

**Isolation, identification and characterization of bacteria and
fungi from the gut of fourth instar larvae of *Aedes aegypti*
(LINNAEUS, 1762) collected from kole wetlands by
culture dependent and metagenomic analysis**

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**Thesis submitted in partial fulfilment of the requirements for the Degree of
DOCTOR OF PHILOSOPHY IN ZOOLOGY
Under the faculty of science
University of Calicut**



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DECLARATION

I, SINI FRANCIS C F, hereby declare that the work embodied in the thesis “**Isolation, identification and characterization of bacteria and fungi from the gut of fourth instar larvae of *Aedes aegypti* (LINNAEUS, 1762) collected from kole wetlands by culture dependent and metagenomic analysis**” submitted to the University of Calicut in partial fulfilment of the requirements for the Degree of Doctor of Philosophy in Zoology is a bonafide record of the research work carried out by me under the supervision of Dr. Honey Sebastian, Professor, Department of Zoology, Vimala college and Dr. Aneesh E.M., Associate Professor, Department of Zoology, University of Calicut and Dr. Sudhikumar A.V, Professor, Research and Post-Graduate Department of Zoology, Christ College, Irinjalakuda, Thrissur as Co-guide and no part of the thesis has formed the basis for the award of any degree, diploma or other similar titles of any university. The contents of the thesis have undergone a plagiarism check using authenticate software at C.H.M.K Library University of Calicut, and the similarity index was found to be within the permissible limit. I also declare that this thesis is free from AI generated content.

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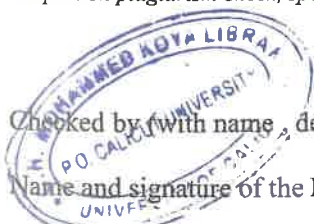
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CONTENTS

	Title	Page No.
	ABSTRACT	
CHAPTER 1: INTRODUCTION		1
CHAPTER 2: REVIEW OF LITERATURE		29
OBJECTIVES		57
CHAPTER 3: To Isolate, characterize and identify bacterial inhabitants from gut of fourth instar <i>Aedes aegypti</i> larva collected from kole wetlands by culture dependent method and metagenomic analysis.		61
	Introduction	63
	Materials and Methods	65
	Results	70
	Discussion	105
	Conclusion	113
CHAPTER 4: To isolate, characterize and identify fungi from fourth instar <i>Aedes aegypti</i> larva collected from kole wetlands by culture dependent and metagenomic analysis.		115
	Introduction	117
	Materials and Methods	119
	Results	125
	Discussion	145
	Conclusion	152
CHAPTER 5: To find out the inter and intraspecific genetic divergence between bacterial and fungal isolates from the fourth instar <i>Aedes aegypti</i> larvae collected from kole wetlands.		153
	Introduction	155
	Materials and Methods	156
	Results	158
	Discussion	167
	Conclusion	174
SUMMARY		175
	Summary	177
	Recommendations	178
REFERENCES		179
PUBLICATIONS		211

LIST OF TABLES

- Table 3.1 Composition profile
- Table 3.2 OTU Table
- Table 3.3 Microbial population according to Phylum
- Table 3.4 Microbial population according to Class
- Table 3.5 Microbial population according to Order
- Table 4.1 Alpha diversity indices of fungi

LIST OF FIGURES

- Fig. 3.1 Bacteria isolated during different seasons
- Figure 3.2 Evolutionary analysis of bacteria belongs to *Bacillus* genera by Maximum Likelihood method
- Figure 3.3 Evolutionary analysis of bacteria belongs to *Enterobacter* genera by Maximum Likelihood method
- Figure 3.4 Evolutionary analysis of bacteria belongs to *Rahnella* genera by Maximum Likelihood method
- Figure 3.5 Evolutionary analysis of bacteria belongs to *Stutzerimonas* genera by Maximum Likelihood method
- Figure 3.6 Evolutionary analysis of bacteria belongs to *Aeromonas* genera by Maximum Likelihood method
- Figure 3.7 Evolutionary analysis of bacteria belongs to *Klebsilla* genera by Maximum Likelihood method
- Figure 3.8 Evolutionary analysis of bacteria belongs to *Priestia* genera by Maximum Likelihood method
- Figure 3.9 Top 10 phyla abundance distribution

