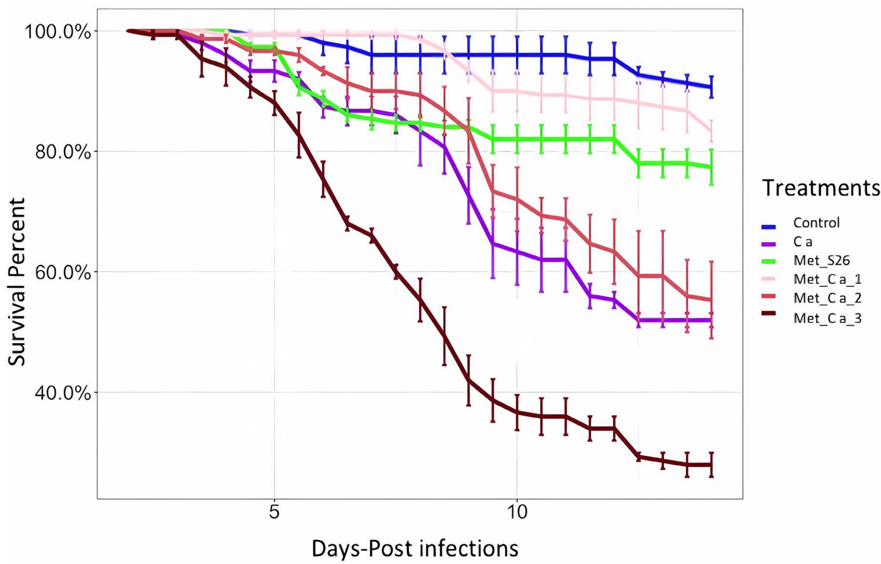

Figure 1. Survival curves of *Aedes aegypti* following infection with various microorganism suspensions. (C a = 10^7 bacterial cells/mL, Met_S26 = 10^7 spores/mL, Met_C a_1 = 10^6 spores/mL + 10^7 bacterial cells/mL, Met_C a_2 = 10^7 spores/mL + 10^7 bacterial cells/mL, Met_C a_3 = 10^8 spores/mL + 10^7 bacterial cells/mL).

The treatment with the highest concentration of *Metarhizium pingshaense* strain S26 (10^8 spores/mL) in combination with *Chromobacterium anophelis* (10^7 bacterial cells/mL) achieved the shortest LT50, at 9 days (95% CI: 8.5 - 9.5 days). This suggests a synergistic interaction between the two agents at high doses, accelerating mortality. The single infection with *C. anophelis* at 10^7 bacterial

cells/mL reached its LT50 on day 12 (95% CI: 11.5 - 12.5 days), indicating its effectiveness as a stand-alone treatment but with a slower impact on mosquito survival compared to the double infection. Meanwhile, the co-infection with *C. anophelis* at 10^7 bacterial cells/mL and Met_S26 at 10^7 spores/mL resulted in an LT50 on day 13 (95% CI: 12.5 - 13.5 days), reflecting a less pronounced effect, potentially due to suboptimal concentrations of the fungal spores. Effect sizes were quantified using Cox proportional hazards modeling, which showed a hazard ratio (HR) of 2.3 (95% CI: 1.8 - 3.0, $p < 0.001$) for the high-concentration double infection relative to untreated controls, indicating a more than twofold increase in mortality risk. For the single infection with *C. anophelis* (10^7 bacterial cells/mL), the HR was 1.5 (95% CI: 1.2 - 2.0, $p < 0.01$), while the co-infection at lower fungal concentrations yielded an HR of 1.3 (95% CI: 1.1 - 1.7, $p < 0.05$).

Figure 2 illustrates the prevalence of hyphal outgrowth from Metarhizium anisopliae strain S26 (Met_S26) on dead mosquitoes placed on culture media during the two-week observation period. No hyphal growth was observed in the control group or in mosquitoes infected solely with *Chromobacterium anophelis* (*C. anophelis*). In contrast, hyphal outgrowth was consistently detected in mosquitoes exposed to Met_S26 spores, indicating fungal colonization of the host tissue.

