ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE (CVMA)

Innovative Biocontrol Strategies for Anopheline Mosquitoes: Exploring Synergistic Interactions between *Bacillus cereus* and *Enterobacter cloacae*

Master's Thesis

By

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GSR/9494/16
Health Biotechnology

May 20, 2025

AA, Ethiopia

Title: Innovative Biocontrol Strategies for Anopheline Mosquitoes: Exploring Synergistic Interactions between *Bacillus cereus* and *Enterobacter cloacae*

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May 20, 2025

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MASTER'S THESIS APPROVAL SHEET

Declaration/Approval by Student

I, Michael Abera, declare that this thesis is my original work and has not been presented for the award of any other degree in any other institutions. All the sources for the materials used for the thesis have been duly acknowledged.

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Declaration/Approval by the Supervisor

I certify that **Mr. Michael Abera's M.Sc. thesis** entitled **"Innovative Biocontrol Strategies for Anopheline Mosquitoes: Exploring Synergistic Interactions between *Bacillus cereus* and *Enterobacter cloacae*"** has been carried out under my direct supervision and recommend the thesis to be accepted as fulfilling the requirement for the degree of **Master's of Science in Biotechnology (Health Biotechnology)**.

Supervisor	**Signature**	**Date**
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We certify that **Mr. Michael Abera's M.Sc. thesis** entitled **"Innovative Biocontrol Strategies for Anopheline Mosquitoes: Exploring Synergistic Interactions between *Bacillus cereus* and *Enterobacter cloacae*"** is the final version. Therefore; we, as the examining board, approved it as the final document to be accepted as fulfilling the requirements for the degree of **Master's of Science in Biotechnology (Health Biotechnology)**.

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List of Acronyms

3-HK: 3-hydroxykynurenine

ACT: Artemisinin-based Combination Therapies

COVID-19: Corona Virus Disease of 2019

DDT: Dichloro-Diphenyl-Trichloroethane

LLIN: Long Lasting Insecticidal Nets

PPM: Parts Per Million

PGP: Plant Growth Promoting

RBC: Red Blood Cell

SMI: Sterile Male Insect

TMT: Toxic Male Technique

WHO: World Health Organization

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Abstract

The impact of anopheline mosquitoes on humans needs no introduction; being vectors of malaria in humans. While the pathogenesis of *Plasmodium* in the mosquito as an adult has been well documented, not much research has been conducted on the biocontrol on the larval stage of the mosquitoes to hinder the disease's spread.

Current methods to control the anopheline mosquitoes are chemical, using nonspecific insecticides. As such, biocontrol has become a more preferred avenue but a primary concern is the lack of persistence of said biocontrol methods in the administered environments. Ergo, the objective of this study was to evaluate the larvicidal and persistence effects of a combined formulation of the *Bacillus cereus* and *Enterobacter cloacae* bacterial species on mosquitoes to cull the Anopheles to prevent them from reaching their adult stage. Co-cultures of the bacteria were prepared and tested on insectarium bred larvae at three different concentrations (5, 7 and 10 mL), with singular bacterial cultures serving as controls.

The results were the elimination of first instars or stopping their development entirely. At 7 mL and above, the biofilm produced by the bacteria prevented eggs from hatching and suffocated the young larvae. The persistence of the co-cultures at effective doses also exceeded the previously recorded limit of 14 days for *B. cereus*, with the upper limit being 27 days (seemingly related to the extent of biofilm expansion in containers). A carefully selected bacterial co-culture is therefore a feasible method of *Anopheles* biocontrol, with potential applications on targets like *Culex* and *Aedes.*

Keywords: Anopheline mosquitoes, *Bacillus cereus*, biocontrol persistence, *Enterobacter cloacae*, larvicide, plasmodium, synergistic interactions

1. INTRODUCTION

Malaria is still a problem today like it was several decades ago, with little real impact made on the subject of eliminating the disease or at least its spread. While the distribution of malaria in the world has classified it as a tropical disease, it remains a significant issue nonetheless.

In terms of global malaria cases and deaths, there were 249 million cases in 2022, which far surpassed the pre-COVID-19 levels three years prior (about 233 million cases) with a mortality rate of 14.3 deaths per 100,000 of the population at risk as the death toll was about 608,000. The continent with the highest burden is Africa, with twelve countries taking the lion's share of cases - Burkina Faso, Cameroon, the Democratic Republic of the Congo, Ghana, India, Mali, Mozambique, Niger, Nigeria, Uganda and Tanzania with Sudan as the most recent addition (2022). These countries account for a whopping 70% of all global malaria cases – a fact further exacerbated by the logistical and financial shortcomings of said countries to even offer existing solutions to curb the spread of the disease. Malaria is also spreading in a greater number of countries due to the inclement weather of 2021-24, a good example being Pakistan, which saw an increase of 5 million new cases after a period of intense flooding in 2021-2022. COVID-19 has also played a prominent role in its spread, with the WHO documenting an increase of 32,000 new deaths in 2022 alone compared to 2019[1].

Current methods to curb the spread of malaria in terms of culling the vectors themselves are primarily chemical in nature, with a broad range of insecticides being used in a manner quite similar to pesticides in agriculture. However, the evolution of resistance to said chemicals is becoming more prominent with time, a prime example being the resistance of *Anopheles stephensi* in Ethiopia to all current insecticides used in indoor residual spraying (IRS) and long-

lasting insecticidal nets (LLINs) [2]. Another growing concern is that the chemicals used as insecticides are either also used in agriculture or their chemical analogues are, influencing *Anopheles* susceptibility through the process of mutations in certain genes [3]. Non-specific insecticides not only pose health risks to humans and animals due to their potential carcinogenic properties but also adversely affect beneficial insects such as honey bees by disrupting their pollination activities. They also to pose a health risk to humans and animals since they are potential carcinogens as well.

Naturally, this means that biocontrol methods have become more attractive options in terms of target specificity and environmental impact. Over the years, man has leveraged essential oils, phytochemicals and the mosquito's natural enemies such as invertebrate predators, aquatic entomopathogenic bacteria and fungi as a means of either killing the insects outright or reducing their competence as vectors for disease. This entails a significant decrease in fecundity, life span or developmental hindrances in the insects' life cycles. While the concept of biocontrol is sound, the main issues with these techniques are the inability to translate laboratory results appropriately into field ready products and the lack of persistence of said biocontrol agents in the environment.

In this perspective, prior research into the microbiota of the mosquito midgut has revealed that certain members of the *Bacillus* species have potent larvicidal effects, with close to 95% mortality rate at concentrations ranging from 50-60 ppm [4]. Standouts include *Bacillus thuringiensis* and *B. sphaericus*, which have been documented and used for their larvicidal effects [5, 6]. These success stories come with a caveat though, as these bacteria do not last in the environment for a period longer than 14 days after which they lose much of their potency. As of the time of this research, there has been no headway in addressing this issue and ergo, this study aims to ameliorate this issue.

While it is true that the *Bacillus* members of the mosquito midgut do not last for a long time outside of it, there is enough evidence that these bacteria can form symbiotic relationships with others notorious for their ability to do just that. Of particular interest for this study are the *Enterobacter* – microbes which are part of the diverse microbiome of the mosquito midgut while also retaining an impressive ability to exist outside of it. These bacteria can even survive in ordinary seawater lacking nutrients – one study has even shown *E.cloacae* still being culturable after 30 days in sterile saltwater at 20^0C [7]. This remarkable capability can be attributed to the biofilm these bacteria produce, which serves as a protection against biotic and abiotic stress. In addition, *Enterobacter* and *Bacillus* have been discovered to have synergistic symbiotic relationships when mixed as plant growth promoting (PGP) inoculants [8], although this not always beneficial.

In mosquito gut microbiomes, both these bacteria are common residents but their interactions have not been studied well and their interactions remain a mystery [9]. What is known is that *E.cloacae* has shown the ability to repress *Plasmodium* parasite development, despite not knowing what *Bacillus* brings to the equation. As such, the purpose of this study is to address this research gap by elucidating the interactions between selected members of both bacterial families in anopheline larvae in terms of larvicidal capacity and peritrophic matrix composition.

2. STATEMENT OF THE PROBLEM

Malaria is an ever-expanding health issue across the world and current methods to control the pathogen and vector are being hampered by the speed at which they evolve resistances to said methods. As such, it is of paramount importance to develop a cheap, easily scalable and accessible means of halting malarial spread. Biocontrol is one promising avenue of research in

this regard, as the gentic diversity of bacteria in particular far outstrips the adaptive resistance that *Plasmodium* or *Anopheles* can muster.

3. OBJECTIVES

3.1. General Objective

To develop and evaluate an effective biocontrol system utilizing combined formulations of *B. cereus* and *E. cloacae* against *Anopheles* larvae.

3.2. Specific Objectives

To evaluate the larvicidal efficacy of combined formulations of *B. cereus* and *E. cloacae* against third-instar *Anopheles arabiensis* larvae by measuring mortality rates at 24, 48, and 72 hours post-treatment.

To assess the larvicidal potency time of the combined *B. cereus* and *E. cloacae* biocontrol system.

4. LITERATURE REVIEW

4.1. Plasmodium Life Cycle

Malaria is a potentially fatal disease caused by a family of protozoan parasites known as *Plasmodium*. The parasites have two main hosts throughout their life cycle – the first and most important are the anopheline mosquitoes, of which the female plays the biggest role by transmitting the parasite through her saliva during a (typically nocturnal) blood meal. As the blood meal is a means of supplying the insect with a protein rich diet for laying eggs, the males do not play any significant role in parasite dissemination – they feed on plant juices instead. The protozoan goes through two main phases of its life cycle in the insects, the gametocyte and the sporozoite stage before its migration to the mosquito salivary glands where it prepares to invade a human host [34].

The gametocyte stage starts when a female anopheles mosquito bites an infected human, consuming the male (microgametocytes) and female (macrogametocytes) gametocytes in the blood. The blood then travels through the insect's alimentary canal before settling in the gut, where the gametocytes then unite in sexual congress to form zygotes which in turn mature into ookinetes. Finally, these ookinetes penetrate the insect's midgut lining and form oocysts with in it. As one might expect from the name, the oocysts harbor thousands of maturing sporozoites and their release into the mosquito hemolymph after a period of growth heralds the beginning of the sporozoite stage. The titular sporozoites then travel to the salivary glands of their host to effectively prepare themselves for their imminent dispersal into a human host upon the mosquito's subsequent blood meal [34, 15].

In their second host (humans), the *Plasmodium* parasite also has two clinical stages known as the hepatic and erythrocytic stages. The initial stage involves a liver based developmental phase in

which the protozoan sporozoites are transported via the blood to the liver, where they subsequently invade the hepatocytes with ease. In their new refuge, the sporozoites then asexually multiply, hereby being known as schizonts in a process that can take up to 6-15 days depending on which particular species of *Plasmodium* is responsible for the infection. Notable mentions are *P.vivax* and *P.ovale*, which are capable of forming dormant hypnozoites which then reactivate at a later date. The schizonts then rupture, releasing a payload of thousands of merozoites into the bloodstream as the hepatocyte harboring the parasite is destroyed. The new life forms then continue the second phase of their invasion in red blood cells, where they then follow one of two developmental paths. One is the path of the schizont, in which the merozoites undergo their penultimate transformation, turning into trophozoites which ensure the persistence of the infection after maturing into schizonts to give rise to an eternal cycle of merozoites (and schizonts in their turn) to infect other RBCs. The second is the path of the gametocyte, wherein a smaller population of the merozoites differentiates into their final reproductive form to complete the cycle (micro and macrogametocytes) by infecting mosquitoes once more [34, 15].

The disease condition of 'malaria' clinically manifests during the erythrocytic stage (when the RBCs rupture) as the classical symptoms of fever, chills, and anemia. The coordinated timing of the cell ruptures triggers the characteristic paroxysms of the disease, which can become fatal if left unmanaged; symptom presentation varies across species, with *P.falciparum* exhibiting the highest mortality rate (Figure 1) [34, 15].

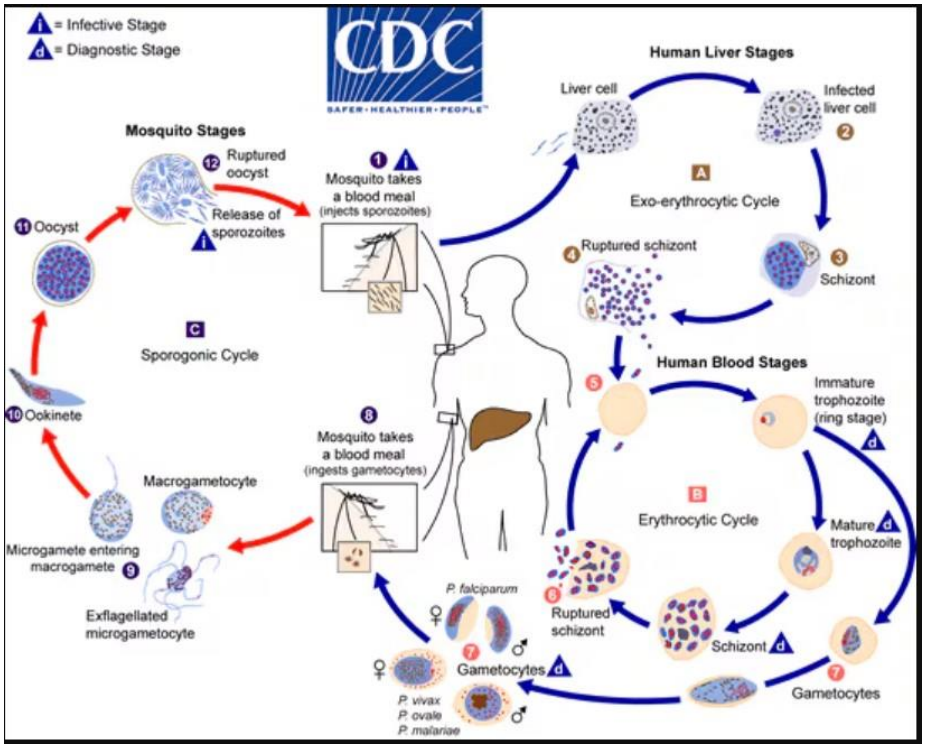

Figure 1: The Malarial Life Cycle

(Adapted from Centers for Disease Control and Prevention, 2024. [14])

4.2. Malaria Global Statistics

Globally speaking, malaria has around 249 million cases worldwide, a substantial increase due to the COVID-19 pandemic (the 2019 case number was about 233 million). In the same year, the disease claimed the lives of approximately 608,000 people, with an estimated mortality rate of 14.3 deaths for every 100,000 people at risk of being exposed to the disease according to the WHO report of 2023 [1]. When considering its geographic distribution, the WHO region of Africa accounts for a staggering 94% of all cases in the world (which equates to roughly 233 million) and 95% of all deaths (somewhere in the neighborhood of 580,000). The four most

affected nations are Nigeria with 27% of cases, the Democratic Republic of the Congo (12%), Uganda (5%), and Mozambique (4%) (Figure 2) [1].

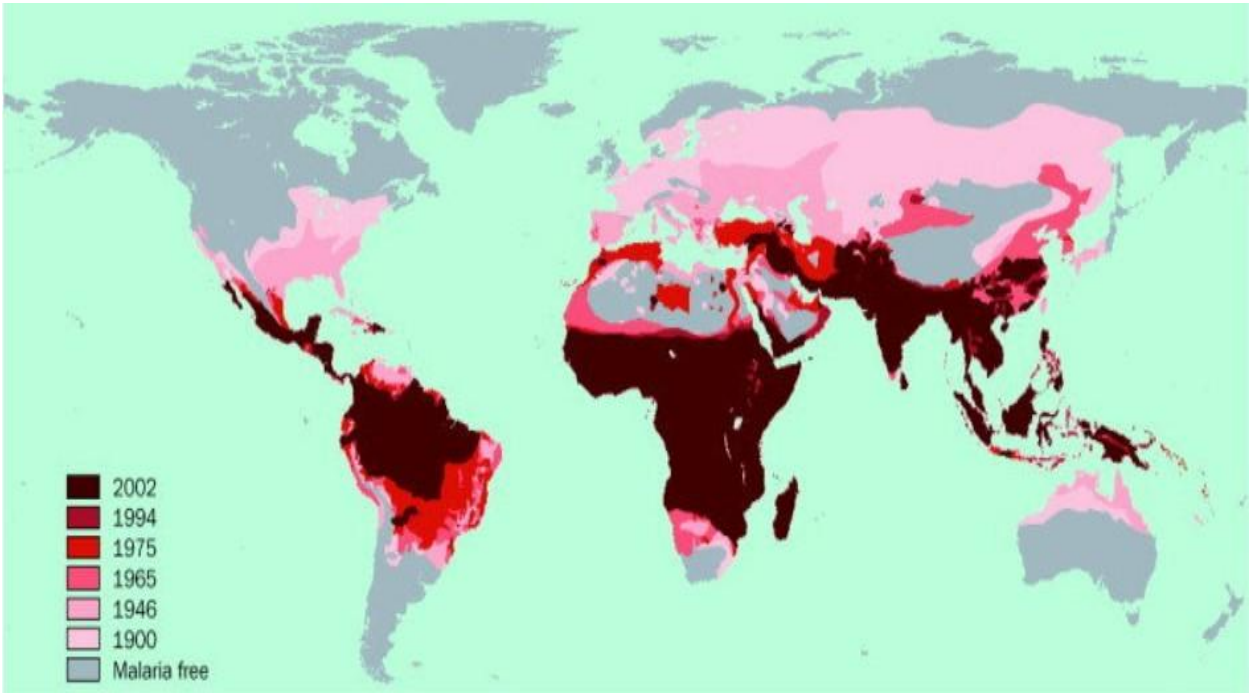

Figure 2 : The global distribution of malaria since preintervention (˜1900–2002)
(Adapted from Hay et al, (2004). [46])

Following up on that, the high burden countries are about twelve in total - Burkina Faso, Cameroon, the Democratic Republic of the Congo, Ghana, India, Mali, Mozambique, Niger, Nigeria, Uganda and Tanzania with Sudan as the most recent addition in 2022 (Figure 3).