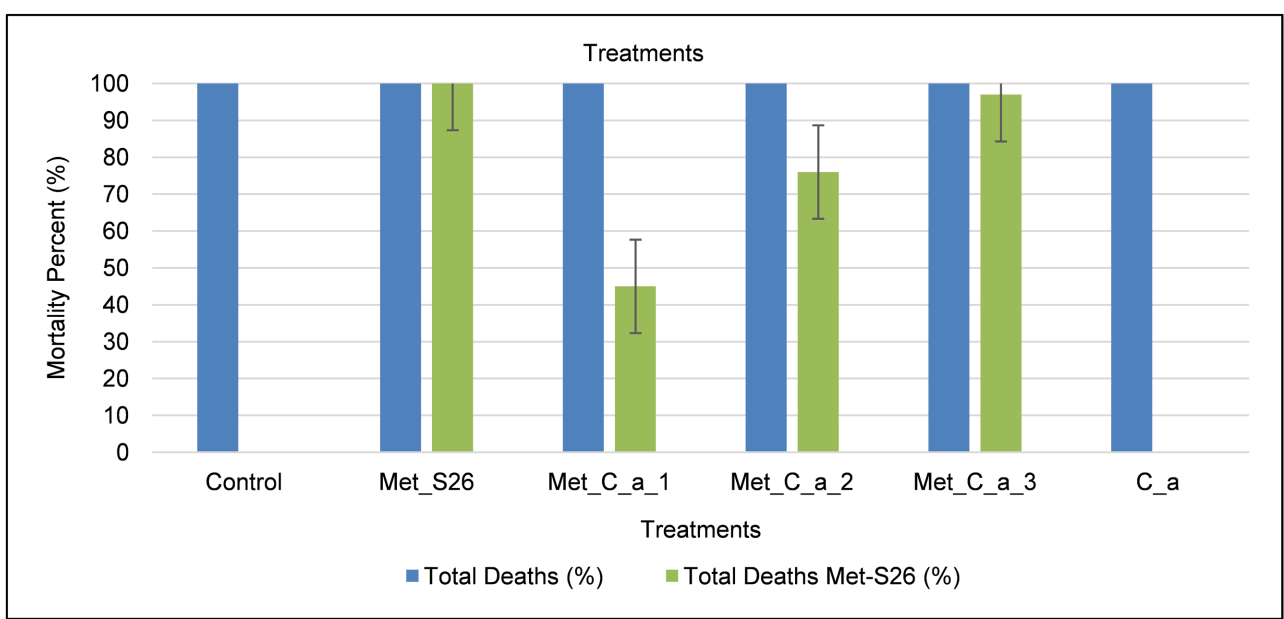

Figure 2. Prevalence of *Metarhizium pingshaense* hyphal outgrowth in dead mosquitoes collected during the survival test.

Specifically, hyphal growth was detected in 45% of mosquitoes co-infected with 10^6 spores/mL of Met_S26 and 10^7 bacterial cells/mL of *C. anophelis* (95% CI: 35% - 55%). This prevalence increased to 76% in mosquitoes co-infected with 10^7 spores/mL of Met_S26 and 10^7 bacterial cells/mL of *C. anophelis* (95% CI: 68% - 84%). The highest prevalence of hyphal outgrowth was observed in mosquitoes co-infected with 10^8 spores/mL of Met_S26 and 10^7 bacterial cells/mL of *C. anophelis*, reaching 98% (95% CI: 94% - 100%), suggesting a dose-dependent

relationship between fungal concentration and colonization. Statistical analysis using Fisher's Exact Test revealed significant differences in hyphal outgrowth prevalence between treatment groups ($p < 0.001$). The odds ratio (OR) for hyphal growth in mosquitoes co-infected with 10^7 spores/mL and 10^7 bacterial cells/mL relative to the control group was 12.3 (95% CI: 7.5 - 19.8), indicating a significantly higher likelihood of fungal colonization. For the high-concentration co-infection (10^8 spores/mL and 10^7 bacterial cells/mL), the OR was 23.1 (95% CI: 16.0 - 34.3), further supporting the enhanced fungal colonization at higher fungal doses. Biologically, the observed dose-dependent increase in hyphal outgrowth is consistent with the hypothesis that higher concentrations of Met_S26 spores facilitate greater fungal colonization of the mosquito body, likely due to an increased initial fungal load that overwhelms the insect's immune defenses. The near-complete colonization at the highest concentration (98% prevalence) underscores the potential of Met_S26 as an effective biocontrol agent, particularly when combined with *C. anophelis*, which may weaken the mosquito's immune system. In contrast, the absence of hyphal growth in the control and single-infection groups suggests that *C. anophelis* alone does not promote fungal growth, emphasizing the crucial role of Met_S26 in this interaction.

3.2. Impact of Co-Infection on *Aedes aegypti* Fecundity

For the four groups of 60 mosquitoes subjected to individual oviposition, 58 mosquitoes per group were analyzed, as two mosquitoes from one treatment drowned before laying eggs. Figure 3 presents the average number of eggs laid per treatment.