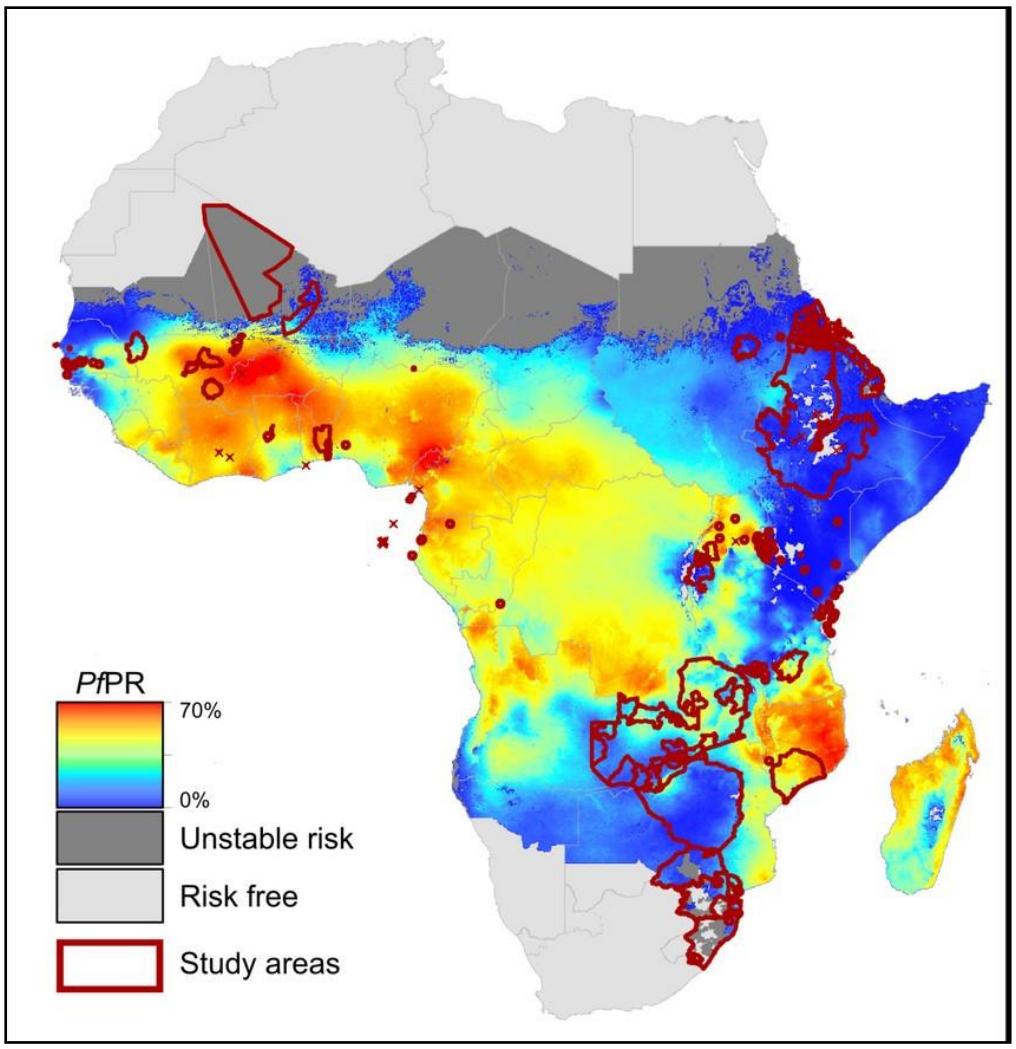

Figure 3: African Malaria Distribution

(Adapted from Gehring et al, (2014). [47])

The pattern of malaria cases has a decidedly upward trend, with an increase of 5 million cases from 2021 to 2022, a fact that is extremely pronounced in countries like Pakistan, which saw a period of extreme flooding in this period [10]. Given the radical shift of inclement weather in 2023 to 2024, this number can only be expected to increase (Sudan and Nigeria both had hydroelectric dam collapses from July to September 2024 alone). COVID-19 has also hampered

malaria control, with the WHO recording 32,000 additional deaths in 2022 compared to 2019 (Figure 4).

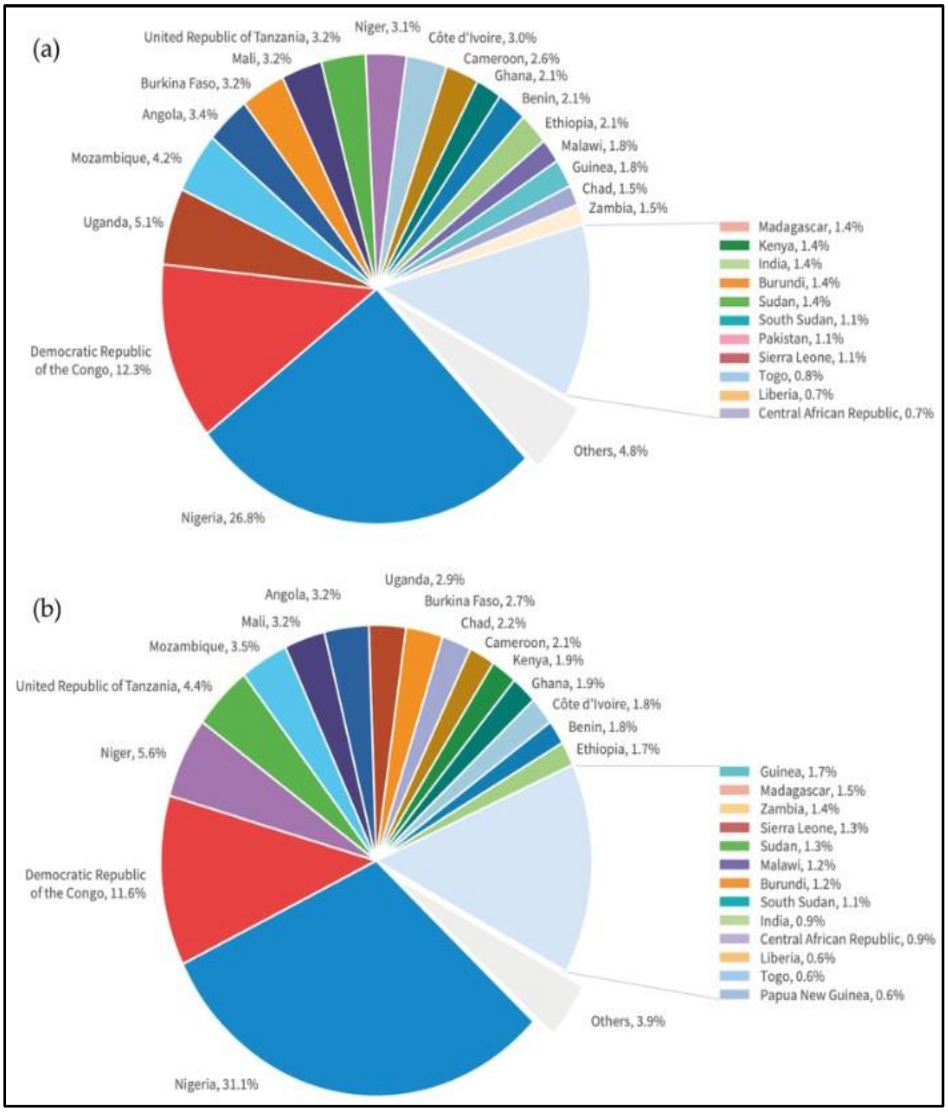

Figure 4: Global Malaria Trends

(Adapted from Mardini et al, (2024). [48])

4.3. Malaria: Disease Types and Current Treatment Methods

The severity of malaria varies depending on the plasmodium species involved, with five primary types responsible for human infection [40]:

I. *Plasmodium falciparum*: The most dangerous variant of the protozoan as it is responsible for most of the malarial deaths worldwide. Its clinical features are severe malaria that leads to cerebral malaria, anemia, and multi-organ failure.

II. *Plasmodium vivax*: The second most prevalent variant, known for its recurring infections – the organism uses hypnozoites to effectively create sleeper infections that flare up intermittently. It causes milder disease symptoms compared to *P.falciparum* but relapses are common.

III. *Plasmodium ovale*: The other hypnozoite former, this parasite has a similar pathogenic profile to *Plasmodium vivax* but causes a milder form of the disease.

IV. *Plasmodium malariae*: A lesser known and rarer variant of *Plasmodium* (despite the species name), it is notable for causing long term infections that have been associated with nephrotic syndrome in select cases.

V. *Plasmodium knowlesi*: A zoonotic variant of *Plasmodium* that primarily infects macaques but also can establish itself in humans – this is a significant source of infection in Southeast Asia. The malaria produced by this protozoan is also quite severe.

In addition to the complex life cycle in two hosts, *Plasmodium* has a remarkable degree of genetic flexibility that confers environmental adaptations as well as drug resistance. Regarding their distribution, *P. falciparum* dominates sub-Saharan Africa so much so that sickle cell anemia is regarded as an adaptation response to its prevalence, whereas *P. vivax* is more widely established across Asia and Latin America [34, 40].

The current methods of treating an established malarial case involve the use of antimalarial drugs. These drugs tend to have a primary focus on targeting and hindering the hepatic and erythrocytic stages of the parasite's life cycle, ignoring the mosquito based stages (even though *Plasmodium* is an unwanted infection in the insect as well). There are five main drug families and/or therapies to consider:

Artemisinin-based **C**ombination **T**herapies (ACTs) are the first line and the most potent means of clearing out large parasitic loads in the host, mostly used as treatment for uncomplicated *P. falciparum* malaria. The active component Artemisinin is produced as a metabolite from the sweet wormwood plant, *Artemisia annua* (hence the name). It has three main derivatives artesunate, artemether, and dihydroartemisinin and all of them work to kill both sexual and asexual forms of *Plasmodium* in the blood. All of these compounds operate via a similar mechanism- featuring peroxide groups which are activated by iron (or iron containing moieties), notably hemozoin, a byproduct of hemoglobin digestion by schizonts [3]. Upon encountering hemozoin, artemisinin produces reactive oxygen species (ROS) and carbon radicals which then damage the cellular components of *Plasmodium*, leading to its death – this has a broad range of stages that the drug can safely eliminate. The load of the parasite in a host can be reduced by as much as a 100 million-fold in 3 days since the drug reaches peak plasma level in just a few hours [11].

This drug also has gametocidal activity, which excels in low transmission settings. However, this is no silver bullet – of particular concern nowadays is the development of resistance to this drug (attributed to the *pfkelch13* gene), which is why the WHO recommends the use of artemisinin in conjunction with other drugs, hence the name Artemisinin-based Combination Therapies (a combination of Artesunate-amodiaquine, Artemether-lumefantrine or Dihydroartemisinin-

piperaquine) [12, 13]. The persistent prevalence of malaria in impoverished regions can be attributed to both ongoing health challenges and logistical hurdles in distribution, which hinder effective intervention efforts. The persistent prevalence of malaria in impoverished regions can be attributed to both ongoing health challenges and logistical hurdles in distribution, which hinder effective intervention efforts.

Common artemisinin – drug formulations available on the market and their mode of action are listed below [35]:

a) Chloroquine: A preferred method of treatment for *P. vivax* and *P. malariae*, it acts by inhibiting the parasites' ability to digest hemoglobin in RBCs.
b) Quinine: The most colloquially recognized antimalarial drug, it is used to treat severe malaria since it disrupts the parasite's means to digest and use hemoglobin.
c) Atovaquone-proguanil: This drug combination works to interfere with the mitochondria of the parasites, preventing their growth.
d) Primaquine: The answer to relapsing malaria, it eliminates the growth advantage of *P.vivax* and *P.ovale* by targeting their hepatic life stage.

These drugs follow three primary modes of action – the killing of liver resident parasites, the targeting of erythocytic stages and the prevention of transmission through the reduction of blood parasitic load (Figure 5) [35].

Class	Drug	Use
4-Aminoquinoline	Chloroquine	Treatment of non-falciparum malaria
	Amodiaquine	Partner drug for ACT
	Piperaquine	ACT partner drug with dihydroartemisinin as ACT
8-Aminoquinoline	Primaquine	Radical cure and terminal prophylaxis of *Plasmodium vivax* and *Plasmodium ovale*; gametocytocidal drug for *Plasmodium falciparum*
		Radical cure of *P. vivax* and *P. ovale*
	Quinine	Treatment of *P. falciparum* and severe malaria
Arylamino alcohol	Mefloquine	Prophylaxis and partner drug for ACT for treatment of falciparum
	Lumefantrine	Combination with artemether as ACT
Sesquiterpene lactone endoperoxides	Artemether	ACT: combination with lumefantrine
	Artesunate	ACT; treatment of severe malaria
	Dihydroartemisinin	ACT: combination with piperaquine
Mannich base	Pyronaridine	Combination with artesunate as ACT
Antifolate	Pyrimethamine/sulfadoxine	Treatment of some chloroquine-resistant parasites; Combination with artesunate as ACT
Naphthoquinone/antifolate	Atovaquone/proguanil	Combination for prophylaxis and treatment of *P. falciparum* (Malarone)
Antibiotic	Doxycycline	Chemoprophylaxis; treatment of *P. falciparum*
	Clindamycin	

ACT = artemisinin-based combination therapy.

Figure 5: Commonly Used Antimalarial Drugs

(Adapted from Cui, L. et al, (2015). [49])

4.4. Biology and Management of Disease Vector Mosquitoes

When addressing the primary host of the parasites, it is essential to consider the unique biology and ecology of mosquitoes as these factors fundamentally influence the effectiveness of any control strategy. The anopheles mosquito is one of the three primary disease carrying species that significantly impact humans, alongside *Culex* and *Aedes* [36].

The *Culex* is known for transmitting the West Nile virus and Japanese encephalitis while the *Aedes* does the same for dengue, Zika, yellow fever, and chikungunya. *Anopheles* surprisingly carries only one main disease – malaria and so, a replete knowledge of the feeding habits, larval development and egg laying preferences of its member species is indispensable (Figure 6) [36,41].

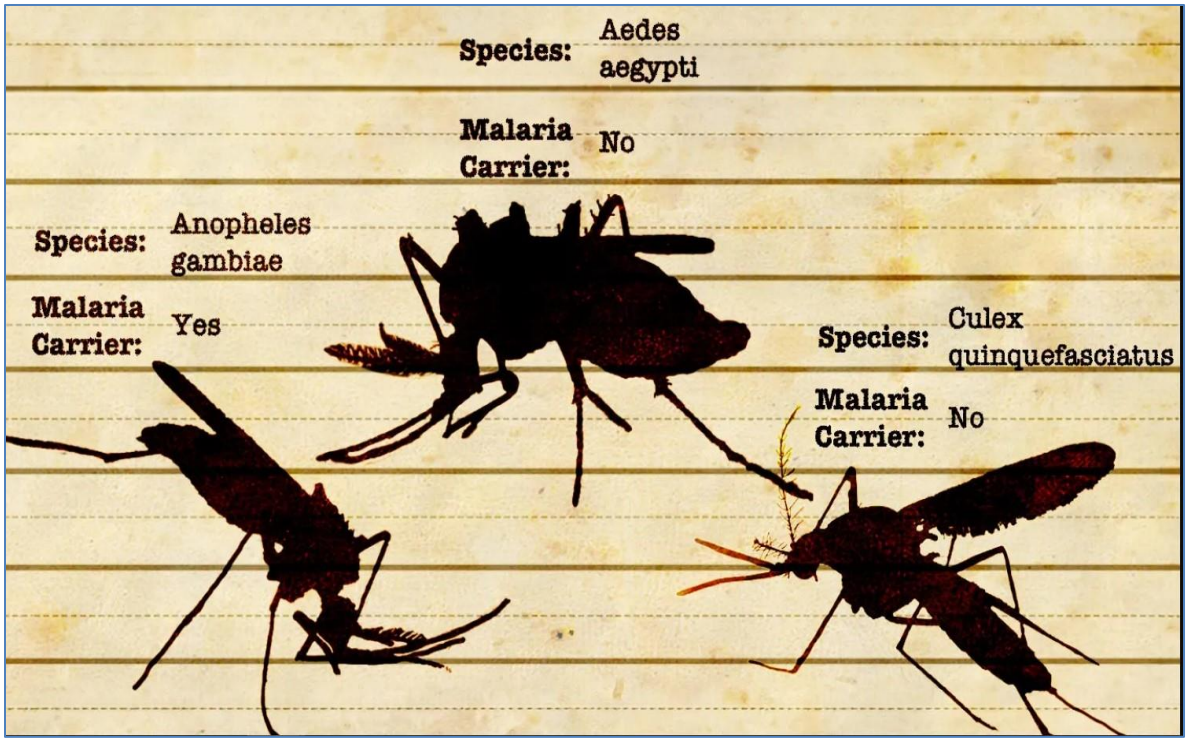

Figure 6: The Trinity of Mosquito Vectors (left to right – *Anopheles*, *Aedes* and *Culex*)
(Adapted from Welcome Sanger Institute Blog , markthomsonsanger. (2023). [50])

The *Anopheles* is a nocturnal feeder, mostly partaking of human blood during the evening and night. The females use this blood as a nutritional supplement to lay their eggs, which is done on the surface of stagnant water with the eggs laid singly. The larvae (wrigglers) that hatch from these eggs after 2-3 days float horizontally relative to the water surface and lack a respiratory siphon. They then float and feed at the surface of the water eventually going through four instars, which are molting phases followed by pupation [14]. The pupae then remain dormant for a while before releasing the adult mosquito, with the entire life cycle taking around 5-14 days depending on species and temperature (Figure 7) [15].

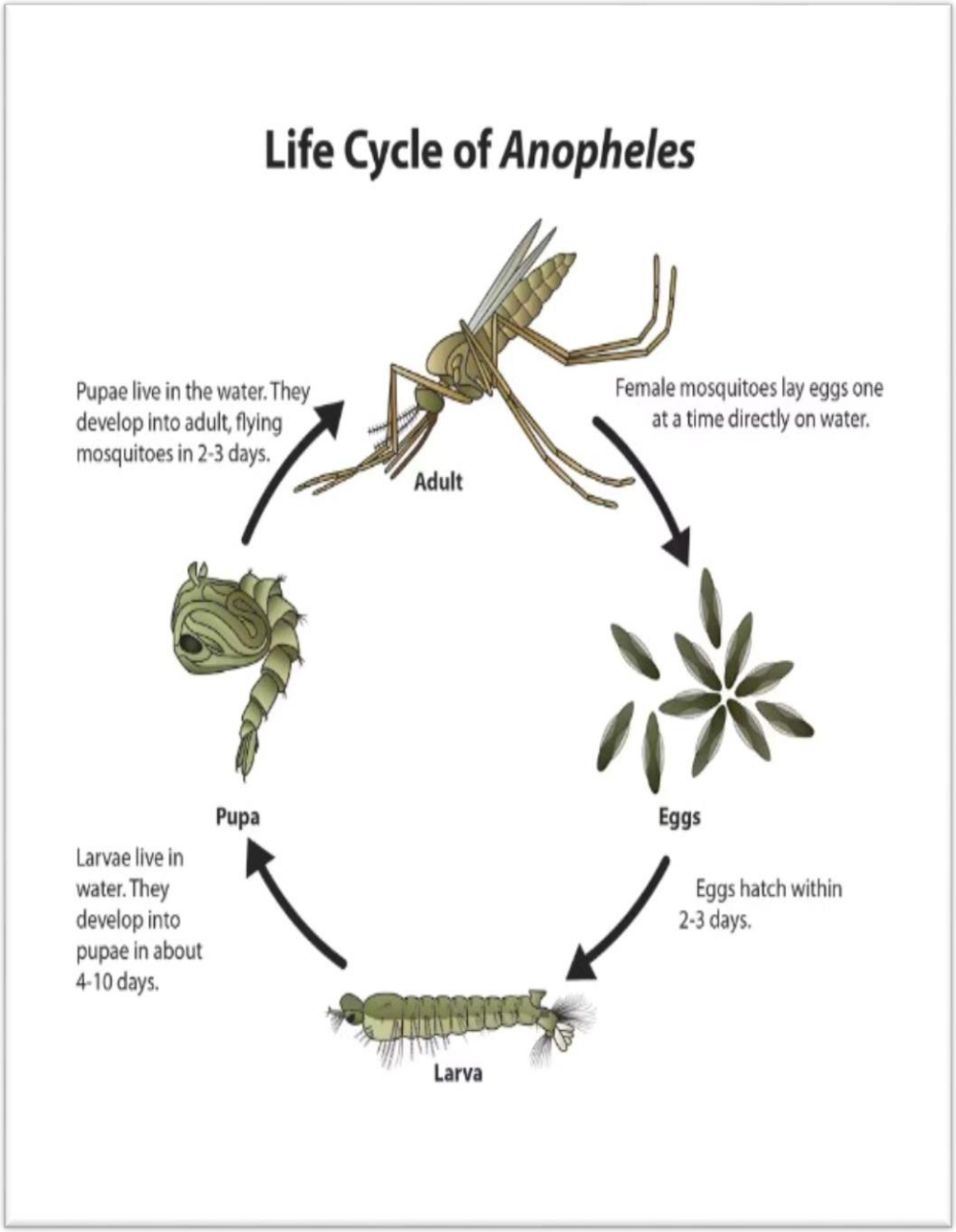

Figure 7: Anopheline Life Cycle

(Adapted from CDC. (2024). [14])

On the topic of species, the *Anopheles* has six different ones of significance as vectors of disease [34], with *Anopheles gambiae*, *Anopheles arabiensis* and *Anopheles stephensi* being of particular

interest to this study given their proclivity to living in Sub-Saharan Africa and/or urbanized areas. Since these mosquitoes pose a health hazard for any human settlement they colonize, the insects must be culled when discovered. As such, there are several methods employed to control their populations, both chemical and biological. However, the existing methods have their disadvantages as explained below.

4.5. Pesticides and biological method based mosquito control: environmental and human health consequences

The standard method of mosquito control in human settlements (both urban and rural) primarily revolves around chemical solutions like pesticides. These pesticides can be broadly divided into four main categories (pyrethroids, organophosphates, carbamates and DDT) although the use of the last class has been discontinued due to environmental, resistance and health concerns [37].

Pyrethroids are commonly used as an agent in indoor residual spraying (IRS) and the impregnation of bed nets. Notable examples include permethrin, deltamethrin among others [37]. Organophosphates are used as larvicides and IRS components with common examples such as malathion and fenitrothion. A key concern associated with the use of these chemicals however is the potential development of resistance among specific mosquito populations [37].

Carbamates are pesticides with variable results depending on which mosquito population is resistant to it or not but is also used in IRS. Bendiocarb is a good representative of this pesticide class [37]. DDT (Dichloro-Diphenyl-Trichloroethane), the final class, needs no introduction, as this is a chemical that has been discontinued after historic use due to environmental, health and resistance concerns. Interestingly, mosquitoes resistant to DDT are especially prevalent in Africa [37].

The primary disadvantage of chemical insecticides is the development of resistance in mosquitoes. This issue can be broken down into three categories [37]:

a) Multiple-Insecticide Resistance: A trait of *A.gambiae*- for example, the populations in Côte d'Ivoire are resistant to all pesticide classes, making them a significant threat to mankind [16].

b) Knockdown Resistance: Mutations in certain genes, such as the *L1014F* gene confer the carrier mosquito with resistance to pesticides like pyrethroids and DDT [3, 17].

c) Agricultural Practices: The fact that mosquito pesticides have chemical analogs in agricultural pesticides means that the varying doses used on crops and weeds inadvertently exacerbate resistance in Anopheles populations, with a selection pressure for resistant ones.

These problems are currently addressed with insecticide resistance management, the use of synergists, the development of new insecticides and monitoring and surveillance [17, 18].

On the biocontrol side of things, anopheline mosquitoes are regulated by the following techniques:

Invertebrate predators: Backswimmers (*Notonectidae*), dragonfly larvae (*Odonata*), and certain beetles (*Coleoptera*) are known to be avid predators of *Anopheles* larvae, with backswimmers taking the center stage in larval consumption. The efficacy of this method is determined by larval instar level, water volume, and the number of predators introduced with a laboratory study conducted in Ethiopia documented the potency of a diverse selection of predators introduced to a larval population [19].

Mosquitofish (*Gambusia affinis*): A fish that consumes mosquito larvae at a significant rate – using this predator requires ecological consideration to keep it from displacing natural populations and skewing their biodiversity. Alternatives are the fathead minnows (*Pimephalespromelas*) and bluegill (*Lepomis macrochirus*) since they don't run the risks that come with introducing non-native species [20].

Entomopathogenic Microorganisms: Bacteria and fungi are capable of interacting with the midgut of mosquitoes altering their fitness or outright killing them in some cases. The insects have a resident microbiome that allows them to develop normally but certain microbes like *Bacillus thuringiensis israelensis* (Bti), kill larvae specifically [21]. The issue here is the lack of persistence in the environment (as an example, *Bacillus thuringiensis* only remains potent for 14 days) [5, 6].

Integrated Mosquito Management (IMM): A combination of biocontrol methods in conjunction with chemical pesticides as a means to balance efficacy with minimal resistance - this includes surveillance, source reduction, and public education alongside biocontrol measures [20].

Environmental management: This is another name for habitat management, which boils down to draining stagnant water or promoting the presence of natural predators in these environments.

4.6. Potential Management Methods: Biocontrol Applications in the Mosquito Midgut

Biocontrol methods are environmentally friendly and do not run the risk of grooming the population of *Anopheles* into becoming resistant variants. However, the process of bringing these methods from the laboratory to the field is a logistical challenge and even upon successful

introduction, certain biocontrol agents (like entomopathogenic microbes) do not persist in the environs for long, limiting their use (see an image of the midgut in Figures 8 and 9) [22].

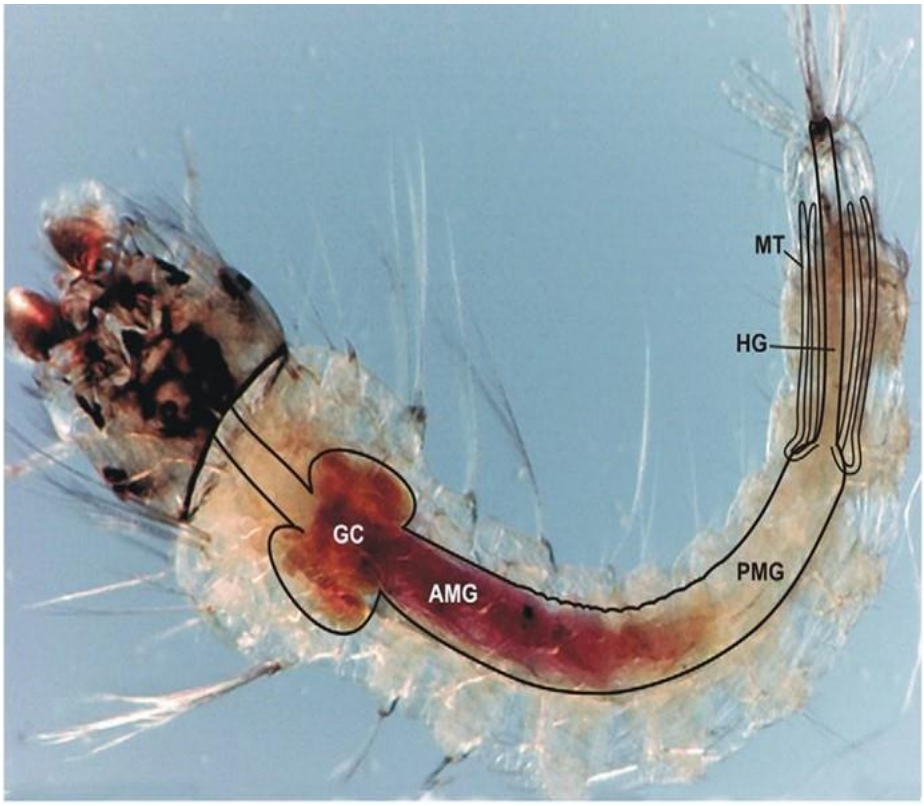

Figure 8: A living fourth instar *Anopheles gambiae* larvae

(Adapted from Kalappa et al, (2018). [22])

The figure above depicts a living fourth instar *Anopheles gambiae* larva that had been fed pH sensitive dye (m-cresol purple) to highlight the gut luminal pH gradient. The structure and compartments of the alimentary canal are outlined in black [22].

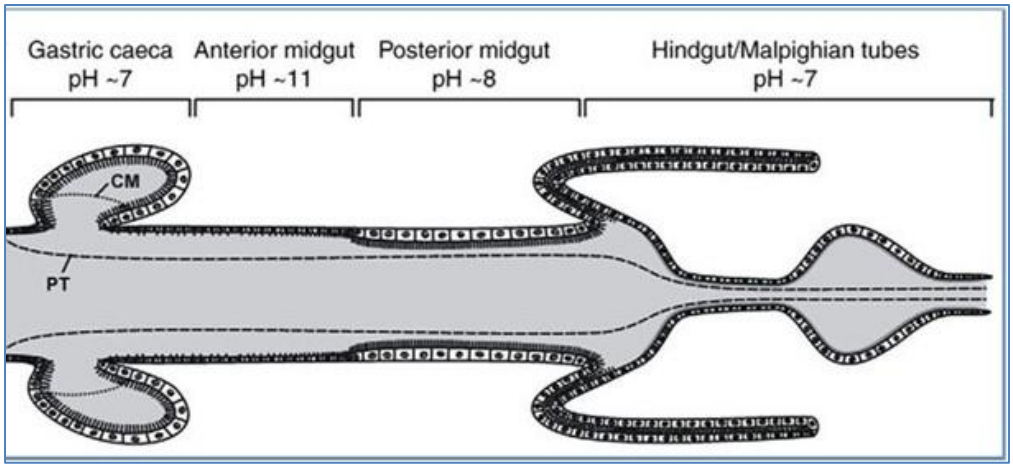

Figure 9 : A stylized diagram of the gut with the anterior to the left

(Adapted from Kalappa et al, (2018). [22])

The figure above depicts a stylized diagram of the gut with the anterior to the left. The brackets above indicate the extent of each region, and the approximate pH of each is indicated. The abbreviations in the image are: GC, gastric caeca; AMG, anterior midgut; PMG, posterior midgut; HG, hindgut; MT, Malpighian tubules; CM, caecal membrane; PT, peritrophic matrix [22].

The composition and diversity of the microbiome are critical factors shaping the spectrum of benefits conferred to the larvae. Newly hatched larvae acquire their core microbiota upon their first feeding or ingestion of surrounding water. There is no evidence of vertical transmission of microbes from the mother, although new larvae can acquire microbes from the broken egg shell fragments that they can potentially consume. This means the level of variety between larvae (even if they are of the same species) is staggering – it is entirely determined by the environment and there is no ‘common core’ microbiome to expect in the larval midgut [23].

Dominant bacterial genera include *Pseudomonas*, *Actinobacteria*, *Enterobacteriaceae*, *Serratia marcescens* and *Flavobacterium* with each member providing a different benefit [38].

Actinobacteria are most common in the midgut of *Anopheles atroparvus*. These bacteria generally work with plant biomass decomposition in aquatic environments and may provide functional advantages such as digestion and nutrient absorption augmentation. The *Pseudomonas* species on the other hand, have been identified as common microbiota across various *Anopheles* species. They possess high metabolic versatility and have potential roles in nutrient cycling and pathogen suppression, particularly in improving the condition of the peritrophic matrix.

The *Enterobacteriaceae* family (like *Enterobacter agglomerans*), has been associated with the midgut microbiota of *Anopheles* mosquitoes. They can produce reactive oxygen species that inhibit the development of Plasmodium parasites, thereby affecting the mosquito's vector competence. In a similar vein, *Serratia marcescens* is a dominant isolate in the midgut of both larvae and adult Anopheles mosquitoes and is suspected to have a hand in managing the immune response of the mosquito and the development of *Plasmodium*.

The *Flavobacterium* genus has also been identified in the midgut of *Anopheles* mosquitoes and they contribute to the overall microbiota diversity and potentially influence larval health and development.

Overall, the functional roles of midgut bacteria can be divided into nutritional support (organic material breakdown and improved nutrient absorption) immune modulation (immune system stimulation) and pathogen suppression (the production of anti – *Plasmodium* metabolites that inhibit parasitic growth and/or development). For this study, the primary focus is on examining the impact of *Bacillus* bacteria within the midgut alongside exploring their interaction with

Enterobacteria and their persistence within the local micro-environment [39]. The influence of midgut microbiota on *Anopheles* mosquitoes primarily revolve around signaling pathways, metabolite degradation (like tryptophan) and the production of toxins with larvicidal effects on the mosquitoes themselves [24, 25, 27]. While a vast majority of their effects have not been fully clarified, certain mechanisms have been shown to affect *Anopheles* vector competency as explained below:

Tryptophan catabolism in *Anopheles* larvae (the Kynurenine Pathway) influences both mosquito metabolism and vector competence for malaria transmission. The primary catabolic route for tryptophan in Anopheles mosquitoes is through the kynurenine pathway. Tryptophan is oxidized to kynurenine and subsequently to 3-hydroxykynurenine (3-HK) since 3-HK can generate reactive species under physiological conditions, so it is often converted to xanthurenic acid, a more stable form [24, 25]. This provides metabolites that play important roles in pigmentation and immune response control. Microbiota can affect this process in various ways – in *Anopheles stephensi* their participation helps regulate the levels of tryptophan and its metabolites and their elimination leads to the tryptophan and associated metabolite accumulation which can impair the structure of the peritrophic matrix and enhance susceptibility to *Plasmodium berghei* infections [25].

The *Bacillus* bacteria are one of the members of the midgut microbiota and these diverse microbes are significant biocontrol agents for reasons that vary wildly based on species:

For instance, *Bacillus thuringiensis israelensis* (Bti) , a gram-positive, spore-forming bacterium, produces crystalline and cytolytic proteins during sporulation, which are

highly toxic to mosquito larvae [26]. It has exhibited a 98% decrease in *Anopheles gambiae* over 48 hours when applied to rice fields [27].

Bacillus sphaericus (Bs) has been used to control *Anopheles gambiae* and other mosquitoes in rice fields and swamps. Treatments with a granular formulation of Bs reduced larval populations of *A. gambiae* by 98% after 48 hours, but repetitive applications every 15 days were required to maintain control. The persistence of Bs spores was more apparent in rice fields compared to swamps [42].

Bacillus cereus has shown larvicidal activity against *Anopheles* larvae, with 50% mortality at 27.93 ppm and 95% mortality at 57.43 ppm. The residual effect of *B. cereus* at the LC95 concentration results in 96.7% mortality of *Anopheles* larvae within the first 48 hours, decreasing to 70% by day 14 [4].

The main constraints with *Bacillus*-based biocontrol systems are the lack of long-lasting residual effects and overcoming logistical problems concerning the vastness of breeding habitats. In previous research on this topic, integrated vector management programs have been postulated to show promise in remediating this issue and this study proposes that one specific bacterial family could ameliorate the persistence issue of *Bacillus* – *Enterobacteriaceae* [39].

Enterobacteriaceae have the remarkable ability to survive for prolonged periods in aquatic environments. For example, *E. cloacae* can survive for extended periods in seawater, even in the absence of nutrients. One study found *E. cloacae* maintained cultivability for over 30 days in sterile seawater at 20°C, something that is attributed to its ability to form biofilms against environmental stress [7]. In freshwater, *E. aerogenes* and *E. cloacae* have been detected in rivers and lakes, indicating their ability to survive in these habitats despite concerns about their

potential as antimicrobial drug resistance gene reservoirs [28]. In soil sediment, *Enterobacter* species can accumulate in sediments of aquatic bodies like lagoons and estuaries. A study found that *Enterobacter* was one of the dominant *Enterobacterales* genera in lagoon sediments with fish farming activity since sediments provide protection and nutrients that allow *Enterobacter* to persist for longer periods with resuspension of contaminated sediments releasing *Enterobacter* into the water [29].