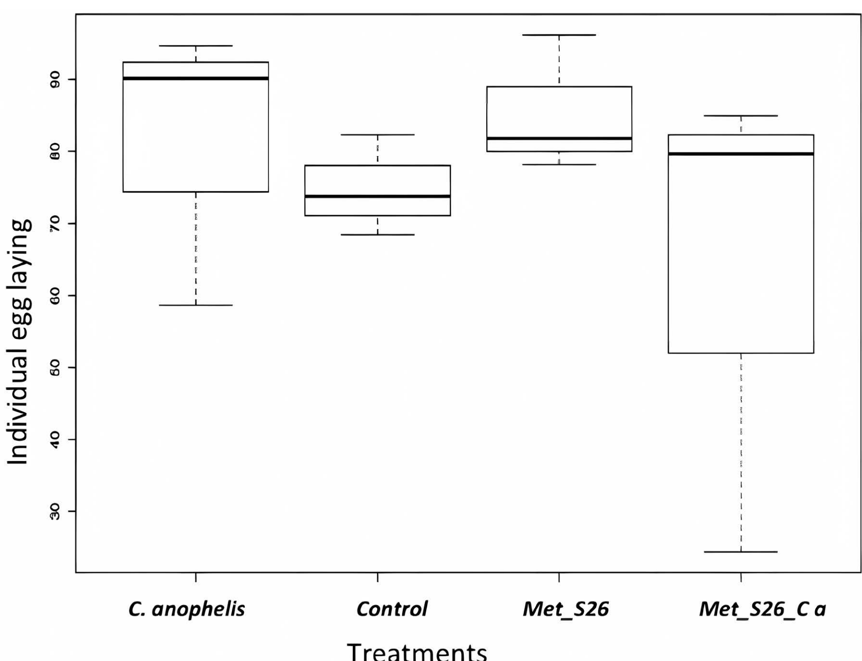

Figure 3. Fecundity of *Aedes aegypti* following infection with various microorganism suspensions (*C. anopehelis* = 10^7 bacterial cells/mL, Met_S26 = 10^7 spores/mL, Met_S26_C a = 10^7 spores/mL + 10^7 bacterial cells/mL).

Females infected with 10^7 bacterial cells/mL of *Chromobacterium anophelis* (*C. anophelis*) laid an average of 90 eggs each (95% CI: 85 - 95), while those infected with 10^7 spores/mL of *Metarhizium anisopliae* strain S26 (Met_S26) laid an average of 82 eggs each (95% CI: 76 - 88). Females co-infected with 10^7 bacterial cells/mL of *C. anophelis* and 10^7 spores/mL of Met_S26 laid an average of 79 eggs each (95% CI: 73 - 85), and the control group laid an average of 74 eggs each (95% CI: 69 - 79).

Statistical analysis using one-way ANOVA with Tukey's post-hoc test revealed no significant differences in egg-laying between any of the infected groups or between the infected groups and the control group (F (3, 228) = 1.45F (3, 228) = 1.45F (3, 228) = 1.45, p = 0.23 p = 0.23 p = 0.23), indicating that the treatments did not significantly impact egg production. Effect size was calculated using Cohen's d, with no substantial differences observed between the treatment groups and the control group. The effect size for the group infected with *C. anophelis* (10^7 bacterial cells/mL) compared to the control group was d = 0.24 d = 0.24 d = 0.24, indicating a small effect, while the effect size for the group infected with Met_S26 (10^7 spores/mL) was d = 0.29 d = 0.29 d = 0.29, and the co-infection group had an effect size of d = 0.35 d = 0.35 d = 0.35.

3.3. Impact of Co-Infection on *Aedes aegypti* Egg Fertility

Figure 4 presents the number of eggs hatched into larvae for each treatment group following individual egg-laying.

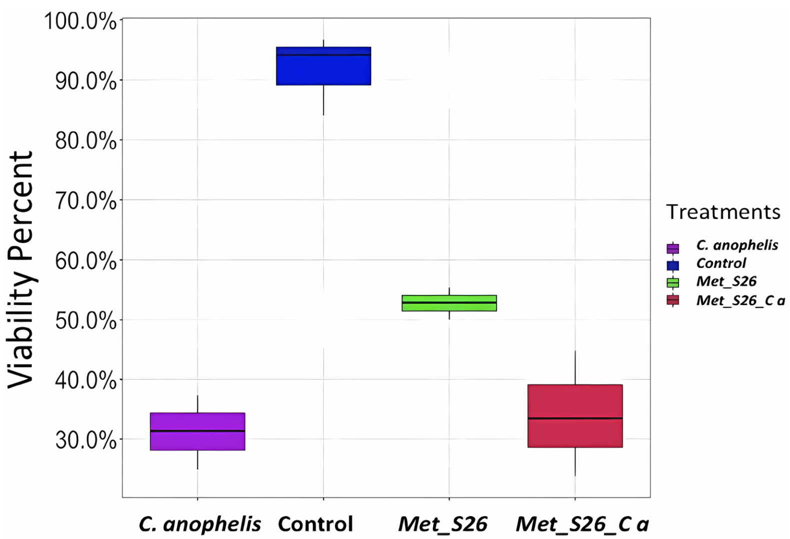

Figure 4. Fertility of *Aedes aegypti* eggs following infection with various microorganism suspensions.

Females infected with *Chromobacterium anophelis* at 10^7 bacterial cells/mL and those co-infected with *C. anophelis* at 10^7 bacterial cells/mL and *Metarhizium anisopliae* strain S26 (Met_S26) at 10^7 spores/mL had similar hatching rates of 32% (95% CI: 27% - 37%) and 34% (95% CI: 29% - 39%), respectively. These

hatching rates were significantly lower than that of females infected with Met_S26 at 10^7 spores/mL, which achieved a 54% hatching rate (95% CI: 48% - 60%), and substantially lower than the control group, which achieved a 95% hatching rate (95% CI: 92% - 98%).

Statistical analysis using one-way ANOVA with Tukey's post-hoc test revealed significant differences in hatching rates between the infected groups and the control group (F (3, 228) = 86.72F (3, 228) = 86.72F (3, 228) = 86.72, p < 0.001 p < 0.001 p < 0.001). The control group had a significantly higher hatching rate than all other treatment groups, and the Met_S26 group (10^7 spores/mL) exhibited a significantly higher hatching rate than both the *C. anophelis* and co-infection groups. Effect sizes were calculated using Cohen's d. The effect size for the control group compared to the *C. anophelis* infection group was d = 3.91 d = 3.91 d = 3.91, indicating a large effect, while the effect size for the Met_S26 (10^7 spores/mL) infection compared to the *C. anophelis* group was d = 1.92 d = 1.92 d = 1.92, suggesting a moderate effect. The co-infection group had a similar effect size of d = 1.89 d = 1.89 d = 1.89 when compared to the control group.

4. Discussion

This study clearly demonstrates the interaction between the entomopathogenic fungus *Metarhizium pingshaense* (Met_S26) and the bacterium *Chromobacterium anophelis* when cohabiting in *Aedes aegypti*. The findings highlight both the virulence of individual infections and the intriguing protective effects observed in co-infected mosquitoes. While both single infections with *Chromobacterium Csp* (as shown by Ramirez *et al.*) and Met_S26 [7] are pathogenic, our longevity analysis revealed that mosquitoes co-infected with both microorganisms survived longer than those infected by the more virulent pathogen alone. For example, mosquitoes co-infected with 10^7 spores/mL of Met_S26 and 10^7 bacterial cells/mL of *C. anophelis* lived longer than those infected only with *C. anophelis* at 10^7 bacterial cells/mL, the more virulent of the two pathogens. This extended survival suggests that the presence of *C. anophelis* may mitigate the virulence of Met_S26, indicating a complex interaction between the two pathogens. This finding contrasts with the typical expectation of enhanced virulence in co-infections, where pathogens are often thought to work synergistically to cause greater harm.

The extended survival of co-infected mosquitoes can be attributed to a potential protective mechanism within the mosquito, possibly involving *C. anophelis*. The bacterium, despite its pathogenic nature, has been reported to protect mosquitoes against other infections. Ramirez *et al.* propose two mechanisms for *Chromobacterium Csp*-mediated protection: the activation of mosquito immune responses leading to the secretion of antimicrobial peptides, and direct interference with pathogen virulence through bioactive compounds such as violacein [6]. Our results provide support for the latter, as we observed inhibition of Met_S26 hyphal growth in some co-infected mosquitoes. This suggests that *C. anophelis* interferes with the fungal pathogen's development, potentially limiting its virulence in the

co-infection scenario. This interference likely reduces the overall impact of the co-infection relative to the more virulent *C. anophelis* mono-infection, providing the mosquito with a survival advantage. This finding is particularly noteworthy in light of previous studies, such as those by Holt *et al.* (2024), which highlighted the role of microbial interactions in mediating the virulence of pathogens in insect hosts.