So, given all of this information, a combined formulation applied to water bodies should, in theory, create a system wherein the persistence of the *Enterobacter* could carry over to *Bacillus*, allowing it to stay potent in the environment longer, a possibility further corroborated by the fact that the two species have been proven to work synergistically to promote plant growth when used as mixed inoculants [30]. This study aims to use this information by taking a closer look at the *Anopheles* mosquito larva midgut and leveraging the existing symbiotic relationships between its members for potential larvicidal or *Plasmodium* inhibitory effects.

5. MATERIALS AND METHODS

5.1. Study Area

The biocontrol of the various anopheline larval stages via the combined influence of single or multiple bacteria, formulated as co-cultures introduced to the aqueous environment in which the larvae develop. The primary goal of this being either the complete elimination of the larvae from their local environment or a satisfactory stalling of their life cycle, so that this method could be considered a viable strategy to curb the spread of malaria in reltively impoverished states, which can afford the standard means to exterminate the disease.

5.2. Study Design

An experimental and quantitative approach, with insectarium reared *A. arabiensis* larvae pooled in groups of 20 per container, with three varying concentration levels of the bacterial co-culture (5, 7 and 10 mL) to test biocontrol potency. Additional factors for comparison were used in the form of single or blank bacterial culture containers as well, with every container's larval load refreshed every 5 days for over a month to test biocontrol persistence.

This experiment was repeated three times to test the effects of temperature on the biocontrol system and to test the efficacy of said system on varying developmental stages of the larvae (first and third instar stages). Data analysis was conducted via the probit finney test, with the working null and alternate hypothesis being that the co-culture used was either similar to a single culture in efficacy or not respectively. Data on larval conditions (live or dead) was collected via manual counting, done by professional entomologists to avoid personal bias and related errors. A total of around 4000 mosquito larvae were tested during these experiments, with outlier data removed based on how sharply it diverged from other data entries.

5.3. Rearing insectarium grown mosquitoes

This work was carried out in collaboration with the Akililu Lemma Institute of Pathobiology to use their laboratory and insectarium facilities.

A. arabiensis larvae were grown in selected water containers after obtaining the eggs from egg-laying adults reared separately and the mugs (for 50 mL of water) serving as containers from the Entomology Research Unit of Akililu Lemma Institute of Pathobiology, AAU. The eggs were obtained from the adult mosquitoes being reared at the insectarium and the larvae that hatched had sterile midguts as the water they hatched into would also be sterile. The tap water used was boiled and cooled to serve as water for egg and larvae incubation. Further assurances to maintain container sterility was the design of the entomology room, which had a rubber-sealed door and one-directional airflow that maintained an average temperature of 25^0C. The standard protocol at this institute was to house the eggs at 23-27^0C in boiled and cooled tap water in contrast to the distilled water used in Mogea et al's method [4] and to feed the emerging larvae with three drops of sterile yeast extract over 7 days, which was the average time for the eggs to develop into mature adults.

Before any further studies, a test run of the selected bacteria was conducted – twelve containers with 20 eggs each suspended in 50 mL of distilled water (with the room's temperature set to 37^0C via a radiator) were prepared for bacterial inoculation. After verifying the larvicidal action of the bacteria, in depth analysis of the effects of the varying concentrations of the bacterial co-cultures at different stages of the *A. arabiensis* larvae was conducted. Twelve coffee mugs, each holding 100 mL of distilled water were prepared, with 20 eggs evenly distributed per container. Subsequently, a second set of 12 mugs was also prepared, this time containing 20 larvae at the third instar stage of development. All sets were maintained at a room temperature of 25 -27^0C,

with the exception of the first preliminary test, that conducted on eggs incubated at 37^0C to serve as a benchmark for the influence of temperature on the co-cultures' activity.

5.4. Preparation of the combined *Bacillus* and *Enterobacter* co-culture

B. cereus and *E. cloacae* samples were first obtained as a donation from Addis Ababa University (AAU) and the Ethiopian Public Health Institute's Microbiology to ensure the identity of the bacteria prior to culturing them as these institutions regularly send their samples for gene sequencing abroad. *B. cereus* was available from environmental soil samples stored in glycerol stocks at -80^0C from prior research at Addis Ababa University (AAU). *E. cloacae* samples were provided by Ethiopian Public Health Institute's Microbiology Research Division, as they had a diverse collection of microbes in storage. The bacteria were first transferred to new media from the glycerol stocks – *B. cereus* was inoculated on Tryptic Soy Agar and *E. cloacae* were inoculated on Eosin Methylene Blue Agar.

B. cereus and *E. cloacae* cultures were then prepared in 18 separate test tubes (nine for each bacterium), with every test tube containing 10 mL of nutrient broth medium. The bacterial cultures were allowed to grow for 48 hours at 37^0C for the preliminary test before being mixed and added to the prepared mosquito containers at varying concentrations (10mL, 7mL and 5mL), with the proportions of the two bacteria being an even 50-50 split (for example, the 10 mL co-culture were 5 mL of *B. cereus* and 5 mL of *E. cloacae*).

Subsequent tests were conducted using the bacterial cultures grown for only 24 hours before their transfer to the mosquito containers. As additional verification of the bacterial load, the colony forming units per mL of the cultures (CFU/mL) were calculated by plating 0.02% of the

stock cultures (with separate 5 mL cultures specifically prepared for this) on two Petri plates of yeast extract agar (25 mL each).

Generation times for the bacteria were accounted for when the two samples were mixed in a co-culture (*E. cloacae*= 51.8 up to 281.5 minutes and *B. cereus*= 28.8 to 36.0 minutes).

5.5. Biological tests

For the larvicidal analysis, three volume variations of *Bacillus* and *Enterobacter* (5 mL, 7 mL and 10 mL) were tested – the bacteria were incubated overnight (24 hours) before being mixed as a co-culture. *A. arabiensis* larvae were pooled in maintenance containers (coffee mugs) with 20 larvae or 20 eggs per container suspended in 100 mL of water (50 mL for the preliminary test). The experimental design was structured as follows: there were 3 different volumes of *E. cloacae* and *B. cereus* co-cultures (with each bacterium contributing 50% of the co-cultures total volume), each with three separate replicates – meaning that there were 9 bacterial co-culture containers per life stage to study (either eggs or third instar larvae). In addition to that, there were 3 additional controls – one container with only *E. cloacae*, another with only *B. cereus* and a final container with only water in addition to their respective mosquito load.

This experiment was done separately to study the effects of the bacteria on *A. arabiensis* eggs that are about to hatch and their third instar larvae. Observations were conducted every 24 hours, with the number of hatched eggs, dead larvae and the number of living ones also being recorded. After that, a persistence test was conducted to know how long the combined formulations could persist in the container until more than 30% of larvae survived the inoculations. Every 3-5 days, the dead larvae or unresponsive eggs were removed from their containers and 20 new larvae or eggs were introduced for the subsequent 3-5 days until the LC_{70} was reached, at which point the

observations ceased. An initial preliminary test was conducted prior to the main test, with the crucial difference being that the preliminary test was done in a room kept at 37^0C, while the main test was conducted at 25^0C, to check the effect of higher temperatures on co-culture biocontrol efficiency (Figure 10).

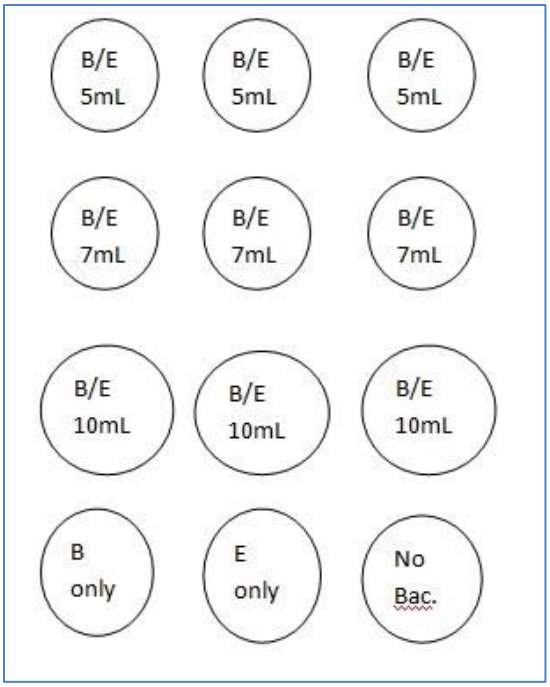

Figure 10 : Experimental Design Framework

In all tests, following the eggs hatching or the detection of larvae in the containers, insects received a sterile yeast extract solution; initially 3 drops-gradually increased to 6 drops for the lower concentrations (5 mL) and adjusted to 4.5 drops for the higher volumes (7 and 10 mL).

5.6. Statistical analysis

The data was properly recorded in a Microsoft Excel spreadsheet at each step of the laboratory work. Determination of the lethal concentration was analyzed with the probit Finney analysis of the larvae mortality [31] and was done via SPPS Statistics 23.

6. RESULTS

6.1 The Effect of Temperature on Biocontrol Efficiency

The eggs suspended in 50 mL of water and subjected to varying concentrations of the bacterial co-cultures (48 hours old) showed increasingly adverse responses with increasing concentration levels. As a benchmark for subsequent tests, there were three replicates for each co-culture concentration level where each bacterial isolate constituted half of the total co-culture concentration (5, 7 and 10 mL from a combination of 2.5, 3.5 and 5 mL of *B. cereus* and *E. cloacae* respectively). Predictably, the 10 mL co-culture concentration showed the most larvicidal activity; with only 8 out of 20 eggs having hatched in every container - by the fourth day, all larvae that did hatch were dead. In total, this experiment was conducted on 240 eggs that hatched into first instar larvae and was a test to check the efficacy of bacterial co-culture larvicidal efficacy at higher temperatures since this would expedite the larvae's development (Table 1, Figure 11).

Figure 11 : Preliminary Test Design

(37^0C, 240 eggs tested over 5 days with 3 varying co-culture concentrations)

The 7 mL concentrations had varying responses: two replicates had 9 and 12 eggs hatch and by the fourth day, only one replicate had a single living larvae – it was also unable to shift to the second instar and died on the fifth day (to put it in perspective, according to the protocols of the resident institute, larvae shift from instar to instar in 3-5 days at the room's current temperature). One exceptional replicate had a film of bacteria that formed on the surface of the container, making it impossible for any eggs to hatch at all – all 20 eggs died prematurely (Table 1).

The 5mL concentration replicates had varying responses based on which bacteria dominated the culture – *B. cereus* dominant containers (discernible by the relative lack of biofilm deposits in the water) had almost all their larvae dead in 3 days. However, all three replicates had eliminated nearly all larvae by day 4, despite the varying numbers of larvae between the containers (Table 1).

Table 1: Temperature Effect Test Results. Numbers indicate viable mosquito eggs/larvae that hatched or are alive.

Days	B/E* 5mL			B/E 7mL			B/E 10mL			B Only[C]#	E Only[C]	No Bac[C]
	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R1	R1
0	20	20	20	20	20	20	20	20	20	20	20	20
1	8	4	9	9	12	0	8	7	8	10	11	6
2	6	3	5	8	9	0	7	2	7	9	10	8
3	3	0	4	5	6	0	2	1	2	7	9	8
4	0	0	1	0	1	0	0	0	0	3	3	7
5	0	0	0	0	0	0	0	0	0	3	3	7
Total	0	0	0	0	0	0	0	0	0	3	3	7

*B/E: Refers to ***B****. cereus/****E****. cloacae*.

#[C]: Refers to **C**ontrols.

6.2 Main Tests

6.2.1 Colony Forming Unit (CFU) Test

Before experimentation, the CFU/mL of the 24-hour cultures of *B. cereus* and *E. cloacae* were enumerated - *B. cereus* had a CFU/mL of 3400 and *E. cloacae* had a CFU/mL of 3100. The speed at which both bacteria grew made it difficult to distinguish individual colonies even at dilution levels around 10^{-6} per milliliter – single colonies would merge even after 24 hours of incubation so only the unmerged colonies were considered for the CFU/mL calculations (Figure 12).

Figure 12 : Colonies of *B. cereus* (left) and *E. cloacae* (right) CFU/mL
(24 hour incubation, 37^0C, yeast extract agar)

6.2.2 First Instar Larvicidal Potency and Persistence Tests

After the mosquito containers were inoculated with the bacterial cultures, the larvicidal potency was directly related to two main factors – biofilm production capacity and bacterial concentrations. Although toxin concentrations were insufficient to directly kill larvae, the biofilm formed on the water's surface served as an asphyxiation barrier by preventing the young larvae from extending their siphons, while also providing a safe haven for bacterial proliferation. The biofilm also improved the persistence levels of the bacteria in the containers – after exceeding the 14-day limit, the bacteria appeared to maintain a steady population size conforming to their initial inoculation levels (with the only exceptions being the 5 mL inoculations) (Table 2).This was demonstrated when the larvae count in each container (Table 2), tended to decrease sharply past day 14 for all the co-culture containers from an average of 0 to 4 larvae for the 7 and 10 mL co-cultures while the 5 mL containers instead saw an unprecedented increase in larvae counts (20+).

Table 2: First Instar Larvicidal and Persistence Test Results

(25^0C, new eggs added every 5 days until day 15)

	B/E 5 mL			B/E 7 mL			B/E 10 mL			B only[C]	E only[C]	No Bac[C]
	R1	R2	R3	R1	**R2***	R3	R1	R2	R3	R1	R1	R1
Day 0	20	20	20	20	20	20	20	20	20	20	20	20
Day 1	14	12	9	15	0	7	14	7	0	10	11	7
Day 2	14	13	9	13	0	7	14	7	0	10	12	10
Day 3	14	13	9	13	0	7	10	7	0	10	12	10
Day 4	12	13	9	13	0	7	4	5	0	10	12	10
Day 5	11	11	9	11	0	3	2	5	0	10	12	10
Day 6	12+11	10+11	12+9	10+11	0	6+3	0	1+5	4+0	14+7	12	10
Day 7	12+11	9+12	21	21	0	6+3	4	1+5	4	21	19	14
Day 8	24	18	17	13	0	6	3	5	1	20	19	14
Day 9	24	18	11	10	0	4	3	5	1	19	19	12
Day 10	24*	18*	10*	2*	0*	2*	1*	5*	0*	17*	27*	12*
Day 11	27	26	7	0	0	3	1	2	8	25	27	12
Day 12	27	26	7	11	0	1	1	2	7	25	27	9
Day 13	27	26	7	11	0	1	0	0	6	25	27	9
Day 14	27	26	7	11	0	1	0	0	4	25	27	9
Day 15	27	26	7	11	0	0	0	0	4	-	-	-
Day 16	27	26	7	11	0	0	0	0	4	-	-	-
Day 17	27	26	7	11	0	0	0	0	4	-	-	-
Day 18	27	26	7	11	0	0	0	0	4	-	-	-
Day 19	27	26	7	11	0	0	0	0	4	-	-	-
Day 20	27	26	7	11	0	0	0	0	4	-	-	-
Day 21	27	26	7	11	0	0	0	0	4	-	-	-

Day 22	27	26	7	11	0	0	0	0	4	-	-	-
Day 23	27	26	7	11	0	0	0	0	4	-	-	-
Day 24	27	26	7	11	0	0	0	0	4	-	-	-
Day 25	27	26	7	11	0	0	0	0	4	-	-	-
Day 26	27	26	7	11	0	0	0	0	4	-	-	-
Day 27	27	26	7	11	0	0	0	0	4	-	-	-

***Exceptional replicate; discarded as an outlier for subsequent probit analysis.**

The hatchability of the bacteria-free control containers stayed a steady 50% and the single bacteria controls maintained a constant number of first instar larvae that shifted minutely with every new addition of eggs.

6.2.3 Third Instar Larvicidal and Persistence Tests

The first round of inoculations - 10mL, 7mL and 5 mL didn't affect the development of the third instar larvae in the slightest. Since volume levels appeared to play a large role in determining larvicidal potential in previous tests, a second round of inoculations at 30 mL, 20 mL and 15 mL were conducted but to no significant effect – the results were identical to the first larval test. Ergo, this approach was abandoned in favor of the egg tests (Tables 3 and 4).

Table 3: Primary Third Instar Larvae Death Tests*

	B/E 5 mL			**B/E 7 mL**			**B/E 10 mL**			**B only[C]**	**E only[C]**	**No Bac[C]**
	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R1	R1
Day 0	0	0	0	0	0	0	0	0	0	0	0	0
Day 1	0	0	1	0	0	0	0	0	0	0	0	0
Day 2	0	1	3	0	0	0	0	0	0	1	2	0
Day 3	0	1	3	0	0	0	0	0	0	1	3	0

Table 4: Secondary Third Instar Larvae Death Tests*

	B/E 15 mL			B/E 20 mL			B/E 30 mL			B only[C]	E only[C]	No Bac[C]
	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R1	R1
Day 0	0	0	0	0	0	0	0	0	0	0	0	0
Day 1	0	0	0	0	0	0	0	0	0	0	0	0
Day 2	0	0	0	0	0	0	0	0	0	0	0	1

*Note: Numbers measure the number of dead larvae.

All 20 larvae in every container became pupas in 2 days and full-grown adults after another 2-3 days (Figure 13).

Figure 13 : **20 Egg (left) and 20 Larvae Tests (right)** (25^0C, 240 mosquitoes in total per test)

The larvicidal efficacy of each bacterium can be seen in the control containers– the left cup (blank) had no bacteria so the eggs added to it hatched and developed into adults in 5 days. The central cup (*E. cloacae*) didn't show larvicidal potency so much as the stagnation of larval development – biofilm production suffocated some larvae but most stayed in their first or second instar forms. The right cup (*B. cereus*) showed true larvicidal activity, with a 95% larval mortality rate observed after 5 days as well – this activity petered off quickly after day 14 however (Figure 14).

Figure 14 : Control Cups [Eggs] after 14 days (Left to Right - Blank, *E. cloacae*, *B. cereus*)

The larvicidal potency over time was primarily affected by the concentration of *E. cloacae* in the containers – the higher it was, the higher the level of biofilm produced, which in turn improved *B. cereus* potency past the previous 14-day limit (Figure 15).

Figure 15 : **Larvicidal experiments B/E Cups at 14 days of incubation (Left to Right: 5, 7 and 10 mL*).** *The biofilm deposits (the yellow or brown colors) can be seen to increase with rising co-culture concentrations.

The biofilm production of individual cultures was also compared to the observed contribution of individual bacteria to the film forming at the bottom of every cup. *E. cloacae* was superior, with a thick white turbidity forming at the bottom of the test tubes – upon disturbing the solution, a murky orange color formed (the same color at the bottom of the containers). A composition of *B.*

cereus also forms a little biofilm in comparison (control cups with this bacteria form a light yellow film) (Figure 16).

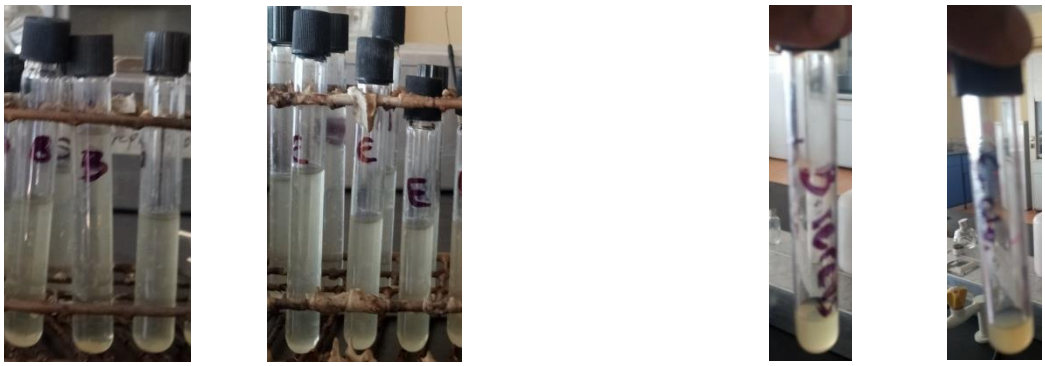

Figure 16 : Biofilm production after 1 (left) and 14 days (right) (left to right: *B. cereus*, *E. cloacae*)

6.3 Probit Analysis

6.3.1 Temperature Effect Test

Before proceeding to the main data analysis, the data was first processed for validity and significance (Table 5).

Table 5: Initial Data Integrity and Convergence Check

Data Information		
		N of Cases
Valid		60
Rejected	Out of Range[a]	0
	Missing	0
	Number of Responses > Number of Subjects	0
Control Group		5
Bacteria	0	5
	1	10
	2	45

a. Cases rejected because of out of range group values.

Convergence Information		
	Number of Iterations	Optimal Solution Found
PROBIT	11	Yes

Based off these initial results, the data was deemed suitable for parameter estimation, from which a regression equation could be estimated (Table 6).

Table 6: Chi Square Test, Parameter Estimates and Regression Formula

Parameter Estimates							
	Parameter		Estimate	Std. Error	Z	Sig.	95% Confidence Interval
							Lower Bound
PROBIT[a]	Concentrations		.000	.025	.015	.988	-.048
	Intercept[b]	0	-.358	.128	-2.794	.005	-.487
		1	-.416	.265	-1.573	.116	-.681
		2	-1.030	.189	-5.444	.000	-1.219

Parameter Estimates			
	Parameter		95% Confidence Interval
			Upper Bound
PROBIT[a]	Concentrations		.049
	Intercept[b]	0	-.230
		1	-.152
		2	-.841

		Chi-Square	df[d]	Sig.
PROBIT	Pearson Goodness-of-Fit Test	241.068	56	.000[c]

a. PROBIT model: PROBIT(p) = Intercept + BX

b. Corresponds to the grouping variable Bacteria.

c. Since the significance level is less than .050, a heterogeneity factor is used in the calculation of confidence limits.
d. Statistics based on individual cases differ from statistics based on aggregated cases.

Using the regression equation based off the parameter estimates (Table 6), relative median potencies and a probit transformed response of LC_{50} concentrations were extrapolated (Table 7, Figure 17).

Table 7: Relative Median Potency for Bacterial Combinations

Relative Median Potency Estimates					
	(I) Bacteria	(J) Bacteria	95% Confidence Limits		
			Estimate	Lower Bound	Upper Bound
PROBIT	0	1	-153.851	.	.
		2	-1787.988	.	.
	1	0	153.851	.	.
		2	-1634.137	.	.
	2	1	1634.137	.	.
		0	1787.988	.	.

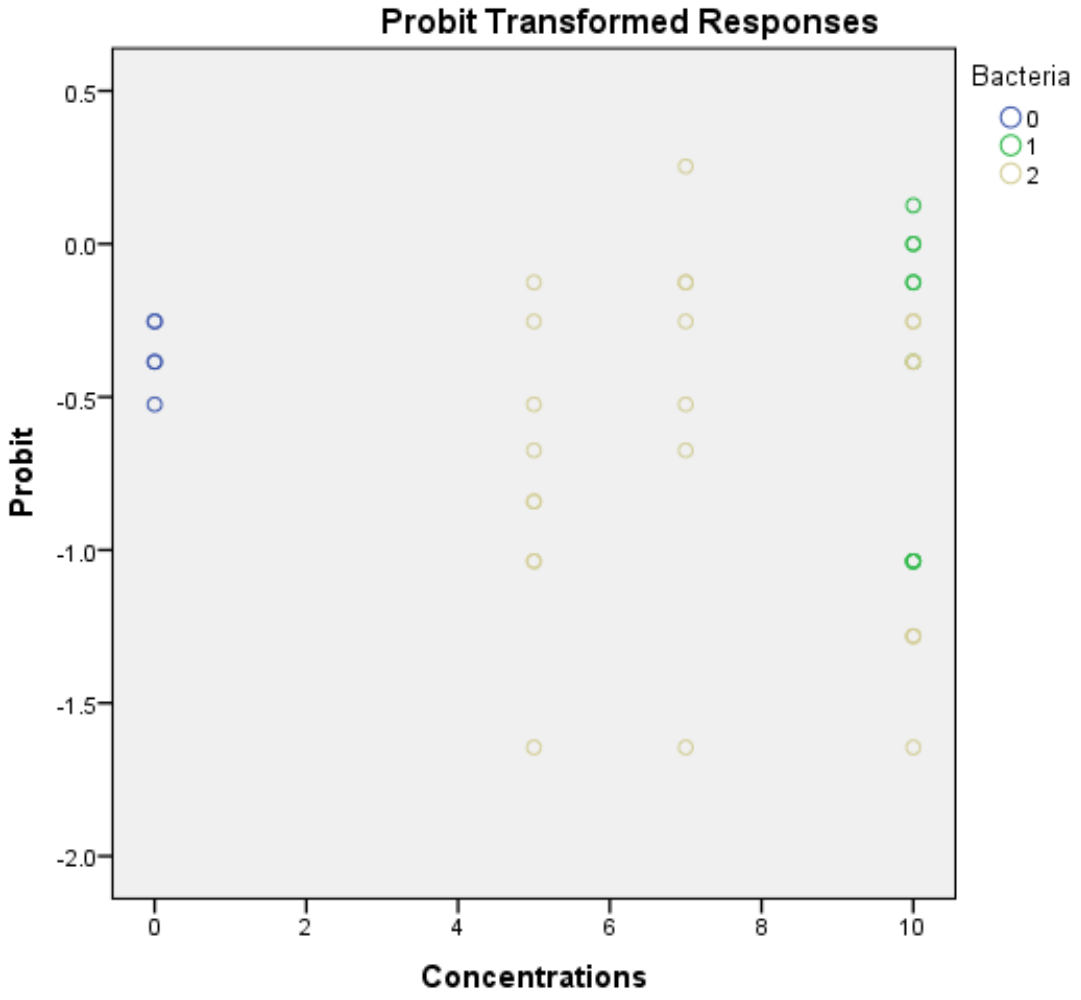

Figure 17 : Temperature Effect Test LC_{50} probit analysis*

*Refer to the appendix part 1, section 4 on confidence limits for the respective test.

Based on the probit analysis (at a significance level of 0.05) of the preliminary egg test, LC50 of the co- culture (2 bacteria) was estimated to be 2742.15 mL. The LC_{50} of the single bacterial cultures was estimated to be 1108.675 mL (refer to the appendix section 1 on confidence limits for varying combinations of bacteria [0, 1 or 2] for the temperature effect test).

6.3.2 First Instar Larvicidal and Persistence Test Analysis Results

Before proceeding to the main data analysis, the data was first processed for validity and significance (Table 8).

Table 8: Initial Data Integrity and Convergence Test *

*One replicate from the B/E 7mL group was omitted as an outlier for this analysis.

Data Information		
		N of Cases
Valid		244
Rejected	Out of Range[a]	0
	Missing	0
	LOG Transform Cannot be Done	0
	Number of Responses > Number of Subjects	0
Control Group		14
Bacteria	0	0
	1	28
	2	216

a. Cases rejected because of out of range group values.

Convergence Information		
	Number of Iterations	Optimal Solution Found
PROBIT	14	Yes

Based off these initial results, the data was deemed suitable for parameter estimation, from which a regression equation could be estimated (Table 9).

Table 9: Parameter Estimates and Regression Formula

Parameter Estimates							
	Parameter		Estimate	Std. Error	Z	Sig.	95% Confidence Interval
							Lower Bound
PROBIT[a]	Concentrations		-4.422	.127	-34.818	.000	-4.671
	Intercept[b]	1	4.312	.132	32.561	.000	4.180
		2	2.739	.102	26.827	.000	2.637

Parameter Estimates			
	Parameter		95% Confidence Interval
			Upper Bound
PROBIT[a]	Concentrations		-4.173
	Intercept[b]	1	4.445
		2	2.841

Chi-Square Tests				
		Chi-Square	df[b]	Sig.
PROBIT	Pearson Goodness-of-Fit Test	1825.539	241	.000[a]

a. PROBIT model: PROBIT (p) = Intercept + BX (Covariates X are transformed using the base 10.000 logarithm.)

b. Corresponds to the grouping variable Bacteria.

c. Since the significance level is less than .050, a heterogeneity factor is used in the calculation of confidence limits.

d. Statistics based on individual cases differ from statistics based on aggregated cases.

Using the regression equation based off the parameter estimates (Table 6), relative median potencies and a probit transformed response of LC_{50} concentrations were extrapolated (Table 10, Figure 18).

Table 10: Relative Median Potency for Bacterial Combinations

Relative Median Potency Estimates						
	(I) Bacteria	(J) Bacteria	95% Confidence Limits			95% Confidence Limits with LOG Transform[a]
			Estimate	Lower Bound	Upper Bound	Estimate
PROBIT	1	2	2.269	1.850	2.967	.356
	2	1	.441	.337	.541	-.356

Relative Median Potency Estimates				
	(I) Bacteria	(J) Bacteria	95% Confidence Limits with LOG Transform	
			Lower Bound	Upper Bound
PROBIT	1	2	.267	.472
	2	1	-.472	-.267

a. Logarithm base = 10.

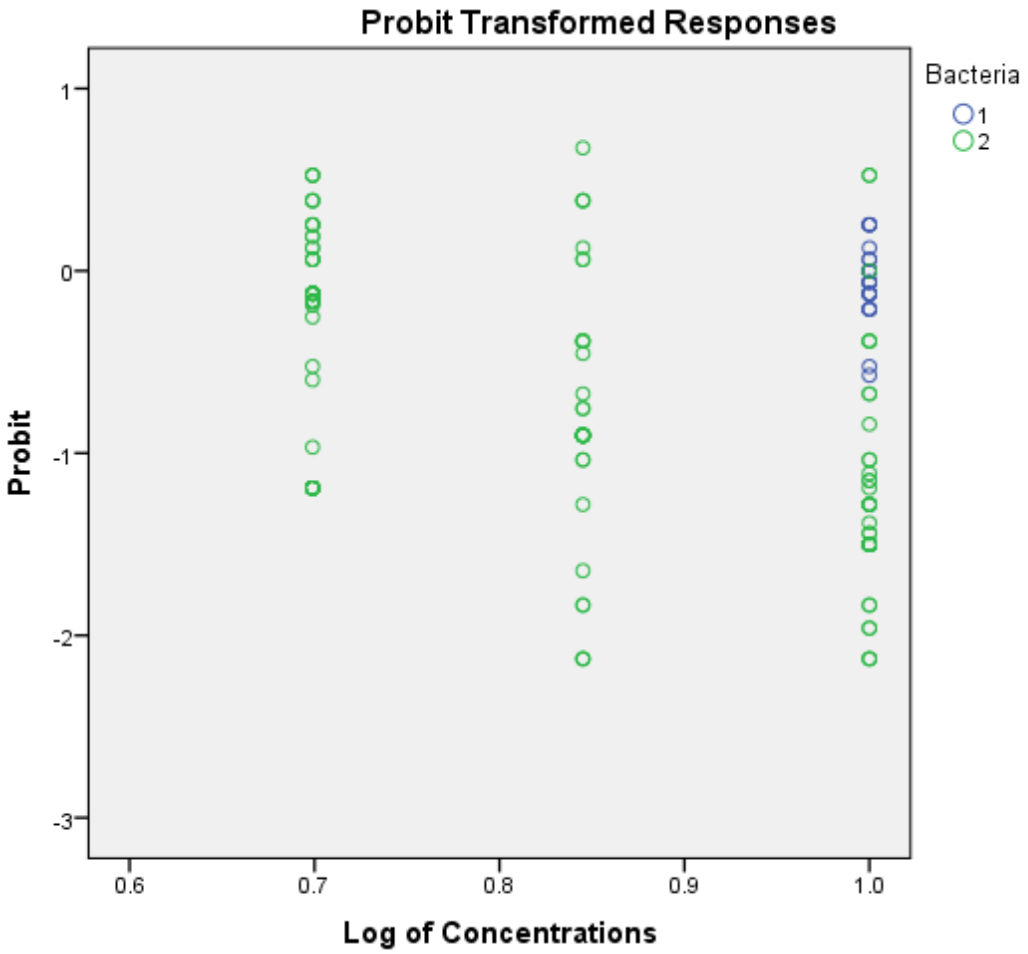

Figure 18 : First Instar Larvicidal Test LC_{50} probit analysis*

*Generated from confidence limits for the respective test – refer to the appendix part 2, section 4 for the full details.

Based on the probit analysis (at a significance level of 0.05) of the main larvicidal and persistence test analysis, LC_{50} of the co- culture (2 bacteria) was estimated to be 4.162 mL with a lower bound of 3.183 mL and an upper one of 4.452 mL. LC50 for single bacteria was estimated to be 9.444 mL with a lower bound of 8.468 mL and an upper one of 10.502 mL (refer to the confidence limits of the respective test in the appendix section 2 for full details). According to the parameter estimates, if only using one bacterium, the number of living larvae that would

survive inoculation increased by 4.445 and if using two of them, it would then be increased by 2.841. The concentration of the bacterial culture reduced the number of live larvae by an average of 4.173 for every milliliter added to the containers, with the regression equation based on parameter estimates given as follows : Live Larvae = -4.173[Concentration] + 4.445 (if using one bacterium) or + 2.841 (if using two).

6.3.3 Biocontrol Persistence Test Results

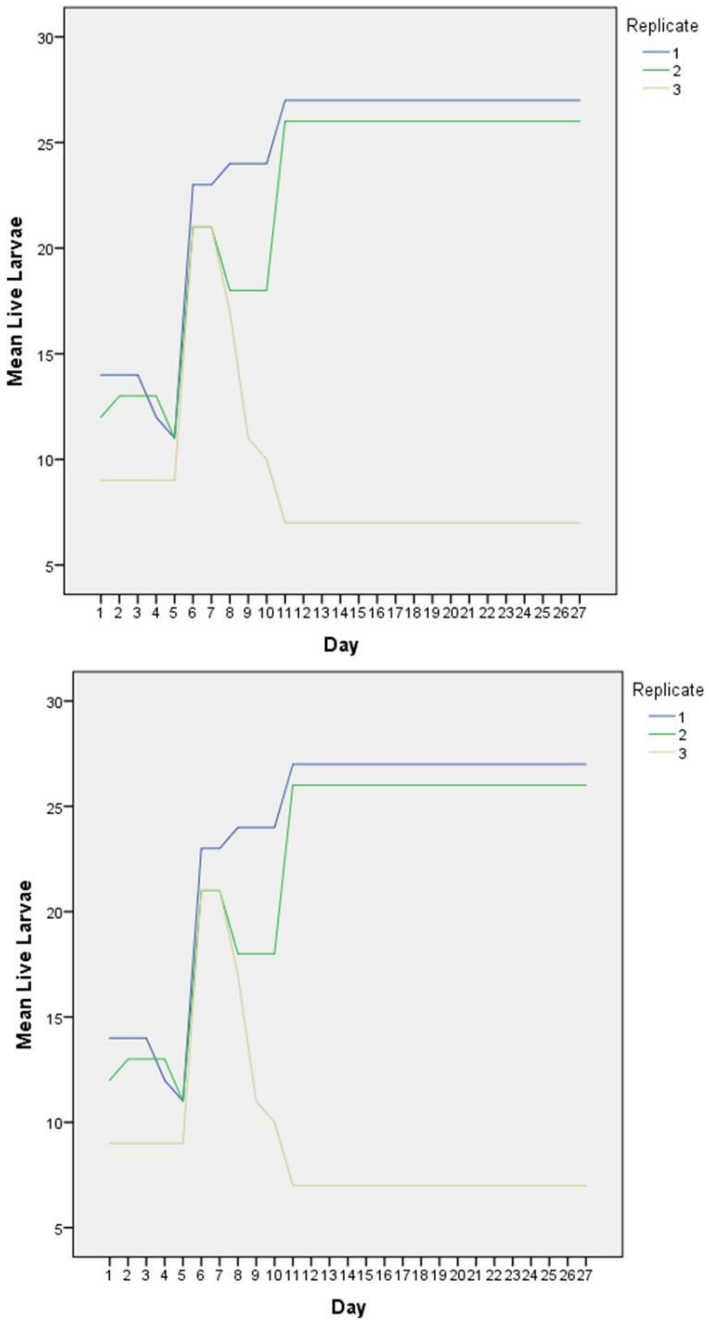

Figure 19: 5mL (Top) and 7mL (Bottom) Biocontrol Persistence Results*

* Replicate 2 in the 7mL test is an outlier and wasn't included in the probit analysis but is shown here as a reference.