In terms of mosquito fertility, co-infection reduced egg hatching rates similarly to single infections, but more larvae were observed in co-infected mosquitoes compared to those infected solely with *C. anophelis*. This suggests a protective effect of *C. anophelis* against Met_S26, which appears to aid embryonic development despite the presence of the fungus. The ability of *C. anophelis* to support the development of embryos in the presence of the fungal pathogen is consistent with its known role in modulating the immune environment of mosquitoes, potentially enhancing the survival of developing eggs. Previous studies have shown that bacteria in the mosquito gut can influence reproductive success by promoting beneficial immune responses [11]. In our study, *C. anophelis* might be modulating immune responses or creating a more favorable environment for embryonic development, counteracting the detrimental effects of the fungal infection. These results align with research by Ippolito *et al.* (2018) [12], who demonstrated that certain bacterial species could influence reproductive fitness in *Anopheles* mosquitoes, but the specific interactions between fungal pathogens and bacteria remain underexplored. Our study thus adds valuable insight into the broader ecological dynamics of mosquito-microbe interactions and their potential applications in integrated pest management.

Our study contributes to the growing body of literature on mosquito-pathogen interactions by highlighting the potential for microbial co-infections to influence mosquito survival, fertility, and disease transmission. It also provides experimental evidence supporting the idea that *C. anophelis* can act as a modulator of Met_S26 virulence, suggesting new avenues for biocontrol strategies. However, our findings are constrained by several factors. First, the study was conducted under controlled laboratory conditions, which may not fully replicate the complexity of natural environments where ecological factors such as temperature, humidity, and mosquito density could influence microbial interactions. The long-term ecological consequences of co-infections in the wild remain uncertain. Additionally, while we focused on *C. anophelis* and Met_S26, other microbial species could play an equally important or synergistic role in shaping mosquito fitness and disease transmission dynamics.

Another limitation is the focus on a single mosquito species, *Aedes aegypti*, which, while highly relevant to vector control efforts, may not generalize to other mosquito species or regions with different microbial communities. Further studies examining the interactions between Met_S26, *C. anophelis*, and other mosquito species, particularly in the context of field conditions, are necessary to fully understand the potential of these microbial agents for vector control.

5. Conclusions

This study provides valuable insights into the complex interactions between the entomopathogenic fungus *Metarhizium pingshaense* (Met_S26) and the bacterium *Chromobacterium anophelis* within *Aedes aegypti* mosquitoes. Our findings demonstrate that while both pathogens are virulent individually, co-infection with both microorganisms leads to longer mosquito survival compared to infection with the more virulent pathogen alone. This extended survival suggests a protective role of *C. anophelis*, likely through direct interference with the fungal pathogen's growth, aligning with previous research that highlights *C. anophelis*'s ability to modulate mosquito immune responses and suppress other pathogens. Furthermore, we observed that *C. anophelis* not only mitigated the virulence of Met_S26, but also appeared to support mosquito fertility by promoting higher larval survival rates compared to single fungal infections. These findings suggest that microbial interactions, specifically between *C. anophelis* and Met_S26, can influence mosquito fitness in a way that might reduce the effectiveness of Met_S26 as a pathogen in a co-infected mosquito population, presenting new opportunities for biocontrol strategies.

While the results are promising, the practical application of *C. anophelis* in vector control remains challenging. Further studies are required to optimize methods for introducing *C. anophelis* into natural mosquito populations and to assess its potential as part of integrated pest management strategies. Moreover, the long-term ecological implications of co-infections in the field, as well as the influence of environmental factors on microbial interactions, warrant additional investigation. Our study contributes to the growing body of knowledge on microbial interactions in mosquitoes and offers a novel perspective on the potential of *C. anophelis* as a biocontrol agent. By advancing our understanding of how microbial communities affect mosquito survival and reproduction, we pave the way for more effective vector control strategies aimed at reducing the transmission of malaria and dengue. However, the successful implementation of such strategies will require further research into optimizing microbial treatments and assessing their impact in natural environments.

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Authors' Contributions

E.M.B. and A.D. designed the study. E.M.B., J.E.G. and D.K.T. performed laboratory and field experiment. E.M.B. analysed the data. E.M.B. wrote the first draft of the manuscript. All authors read and approved the final manuscript.

Data Availability

Supplementary Materials and Methods, data and codes are available at: GitHub

Repository. Correspondence and requests for materials should be addressed to E.M.B. and A.D.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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