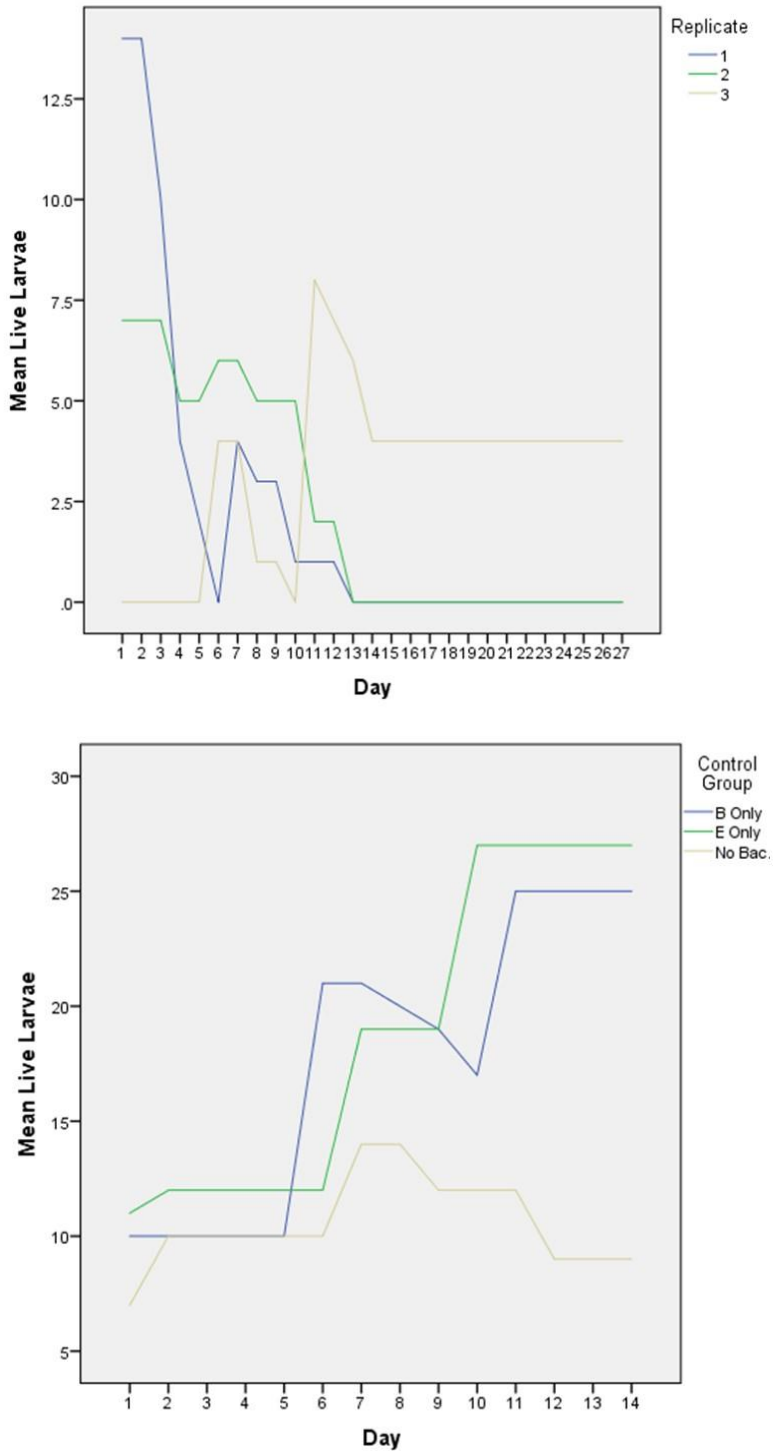

Figure 20 : 10 mL (Top) and Control (Bottom) Biocontrol Persistence Tests

With regard to the persistence of the three formulations, the bacterial co-cultures retained their efficacy patterns (considering their initial concentration levels) for longer than 14 days. Given that two weeks were given as the optimal larvicidal potency period for a pure *B. cereus* culture, the co-cultures appeared to surpass that limit by a minimum of 50% (i.e 21 days) with an average maximum of 27 days depending on their starting concentrations and biofilm deposition. The extent of biofilm deposition determined the co-cultures' better viability as a means of long-lasting biocontrol to limit the propagation of *Anopheles* mosquitoes. The control groups on the other hand showed that their larvicidal potency dropped sharply by day 10, with the bacterial controls having retained a large larval population. The blank control (only water) 'lost' larvae in the form of pupae which later became adults.

7. DISCUSSION

In the course of this experiment, the primary issue addressed was the persistence of the *B. cereus* biocontrol system in an aqueous environment. While the larvicidal potency of this bacterium has been well documented [4], its efficacy is only relevant for 14 days and this was fixed by mixing *B. cereus* with *E. cloacae*, making this combined co-culture retain its larvicidal potency for over 27 days. This improvement (~100% increase in potency time) was due to the synergistic potential between the two bacteria in their co-culture, with *E. cloacae* providing the biofilm that provided the medium to consolidate the *B. cereus* population in the aqueous environment. The biofilm production capacity with regards to bacterial concentrations, which have already been extensively studied for both bacteria prior [32, 33] was used to corroborate these results.

The efficacy of the bacterial co-cultures appeared to be directly influenced by their initial concentrations in the mosquito containers – the higher the inoculum, the more potent the

larvicidal effect. The minimum concentration to achieve 50% mortality was also affected by temperature (comparing the LC_{50} doses for the preliminary test at 37^0C and the main tests at around 23-27^0C) as the mosquito development cycle accelerates with rising temperatures – as the formulations work best on first instar larvae, the contrasting Probit analysis results reflect this (more bacteria are needed at higher temperatures).

In the preliminary test, the higher temperature that the room was maintained sped up the development of the mosquito larvae, as they were able to go from eggs to full adults in just five days. Ergo, the bacteria needed to be present in higher inoculum amounts to appropriately hinder or otherwise eliminate larval stage progression, hence the higher probit parameter estimates for LC_{50}. This test also showed how each bacterial component negatively affects larval development – while *B. cereus* produces larvicidal toxins as previously described in prior research [4], *E. cloacae* has a bigger role in establishing a persistent layer of biofilm in the containers it reaches appreciable concentrations at (3.5 mL and above) – while both bacteria could produce biofilms, the latter was more dominant. These results were in line with previous research in this area, as *E. cloacae* and *B. cereus* are capable of biofilm production – with the former having a more persistent composition than the latter [32,33].

An additional note was that in the preliminary tests, the environmental dominance of both bacteria was demonstrated when the 5-day co-cultures were checked – given the generational time of both bacteria and the fact that they had an 24-hour incubation period before co-culture incubation in the containers, they were both in their logarithmic growth phase. This meant that they rapidly proliferated in the water they were in, eventually reaching and exceeding preferred concentration levels for desired larvicidal potency levels (while not shown in this study, the growth curves of both bacteria in the nutrient broth are well characterized as well) [32,33] .

As such, 37^0C was deemed too extreme for a biocontrol system that would need to function ideally at a temperature range from 20 -27^0C, which was why the subsequent egg and third instar larvae tests were conducted at 25^0C to better represent the average tropical climate [43].

In the main tests, divided into the egg and third instar larvae tests respectively, the results observed differed primarily due to the immunocompetence of the larval stages in question. In the [egg] tests, the larvae that hatched were first instar larvae, with immature midguts and no microbiota to speak of. The [larvae] tests on the other hand, were conducted on third-instar larvae, which were more resilient to the bacterial toxins in their midguts, so much so that the co-cultures didn't affect them at all – as they developed into adults with no issues after 3-4 days. This experiment was repeated twice but with no effect so the co-culture biocontrol method was deemed ineffective on third instar larvae. This could be explained by the fact that the third instar larvae were more immunocompetent than their first instar counterparts – higher volumes, if possible, would be needed to have any effect on these, more developed mosquito progeny.

The first instar larvae on the other hand were indeed affected by the co-cultures, with their mortality being directly proportional to the concentrations of the bacterial co-cultures in the containers they were suspended in. Based on the parameter estimates of the probit Finney analysis, the concentration of the bacterial culture reduced the number of live larvae by an average of 4.173for every milliliter added to the containers, with the regression equation given as follows: Live Larvae = -4.173[Concentration] + 4.445 (if using one bacterium) or + 2.841 (if using two). This meant that the most significant factor was concentration – the presence of two or more bacteria only further facilitated larvae mortality. In practice, this meant that as long as the bacterial co-culture expanded and became a persistent element in the containers, any additional eggs added to them would either not hatch entirely or the new larvae that did hatch

would quickly die out. This was observed from day 27 and onwards since the 10 mL bacterial co-culture containers maintained an acceptable level of larvicidal potency when the other concentration level replicates couldn't keep up with the ever-increasing mosquito egg additions.

The result was heartening nonetheless – with a proper formulation of the bacterial co-culture at 10mL and above, the resulting biocontrol method has the lethality of *B. cereus* [4] and the environmental persistence of *E. cloacae* [29] exceeding the previous limit of 14 days by more than a 100% (the test concluded by day 27 but only because no eggs even hatched in the 10 mL replicates by that point).

Current biocontrol methods for *Anopheles* fall short of this level of efficiency – seeing as most strategies tend to utlilize natural predators and genetic methods, they are at best seen as complementary to chemical intervention. For example, backswimmers have shown high predation rates, with studies reporting an average daily predation of over 70 Anopheles larvae per individual. However, while this is encouraging, this method has the limitations of the generalist predatory nature of these organisms, long life cycles, and the complexity of rearing and releasing them effectively in the field [43].

Other techniques seek to sabotage or undermine the natural mating patterns of the mosquitoes, such as the sterile insect technique (SIT) or the Toxic Male Technique (TMT). While the former is a more traditional method, relying on using sterile males to dominate the pool of available mates for the female, the latter uses genetically modified males who inadvertently poison the females with their toxic semen during mating. TMT reduces the female mosquito's lifespan by about 60% and is more efficient than SIT but is too challenging to be used effectively in Third World nations given the considerable logistic hurdles, only serving as low-risk alternatives to

chemical pesticides despite being touted for quick responses to outbreaks of mosquito-borne diseases [44].

Fungi have also seen some use as *Anopheles* biocontrol with genera like *Lagenidium* gaining some traction for its capacity to infect and kill mosquito larvae. These fungi are non-specific killers though, as they attack all kinds of mosquitoes and not just *Anopheles*, in addition to challenges in mass production, dissemination, and adaptation to polluted breeding environments [45].

This makes this study's product, a low cost and easily scalable co-culture biocontrol product a desirable avenue for mosquito disease vector control in any environment. The bacteria used are ubiquitous, non-fastidious and relatively harmless to most other organisms in their vicinity, making them a prime candidate for worldwide adoption compared to their competitors.

When the bacteria in the containers acclimatize themselves to the new environment and their new source of nutrition (the yeast extract fed to the mosquito larvae), they begin to produce biofilms. These biofilms accumulated at the bottom of the containers but at higher concentrations (certain 7 mL and 10 mL replicates), a thin layer also formed at the surface of the water. These secondary biofilms appeared to halt all hatching processes in their respective containers, with not a single one hatching. If these biofilms formed after hatching had already occurred, they slowly suffocated the newly born larvae within 48 hours or less.

The primary biofilm at the bottom of each container served as a reservoir of bacteria – during the brief grace period when new eggs or larvae were added, the unused yeast extract nourished both active and dormant bacteria in the water and film to presumably maintain their potency over an extended period. As the *B. cereus* component is the primary contributor to the toxins responsible

for the larvicidal effect [4], it is reasonable to conclude that the more avid biofilm producer *E. cloacae* successfully established a harmonious symbiotic relationship with the other bacteria in their shared biofilm and medium.

On a separate note, the propagation rate of both bacteria was augmented when in contact with yeast extracts (both in agar form and drop form) as their biofilm production began in less than 2 days when exposed to them (something that was exemplified in the first CFU tests – there were too many bacteria to enumerate at the 10^{-5} dilution factor if 48 hours had elapsed). In comparison, the nutrient broth cultures had biofilms formed in 4 days at the earliest.

The first instar larvae were also very susceptible to the bacteria in their environment, something that can be attributed to their lack of immunocompetency at this stage in their life cycle. In direct contrast, the development of the third instar larvae was unaffected by the presence of the bacteria even after nearly tripling the concentrations of each inoculation. As the bacterial load wasn't a factor, it can only be assumed that these larvae have more stable midgut dynamics despite having grown in a sterile environment. These larvae do eventually harbor both bacteria from the co-culture in their midguts however – this might affect their fitness as disease vectors but due to resource and logistical constraints, this aspect was not addressed in this study.

8. CONCLUSIONS

A combined formulation of *B. cereus* and *E. cloacae* appears to maintain a steady symbiosis similar to the one they share in mosquito midguts in an aqueous environment. They also have a superior larvicidal and persistence profile when compared to their single bacterial culture counterparts. When introduced to eggs and first instar larvae, the mosquitoes die or remain dormant at their current development stage over 3-5 days if the bacteria are allowed to grow in cultures for 48 hours before inoculation. If the culture time is reduced to 24 hours, the larvicidal ability slows down and the larvae instead seem to stagnate at the first or second instar level. This can be circumvented by enriching the otherwise sterile water the bacteria are in with nutritive factors like yeast extract, sugar and the like. When this is done, biofilm production is accelerated – in cases where there is nutritional abundance, a secondary film forms on the surface of the water and no mosquitoes survive or even hatch after that.

When applying these co-cultures to third-instar larvae, the bacteria don't appear to affect them at all. While this can be attributed to the relative immunological maturity of the larvae at this stage in their lives, the new midgut dynamics they would then possess could affect their fitness as disease vectors.

9. RECOMMENDATIONS

Further improvements on the scope of similarly themed studies could include an expansion on the number of tested mosquito species and bacteria used in co-cultures. Given the three main mosquito genera (and their respective species) and the diverse library of midgut bacteria, a comprehensive test of all possible combinations of bacterial co-cultures on the mosquitoes would generate a more fleshed-out system of biocontrol for disease vector mosquitoes. A study on how the co-culture bacteria can be mass-produced and stored for environmental deployment in separate field tests in known malaria prevalent zones is also recommended as while the current results are encouraging, a better understanding of how these bacteria interact in a competitive environment is crucial.

Another limitation of this study was the relative dearth of mosquito and bacteria variety – when this experiment was initially proposed, six different species of *Anopheles* and three different *Bacillus* (*B. cereus*, *B. thuringiensis* and *B. sphaericus*) species to be used in varying combinations with *E. cloacae*. In addition, after the laboratory experiments were concluded, a second set of field tests were to be conducted all over Addis Ababa and potentially Ethiopia after dissecting and analyzing the effects of the co-cultures on both first and third instar larvae. This was not possible due to the resource constraints involved in designing the study – the monetary and logistical limitations were too significant to overcome.

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11. APPENDICES

Part 1: Temperature Effect on Larvicidal Efficiency Test SPSS Output
Probit Analysis

Notes		
Output Created		18-DEC-2024 11:54:43
Comments		
Input	Active Dataset	DataSet3
	Filter	<none>
	Weight	<none>
	Split File	<none>
	N of Rows in Working Data File	60
Missing Value Handling	Definition of Missing	User-defined missing values are treated as missing.
	Cases Used	Statistics are based on all cases with valid data for all variables in the model.

Syntax		PROBIT LiveLarvae OF TotalEggs BY Bacteria(0 2) WITH Concentrations /LOG NONE /MODEL PROBIT /PRINT FREQ CI RMP /CRITERIA P(.05) ITERATE(20) STEPLIMIT(.1).
Resources	Processor Time	00:00:00.17
	Elapsed Time	00:00:00.13

Section 1: Data Integrity Checks for Temperature Effect Test

Data Information

		N of Cases
Valid		60
Rejected	Out of Range[a]	0
	Missing	0
	Number of Responses > Number of Subjects	0
Control Group		5
Bacteria	0	5
	1	10
	2	45

a. Cases rejected because of out of range group values.

Convergence Information

	Number of Iterations	Optimal Solution Found
PROBIT	11	Yes

Section 2: Goodness of Fit, Parameter Estimates and Regression Formula

Parameter Estimates

Parameter			Estimate	Std. Error	Z	Sig.	95% Confidence Interval Lower Bound
PROBIT[a]	Concentrations		.000	.025	.015	.988	-.048
	Intercept[b]	0	-.358	.128	-2.794	.005	-.487
		1	-.416	.265	-1.573	.116	-.681
		2	-1.030	.189	-5.444	.000	-1.219

Parameter Estimates

Parameter			95% Confidence Interval Upper Bound
PROBIT[a]	Concentrations		.049
	Intercept[b]	0	-.230
		1	-.152
		2	-.841

a. PROBIT model: PROBIT(p) = Intercept + BX

b. Corresponds to the grouping variable Bacteria.

Chi-Square Tests

		Chi-Square	df[b]	Sig.
PROBIT	Pearson Goodness-of-Fit Test	241.068	56	.000[a]

a. Since the significance level is less than .050, a heterogeneity factor is used in the calculation of confidence limits.

b. Statistics based on individual cases differ from statistics based on aggregated cases.

Cell Counts and Residuals

Cell Counts and Residuals

	No.	Bacteria	Concentr-ations	Number of Subjects	Observed Responses	Expected Responses	Residual	Probability
PROBIT	1	0	.000	20	6	7.200	-1.200	.360
	2	0	.000	20	8	7.200	.800	.360
	3	0	.000	20	8	7.200	.800	.360
	4	0	.000	20	7	7.200	-.200	.360
	5	0	.000	20	7	7.200	-.200	.360
	6	1	10.000	20	10	6.800	3.200	.340
	7	1	10.000	20	9	6.800	2.200	.340
	8	1	10.000	20	7	6.800	.200	.340
	9	1	10.000	20	3	6.800	-3.800	.340

	10	1	10.000	20	3	6.800	-3.800	.340
	11	1	10.000	20	11	6.800	4.200	.340
	12	1	10.000	20	10	6.800	3.200	.340
	13	1	10.000	20	9	6.800	2.200	.340
	14	1	10.000	20	3	6.800	-3.800	.340
	15	1	10.000	20	3	6.800	-3.800	.340
	16	2	5.000	20	8	3.040	4.960	.152
	17	2	5.000	20	6	3.040	2.960	.152
	18	2	5.000	20	3	3.040	-.040	.152
	19	2	5.000	20	0	3.040	-3.040	.152
	20	2	5.000	20	0	3.040	-3.040	.152
	21	2	5.000	20	4	3.040	.960	.152
	22	2	5.000	20	3	3.040	-.040	.152
	23	2	5.000	20	0	3.040	-3.040	.152
	24	2	5.000	20	0	3.040	-3.040	.152
	25	2	5.000	20	0	3.040	-3.040	.152
	26	2	5.000	20	9	3.040	5.960	.152
	27	2	5.000	20	5	3.040	1.960	.152
	28	2	5.000	20	4	3.040	.960	.152
	29	2	5.000	20	1	3.040	-2.040	.152
	30	2	5.000	20	0	3.040	-3.040	.152
	31	2	7.000	20	9	3.044	5.956	.152
	32	2	7.000	20	8	3.044	4.956	.152

	33	2	7.000	20	5	3.044	1.956	.152
	34	2	7.000	20	0	3.044	-3.044	.152
	35	2	7.000	20	0	3.044	-3.044	.152
	36	2	7.000	20	12	3.044	8.956	.152
	37	2	7.000	20	9	3.044	5.956	.152
	38	2	7.000	20	6	3.044	2.956	.152
	39	2	7.000	20	1	3.044	-2.044	.152
	40	2	7.000	20	0	3.044	-3.044	.152
	41	2	7.000	20	0	3.044	-3.044	.152
	42	2	7.000	20	0	3.044	-3.044	.152
	43	2	7.000	20	0	3.044	-3.044	.152
	44	2	7.000	20	0	3.044	-3.044	.152
	45	2	7.000	20	0	3.044	-3.044	.152
	46	2	10.000	20	8	3.049	4.951	.152
	47	2	10.000	20	7	3.049	3.951	.152
	48	2	10.000	20	2	3.049	-1.049	.152
	49	2	10.000	20	0	3.049	-3.049	.152
	50	2	10.000	20	0	3.049	-3.049	.152
	51	2	10.000	20	7	3.049	3.951	.152
	52	2	10.000	20	2	3.049	-1.049	.152
	53	2	10.000	20	1	3.049	-2.049	.152
	54	2	10.000	20	0	3.049	-3.049	.152
	55	2	10.000	20	0	3.049	-3.049	.152

	56	2	10.000	20	8	3.049	4.951	.152
	57	2	10.000	20	7	3.049	3.951	.152
	58	2	10.000	20	2	3.049	-1.049	.152
	59	2	10.000	20	0	3.049	-3.049	.152
	60	2	10.000	20	0	3.049	-3.049	.152

Section 3: LC_{50} Confidence Limit Estimations for 0, 1 or 2 bacteria

Confidence Limits					
			95% Confidence Limits for Concentrations		
	Bacteria	Probability	Estimate	Lower Bound	Upper Bound
PROBIT[a]	0	.010	-5241.855	.	.
		.020	-4515.734	.	.
		.030	-4055.034	.	.
		.040	-3708.468	.	.
		.050	-3426.562	.	.
		.060	-3186.617	.	.
		.070	-2976.231	.	.
		.080	-2787.856	.	.
		.090	-2616.537	.	.
		.100	-2458.837	.	.
		.150	-1805.917	.	.
		.200	-1286.997	.	.

		.250	-841.810	.	.
		.300	-442.018	.	.
		.350	-71.552	.	.
		.400	279.985	.	.
		.450	620.101	.	.
		.500	954.824	.	.
		.550	1289.547	.	.
		.600	1629.663	.	.
		.650	1981.200	.	.
		.700	2351.667	.	.
		.750	2751.458	.	.
		.800	3196.645	.	.
		.850	3715.565	.	.
		.900	4368.485	.	.
		.910	4526.185	.	.
		.920	4697.505	.	.
		.930	4885.880	.	.
		.940	5096.265	.	.
		.950	5336.211	.	.
		.960	5618.116	.	.
		.970	5964.683	.	.
		.980	6425.383	.	.
		.990	7151.503	.	.

	1	.010	-5088.003	.	.
		.020	-4361.883	.	.
		.030	-3901.183	.	.
		.040	-3554.616	.	.
		.050	-3272.711	.	.
		.060	-3032.766	.	.
		.070	-2822.380	.	.
		.080	-2634.005	.	.
		.090	-2462.686	.	.
		.100	-2304.986	.	.
		.150	-1652.066	.	.
		.200	-1133.146	.	.
		.250	-687.959	.	.
		.300	-288.167	.	.
		.350	82.300	.	.
		.400	433.836	.	.
		.450	773.952	.	.
		.500	1108.675	.	.
		.550	1443.399	.	.
		.600	1783.515	.	.
		.650	2135.051	.	.
		.700	2505.518	.	.
		.750	2905.310	.	.

		.800	3350.497	.	.
		.850	3869.417	.	.
		.900	4522.337	.	.
		.910	4680.036	.	.
		.920	4851.356	.	.
		.930	5039.731	.	.
		.940	5250.116	.	.
		.950	5490.062	.	.
		.960	5771.967	.	.
		.970	6118.534	.	.
		.980	6579.234	.	.
		.990	7305.354	.	.
	2	.010	-3453.866	.	.
		.020	-2727.746	.	.
		.030	-2267.046	.	.
		.040	-1920.480	.	.
		.050	-1638.574	.	.
		.060	-1398.629	.	.
		.070	-1188.243	.	.
		.080	-999.868	.	.
		.090	-828.549	.	.
		.100	-670.849	.	.
		.150	-17.929	.	.

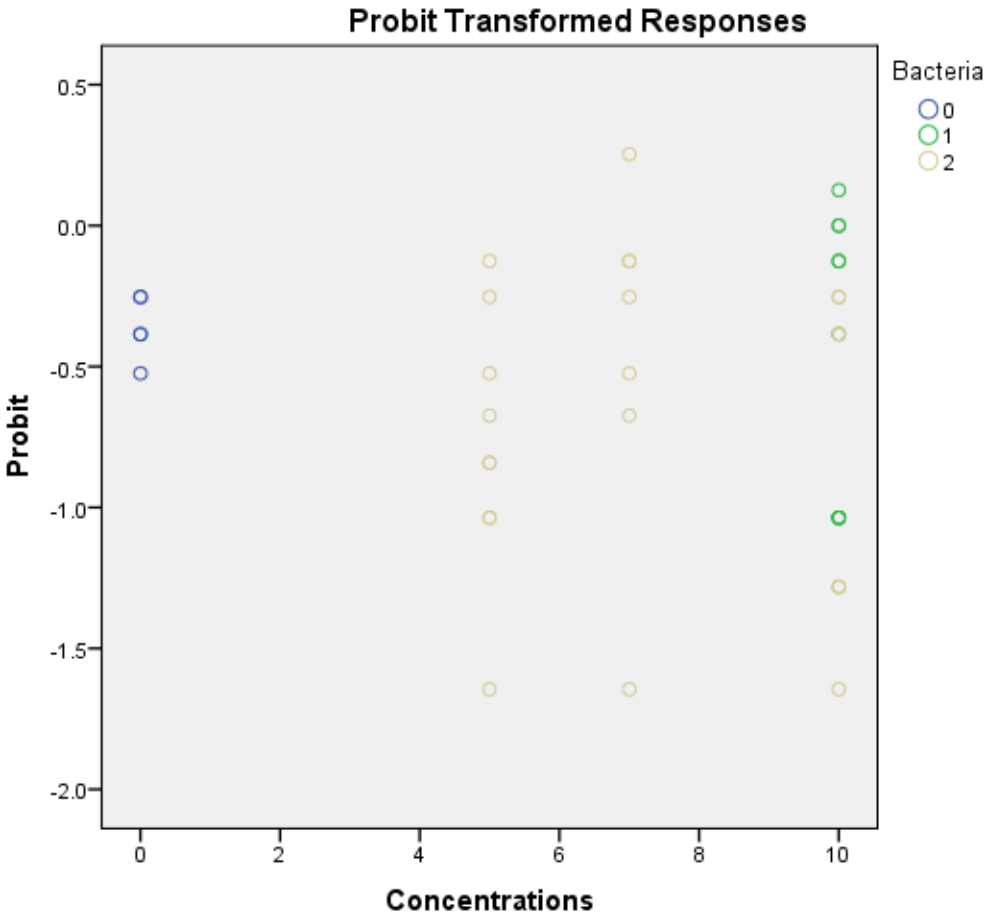
		.200	500.991	.	.
		.250	946.178	.	.
		.300	1345.970	.	.
		.350	1716.436	.	.
		.400	2067.973	.	.
		.450	2408.089	.	.
		.500	2742.812	.	.
		.550	3077.536	.	.
		.600	3417.651	.	.
		.650	3769.188	.	.
		.700	4139.655	.	.
		.750	4539.446	.	.
		.800	4984.634	.	.
		.850	5503.554	.	.
		.900	6156.473	.	.
		.910	6314.173	.	.
		.920	6485.493	.	.
		.930	6673.868	.	.
		.940	6884.253	.	.
		.950	7124.199	.	.
		.960	7406.104	.	.
		.970	7752.671	.	.
		.980	8213.371	.	.

		.990	8939.491	.	.

a. A heterogeneity factor is used.

Section 4: Relative Median Potency Estimates for Bacterial Combinations and Probit Transformed Response

Relative Median Potency Estimates					
	(I) Bacteria	(J) Bacteria	95% Confidence Limits		
			Estimate	Lower Bound	Upper Bound
PROBIT	0	1	-153.851	.	.
		2	-1787.988	.	.
	1	0	153.851	.	.
		2	-1634.137	.	.
	2	1	1634.137	.	.
		0	1787.988	.	.


Probit Transformed Responses
Bacteria
0
1
2
Probit
0.5
0.0
-0.5
-1.0
-1.5
-2.0
0
2
4
6
8
10
Concentrations

Part 2: First-Instar Larvicidal and Persistence Tests SPSS Output
Probit Analysis

Notes

Output Created		11-JUN-2025 16:31:14
Comments		
Input	Active Dataset	DataSet1
	Filter	<none>
	Weight	<none>
	Split File	<none>
	N of Rows in Working Data File	258
Missing Value Handling	Definition of Missing	User-defined missing values are treated as missing.
	Cases Used	Statistics are based on all cases with valid data for all variables in the model.
Syntax		PROBIT LiveLarvae OF TotalEggs BY Bacteria(0 2) WITH Concentrations /LOG 10 /MODEL PROBIT /PRINT FREQ CI RMP /CRITERIA P(0.15) ITERATE(20) STEPLIMIT(.1).
Resources	Processor Time	00:00:00.91
	Elapsed Time	00:00:00.32

Section 1: Data Integrity Checks for First-Instar Larvicidal and Persistence Tests

Data Information

		N of Cases
Valid		244
Rejected	Out of Range[a]	0
	Missing	0
	LOG Transform Cannot be Done	0
	Number of Responses > Number of Subjects	0
Control Group		14
Bacteria	0	0
	1	28
	2	216

a. Cases rejected because of out of range group values.

Convergence Information

	Number of Iterations	Optimal Solution Found
PROBIT	14	Yes

Section 2: Goodness of Fit, Parameter Estimates and Regression Formula

Parameter Estimates

Parameter			Estimate	Std. Error	Z	Sig.	95% Confidence Interval
							Lower Bound
PROBIT[a]	Concentrations		-4.422	.127	-34.818	.000	-4.671
	Intercept[b]	1	4.312	.132	32.561	.000	4.180
		2	2.739	.102	26.827	.000	2.637

Parameter Estimates

Parameter			95% Confidence Interval
			Upper Bound
PROBIT[a]	Concentrations		-4.173
	Intercept[b]	1	4.445
		2	2.841

a. PROBIT model: PROBIT (p) = Intercept + BX (Covariates X are transformed using the base 10.000 logarithm.)

b. Corresponds to the grouping variable Bacteria.

Chi-Square Tests

		Chi-Square	df[b]	Sig.
PROBIT	Pearson Goodness-of-Fit Test	1825.539	241	.000[a]

a. Since the significance level is less than .150, a heterogeneity factor is used in the calculation of confidence limits.

b. Statistics based on individual cases differ from statistics based on aggregated cases.

Cell Counts and Residuals

Cell Counts and Residuals

	Number	Bacteria	Concentrations	Number of Subjects	Observed Responses	Expected Responses	Residual	Probability
PROBIT	1	1	1.000	20	10	9.125	.875	.456
	2	1	1.000	20	10	9.125	.875	.456
	3	1	1.000	20	10	9.125	.875	.456
	4	1	1.000	20	10	9.125	.875	.456
	5	1	1.000	20	10	9.125	.875	.456
	6	1	1.000	40	21	18.250	2.750	.456
	7	1	1.000	40	21	18.250	2.750	.456
	8	1	1.000	40	20	18.250	1.750	.456
	9	1	1.000	40	19	18.250	.750	.456
	10	1	1.000	60	17	27.375	-10.375	.456
	11	1	1.000	60	25	27.375	-2.375	.456

	12	1	1.000	60	25	27.375	-2.375	.456
	13	1	1.000	60	25	27.375	-2.375	.456
	14	1	1.000	60	25	27.375	-2.375	.456
	15	1	1.000	20	11	9.125	1.875	.456
	16	1	1.000	20	12	9.125	2.875	.456
	17	1	1.000	20	12	9.125	2.875	.456
	18	1	1.000	20	12	9.125	2.875	.456
	19	1	1.000	20	12	9.125	2.875	.456
	20	1	1.000	40	12	18.250	-6.250	.456
	21	1	1.000	40	19	18.250	.750	.456
	22	1	1.000	40	19	18.250	.750	.456
	23	1	1.000	40	19	18.250	.750	.456
	24	1	1.000	60	27	27.375	-.375	.456
	25	1	1.000	60	27	27.375	-.375	.456
	26	1	1.000	60	27	27.375	-.375	.456
	27	1	1.000	60	27	27.375	-.375	.456
	28	1	1.000	60	27	27.375	-.375	.456
	29	2	.699	20	14	7.247	6.753	.362
	30	2	.699	20	14	7.247	6.753	.362
	31	2	.699	20	14	7.247	6.753	.362
	32	2	.699	20	12	7.247	4.753	.362
	33	2	.699	20	11	7.247	3.753	.362
	34	2	.699	40	23	14.493	8.507	.362

	35	2	.699	40	23	14.493	8.507	.362
	36	2	.699	40	24	14.493	9.507	.362
	37	2	.699	40	24	14.493	9.507	.362
	38	2	.699	60	24	21.740	2.260	.362
	39	2	.699	60	27	21.740	5.260	.362
	40	2	.699	60	27	21.740	5.260	.362
	41	2	.699	60	27	21.740	5.260	.362
	42	2	.699	60	27	21.740	5.260	.362
	43	2	.699	60	27	21.740	5.260	.362
	44	2	.699	60	27	21.740	5.260	.362
	45	2	.699	60	27	21.740	5.260	.362
	46	2	.699	60	27	21.740	5.260	.362
	47	2	.699	60	27	21.740	5.260	.362
	48	2	.699	60	27	21.740	5.260	.362
	49	2	.699	60	27	21.740	5.260	.362
	50	2	.699	60	27	21.740	5.260	.362
	51	2	.699	60	27	21.740	5.260	.362
	52	2	.699	60	27	21.740	5.260	.362
	53	2	.699	60	27	21.740	5.260	.362
	54	2	.699	60	27	21.740	5.260	.362
	55	2	.699	60	27	21.740	5.260	.362
	56	2	.699	20	12	7.247	4.753	.362
	57	2	.699	20	13	7.247	5.753	.362

	58	2	.699	20	13	7.247	5.753	.362
	59	2	.699	20	13	7.247	5.753	.362
	60	2	.699	20	11	7.247	3.753	.362
	61	2	.699	40	21	14.493	6.507	.362
	62	2	.699	40	21	14.493	6.507	.362
	63	2	.699	40	18	14.493	3.507	.362
	64	2	.699	40	18	14.493	3.507	.362
	65	2	.699	60	18	21.740	-3.740	.362
	66	2	.699	60	26	21.740	4.260	.362
	67	2	.699	60	26	21.740	4.260	.362
	68	2	.699	60	26	21.740	4.260	.362
	69	2	.699	60	26	21.740	4.260	.362
	70	2	.699	60	26	21.740	4.260	.362
	71	2	.699	60	26	21.740	4.260	.362
	72	2	.699	60	26	21.740	4.260	.362
	73	2	.699	60	26	21.740	4.260	.362
	74	2	.699	60	26	21.740	4.260	.362
	75	2	.699	60	26	21.740	4.260	.362
	76	2	.699	60	26	21.740	4.260	.362
	77	2	.699	60	26	21.740	4.260	.362
	78	2	.699	60	26	21.740	4.260	.362
	79	2	.699	60	26	21.740	4.260	.362
	80	2	.699	60	26	21.740	4.260	.362

	81	2	.699	60	26	21.740	4.260	.362
	82	2	.699	60	26	21.740	4.260	.362
	83	2	.699	20	9	7.247	1.753	.362
	84	2	.699	20	9	7.247	1.753	.362
	85	2	.699	20	9	7.247	1.753	.362
	86	2	.699	20	9	7.247	1.753	.362
	87	2	.699	20	9	7.247	1.753	.362
	88	2	.699	40	21	14.493	6.507	.362
	89	2	.699	40	21	14.493	6.507	.362
	90	2	.699	40	17	14.493	2.507	.362
	91	2	.699	40	11	14.493	-3.493	.362
	92	2	.699	60	10	21.740	-11.740	.362
	93	2	.699	60	7	21.740	-14.740	.362
	94	2	.699	60	7	21.740	-14.740	.362
	95	2	.699	60	7	21.740	-14.740	.362
	96	2	.699	60	7	21.740	-14.740	.362
	97	2	.699	60	7	21.740	-14.740	.362
	98	2	.699	60	7	21.740	-14.740	.362
	99	2	.699	60	7	21.740	-14.740	.362
	100	2	.699	60	7	21.740	-14.740	.362
	101	2	.699	60	7	21.740	-14.740	.362
	102	2	.699	60	7	21.740	-14.740	.362
	103	2	.699	60	7	21.740	-14.740	.362

	104	2	.699	60	7	21.740	-14.740	.362
	105	2	.699	60	7	21.740	-14.740	.362
	106	2	.699	60	7	21.740	-14.740	.362
	107	2	.699	60	7	21.740	-14.740	.362
	108	2	.699	60	7	21.740	-14.740	.362
	109	2	.699	60	7	21.740	-14.740	.362
	110	2	.845	20	15	3.181	11.819	.159
	111	2	.845	20	13	3.181	9.819	.159
	112	2	.845	20	13	3.181	9.819	.159
	113	2	.845	20	13	3.181	9.819	.159
	114	2	.845	20	11	3.181	7.819	.159
	115	2	.845	40	21	6.361	14.639	.159
	116	2	.845	40	21	6.361	14.639	.159
	117	2	.845	40	13	6.361	6.639	.159
	118	2	.845	40	10	6.361	3.639	.159
	119	2	.845	60	2	9.542	-7.542	.159
	120	2	.845	60	0	9.542	-9.542	.159
	121	2	.845	60	11	9.542	1.458	.159
	122	2	.845	60	11	9.542	1.458	.159
	123	2	.845	60	11	9.542	1.458	.159
	124	2	.845	60	11	9.542	1.458	.159
	125	2	.845	60	11	9.542	1.458	.159
	126	2	.845	60	11	9.542	1.458	.159

	127	2	.845	60	11	9.542	1.458	.159
	128	2	.845	60	11	9.542	1.458	.159
	129	2	.845	60	11	9.542	1.458	.159
	130	2	.845	60	11	9.542	1.458	.159
	131	2	.845	60	11	9.542	1.458	.159
	132	2	.845	60	11	9.542	1.458	.159
	133	2	.845	60	11	9.542	1.458	.159
	134	2	.845	60	11	9.542	1.458	.159
	135	2	.845	60	11	9.542	1.458	.159
	136	2	.845	60	11	9.542	1.458	.159
	137	2	.845	20	7	3.181	3.819	.159
	138	2	.845	20	7	3.181	3.819	.159
	139	2	.845	20	7	3.181	3.819	.159
	140	2	.845	20	7	3.181	3.819	.159
	141	2	.845	20	3	3.181	-.181	.159
	142	2	.845	40	9	6.361	2.639	.159
	143	2	.845	40	9	6.361	2.639	.159
	144	2	.845	40	6	6.361	-.361	.159
	145	2	.845	40	4	6.361	-2.361	.159
	146	2	.845	60	2	9.542	-7.542	.159
	147	2	.845	60	3	9.542	-6.542	.159
	148	2	.845	60	1	9.542	-8.542	.159
	149	2	.845	60	1	9.542	-8.542	.159

	150	2	.845	60	1	9.542	-8.542	.159
	151	2	.845	60	0	9.542	-9.542	.159
	152	2	.845	60	0	9.542	-9.542	.159
	153	2	.845	60	0	9.542	-9.542	.159
	154	2	.845	60	0	9.542	-9.542	.159
	155	2	.845	60	0	9.542	-9.542	.159
	156	2	.845	60	0	9.542	-9.542	.159
	157	2	.845	60	0	9.542	-9.542	.159
	158	2	.845	60	0	9.542	-9.542	.159
	159	2	.845	60	0	9.542	-9.542	.159
	160	2	.845	60	0	9.542	-9.542	.159
	161	2	.845	60	0	9.542	-9.542	.159
	162	2	.845	60	0	9.542	-9.542	.159
	163	2	.845	60	0	9.542	-9.542	.159
	164	2	1.000	20	14	.923	13.077	.046
	165	2	1.000	20	14	.923	13.077	.046
	166	2	1.000	20	10	.923	9.077	.046
	167	2	1.000	20	4	.923	3.077	.046
	168	2	1.000	20	2	.923	1.077	.046
	169	2	1.000	40	0	1.846	-1.846	.046
	170	2	1.000	40	4	1.846	2.154	.046
	171	2	1.000	40	3	1.846	1.154	.046
	172	2	1.000	40	3	1.846	1.154	.046

	173	2	1.000	60	1	2.769	-1.769	.046
	174	2	1.000	60	1	2.769	-1.769	.046
	175	2	1.000	60	1	2.769	-1.769	.046
	176	2	1.000	60	0	2.769	-2.769	.046
	177	2	1.000	60	0	2.769	-2.769	.046
	178	2	1.000	60	0	2.769	-2.769	.046
	179	2	1.000	60	0	2.769	-2.769	.046
	180	2	1.000	60	0	2.769	-2.769	.046
	181	2	1.000	60	0	2.769	-2.769	.046
	182	2	1.000	60	0	2.769	-2.769	.046
	183	2	1.000	60	0	2.769	-2.769	.046
	184	2	1.000	60	0	2.769	-2.769	.046
	185	2	1.000	60	0	2.769	-2.769	.046
	186	2	1.000	60	0	2.769	-2.769	.046
	187	2	1.000	60	0	2.769	-2.769	.046
	188	2	1.000	60	0	2.769	-2.769	.046
	189	2	1.000	60	0	2.769	-2.769	.046
	190	2	1.000	60	0	2.769	-2.769	.046
	191	2	1.000	20	7	.923	6.077	.046
	192	2	1.000	20	7	.923	6.077	.046
	193	2	1.000	20	7	.923	6.077	.046
	194	2	1.000	20	5	.923	4.077	.046
	195	2	1.000	20	5	.923	4.077	.046

	196	2	1.000	40	6	1.846	4.154	.046
	197	2	1.000	40	6	1.846	4.154	.046
	198	2	1.000	40	5	1.846	3.154	.046
	199	2	1.000	40	5	1.846	3.154	.046
	200	2	1.000	60	5	2.769	2.231	.046
	201	2	1.000	60	2	2.769	-.769	.046
	202	2	1.000	60	2	2.769	-.769	.046
	203	2	1.000	60	0	2.769	-2.769	.046
	204	2	1.000	60	0	2.769	-2.769	.046
	205	2	1.000	60	0	2.769	-2.769	.046
	206	2	1.000	60	0	2.769	-2.769	.046
	207	2	1.000	60	0	2.769	-2.769	.046
	208	2	1.000	60	0	2.769	-2.769	.046
	209	2	1.000	60	0	2.769	-2.769	.046
	210	2	1.000	60	0	2.769	-2.769	.046
	211	2	1.000	60	0	2.769	-2.769	.046
	212	2	1.000	60	0	2.769	-2.769	.046
	213	2	1.000	60	0	2.769	-2.769	.046
	214	2	1.000	60	0	2.769	-2.769	.046
	215	2	1.000	60	0	2.769	-2.769	.046
	216	2	1.000	60	0	2.769	-2.769	.046
	217	2	1.000	60	0	2.769	-2.769	.046
	218	2	1.000	20	0	.923	-.923	.046

	219	2	1.000	20	0	.923	-.923	.046
	220	2	1.000	20	0	.923	-.923	.046
	221	2	1.000	20	0	.923	-.923	.046
	222	2	1.000	20	0	.923	-.923	.046
	223	2	1.000	40	4	1.846	2.154	.046
	224	2	1.000	40	4	1.846	2.154	.046
	225	2	1.000	40	1	1.846	-.846	.046
	226	2	1.000	40	1	1.846	-.846	.046
	227	2	1.000	60	0	2.769	-2.769	.046
	228	2	1.000	60	8	2.769	5.231	.046
	229	2	1.000	60	7	2.769	4.231	.046
	230	2	1.000	60	6	2.769	3.231	.046
	231	2	1.000	60	4	2.769	1.231	.046
	232	2	1.000	60	4	2.769	1.231	.046
	233	2	1.000	60	4	2.769	1.231	.046
	234	2	1.000	60	4	2.769	1.231	.046
	235	2	1.000	60	4	2.769	1.231	.046
	236	2	1.000	60	4	2.769	1.231	.046
	237	2	1.000	60	4	2.769	1.231	.046
	238	2	1.000	60	4	2.769	1.231	.046
	239	2	1.000	60	4	2.769	1.231	.046
	240	2	1.000	60	4	2.769	1.231	.046
	241	2	1.000	60	4	2.769	1.231	.046

	242	2	1.000	60	4	2.769	1.231	.046
	243	2	1.000	60	4	2.769	1.231	.046
	244	2	1.000	60	4	2.769	1.231	.046

Section 3: LC_{50} Confidence Limit Estimations for 1 or 2 bacteria

Confidence Limits								
			95% Confidence Limits for Concentrations			95% Confidence Limits for log(Concentrations)[b]		
	Bacteria	Probability	Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
PROBIT[a]	1	.010	31.711	26.370	40.386	1.501	1.421	1.606
		.020	27.515	23.243	34.253	1.440	1.366	1.535
		.030	25.145	21.445	30.867	1.400	1.331	1.489
		.040	23.498	20.180	28.549	1.371	1.305	1.456
		.050	22.238	19.202	26.798	1.347	1.283	1.428
		.060	21.220	18.405	25.396	1.327	1.265	1.405
		.070	20.365	17.731	24.231	1.309	1.249	1.384
		.080	19.628	17.146	23.235	1.293	1.234	1.366
		.090	18.982	16.630	22.368	1.278	1.221	1.350
		.100	18.406	16.167	21.599	1.265	1.209	1.334
		.150	16.200	14.368	18.710	1.210	1.157	1.272
		.200	14.638	13.063	16.716	1.165	1.116	1.223
		.250	13.418	12.023	15.195	1.128	1.080	1.182
		.300	12.409	11.147	13.963	1.094	1.047	1.145

		.350	11.542	10.380	12.926	1.062	1.016	1.111
		.400	10.776	9.691	12.026	1.032	.986	1.080
		.450	10.082	9.059	11.226	1.004	.957	1.050
		.500	9.444	8.468	10.502	.975	.928	1.021
		.550	8.846	7.908	9.835	.947	.898	.993
		.600	8.277	7.369	9.209	.918	.867	.964
		.650	7.727	6.845	8.612	.888	.835	.935
		.700	7.187	6.326	8.034	.857	.801	.905
		.750	6.647	5.804	7.460	.823	.764	.873
		.800	6.093	5.268	6.876	.785	.722	.837
		.850	5.505	4.699	6.261	.741	.672	.797
		.900	4.846	4.065	5.572	.685	.609	.746
		.910	4.699	3.924	5.419	.672	.594	.734
		.920	4.544	3.776	5.257	.657	.577	.721
		.930	4.380	3.620	5.085	.641	.559	.706
		.940	4.203	3.453	4.900	.624	.538	.690
		.950	4.010	3.271	4.698	.603	.515	.672
		.960	3.795	3.070	4.472	.579	.487	.651
		.970	3.547	2.838	4.210	.550	.453	.624
		.980	3.241	2.557	3.886	.511	.408	.589
		.990	2.812	2.167	3.427	.449	.336	.535
	2	.010	13.976	12.451	16.330	1.145	1.095	1.213
		.020	12.127	10.997	13.822	1.084	1.041	1.141

		.030	11.082	10.160	12.438	1.045	1.007	1.095
		.040	10.356	9.571	11.492	1.015	.981	1.060
		.050	9.801	9.115	10.778	.991	.960	1.033
		.060	9.352	8.743	10.207	.971	.942	1.009
		.070	8.975	8.427	9.733	.953	.926	.988
		.080	8.651	8.153	9.329	.937	.911	.970
		.090	8.366	7.909	8.978	.923	.898	.953
		.100	8.112	7.691	8.668	.909	.886	.938
		.150	7.140	6.829	7.515	.854	.834	.876
		.200	6.451	6.184	6.741	.810	.791	.829
		.250	5.913	5.654	6.169	.772	.752	.790
		.300	5.469	5.198	5.717	.738	.716	.757
		.350	5.087	4.796	5.341	.706	.681	.728
		.400	4.749	4.438	5.014	.677	.647	.700
		.450	4.444	4.113	4.721	.648	.614	.674
		.500	4.162	3.813	4.452	.619	.581	.649
		.550	3.899	3.534	4.201	.591	.548	.623
		.600	3.648	3.271	3.961	.562	.515	.598
		.650	3.406	3.018	3.728	.532	.480	.572
		.700	3.168	2.773	3.499	.501	.443	.544
		.750	2.930	2.530	3.268	.467	.403	.514
		.800	2.685	2.284	3.028	.429	.359	.481
		.850	2.426	2.026	2.772	.385	.307	.443

		.900	2.136	1.743	2.480	.330	.241	.395
		.910	2.071	1.681	2.415	.316	.226	.383
		.920	2.003	1.616	2.346	.302	.208	.370
		.930	1.930	1.547	2.272	.286	.190	.356
		.940	1.852	1.474	2.192	.268	.168	.341
		.950	1.768	1.394	2.104	.247	.144	.323
		.960	1.673	1.307	2.006	.223	.116	.302
		.970	1.563	1.206	1.891	.194	.081	.277
		.980	1.429	1.084	1.749	.155	.035	.243
		.990	1.240	.917	1.546	.093	-.038	.189

a. A heterogeneity factor is used.

b. Logarithm base = 10.

Section 4: Relative Median Potency Estimates for Bacterial Combinations and Probit Transformed Response

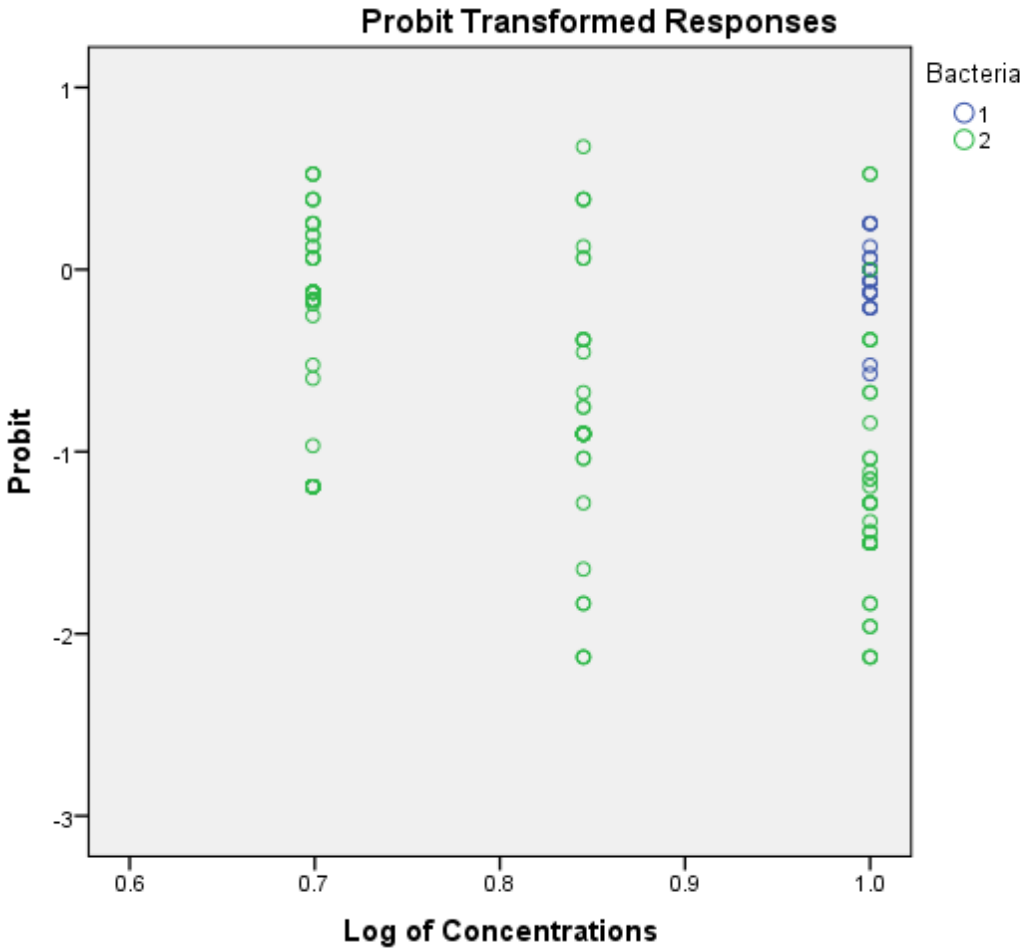
Relative Median Potency Estimates

	(I) Bacteria	(J) Bacteria	95% Confidence Limits			95% Confidence Limits with LOG Transform[a]
			Estimate	Lower Bound	Upper Bound	Estimate
PROBIT	1	2	2.269	1.850	2.967	.356
	2	1	.441	.337	.541	-.356

Relative Median Potency Estimates

	(I) Bacteria	(J) Bacteria	95% Confidence Limits with LOG Transform	
			Lower Bound	Upper Bound
PROBIT	1	2	.267	.472
	2	1	-.472	-.267

a. Logarithm base = 10.


Probit Transformed Responses
Bacteria
1
2
Probit
1
0
-1
-2
-3
0.6
0.7
0.8
0.9
1.0
Log of Concentrations