- Figure 3.10 Diversity profile
- Figure 3.11 Distribution of top 20 significant microbes
- Figure 3.12 Season wise Distribution
- Figure 3.13 Season wise Distribution
- Figure 3.14 20 different phyla
- Figure 3.15 Species wise Hierarchical visualization of gut bacterial communities [Pre-monsoon] through metagenomic analysis
- Figure 3.16 Metagenomic Profile of Top 10 Bacterial Species in Pre-Monsoon
- Figure 3.17 Species wise Hierarchical visualization of gut bacterial communities [Monsoon] through metagenomic analysis
- Figure 3.18 Metagenomic Profile of Top 10 Bacterial Species in Gut Microbiome
- Figure 3.19 Species wise Hierarchical visualization of gut bacterial communities [Post-Monsoon] through metagenomic analysis
- Figure 3.20 Metagenomic Profile of Top 10 Bacterial Species in Post-Monsoon Gut Microbiome
- Figure 4.1. Morphological differences among the identified fungal species
- Figure 4.2 SEM analysis of the isolated fungal strains
- Figure 4.3 Evolutionary analysis of fungi belongs to *Aspergillus* genus by Maximum Likelihood method
- Figure 4.4 Evolutionary analysis of fungi belongs to *Mucor* genus by Maximum Likelihood method
- Figure 4.5 Evolutionary analysis of fungi belongs to *Penicillium* genus by Maximum Likelihood method

- Figure 4.6 Evolutionary analysis of fungi belongs to *Candida* genus by Maximum Likelihood method
- Figure 4.7 Evolutionary analysis of fungi belongs to *Trochoderma* genus by Maximum Likelihood method
- Figure 4.8 Evolutionary analysis of fungi belongs to *Wickerhamomyces* genus by Maximum Likelihood method
- Figure 4.9 Species specific distribution according to the monsoon season
- Figure 4.10 Top ten Species distribution according to the monsoon season
- Figure 4.11 Species specific distribution according to the pre-monsoon season
- Figure 4.12 Top ten Species specific distribution according to the pre-monsoon season
- Figure 4.13 Species specific distribution according to the post-monsoon season
- Figure 4.14 Top ten Species specific distribution according to the post-monsoon season
- Figure 5.1 Genetic variation both within and between bacterial strains
- Figure 5.2 Genetic distance for major bacterial genera
- Figure 5.3 Genetic variation both within and between fungal strains

**കോൾ തണ്ണീർത്തടങ്ങളിൽ നിന്ന് ശേഖരിച്ച നാലാം ഘട്ടത്തിലെ ഈഡിസ്
ഈജിപ്റ്റി (ലിന്നേയസ്, 1762) ലാർവകളുടെ കൂടലിൽ നിന്ന്
ബാക്ടീരിയകളെയും ഫംഗസുകളെയും കൾച്ചർ ആശ്രിതവും
മെറ്റാജീനോമിക് വിശകലനവും ഉപയോഗിച്ച് വേർതിരിച്ചെടുക്കലും
തിരിച്ചറിയലും സ്വഭാവനിർണ്ണയവും**

സംഗ്രഹം

കോൾ തണ്ണീർത്തടങ്ങളിൽ നിന്ന് ശേഖരിച്ച നാലാമത്തെ ഇൻസ്റ്റാർ ഈഡിസ് ഈജിപ്റ്റി ലാർവകളുടെ കൂടലിലെ സൂക്ഷ്മജീവി വൈവിധ്യത്തെക്കുറിച്ച് ഈ പഠനം അന്വേഷിക്കുന്നു. കൾച്ചർ ഡിപെൻഡന്റ് രീതികൾ. മെറ്റാജീനോമിക് വിശകലനം എന്നിവ സംയോജിപ്പിച്ച് കൊണ്ടുള്ള സമീപനം ഉപയോഗിക്കുന്നു. എൻറോബാക്റ്റർ റോഗൻകാംപൈ, ബാസില്ലസ് പസിഫിക്കസ്. റാണെല്ല അക്വാട്ടിലിസ്. ക്ലൈഡിയെല്ല ക്വാസിവാരികോള, എന്നിവയുൾപ്പെടെ ആകെ 15 വ്യത്യസ്ത ബാക്റ്റീരിയൽ സ്പീഷീസുകളെ വേർതിരിച്ചു. 16s rRNA സീക്വൻസിംഗിലൂടെ ഉയർന്ന ശ്രേണി സമാനതകൾ സ്ഥിരീകരിച്ചു. ഫൈലോജനറ്റിക് വിശകലനങ്ങൾ ബാക്റ്റീരിയൽ ജനുസുകളിൽ. പ്രത്യേകിച്ച് ബാസില്ലസ്. എൻറോബാക്റ്റർ. റാണെല്ല. എന്നിവയിൽ ഗണ്യമായ ജനിതകവൈവിധ്യം സൂചിപ്പിക്കുന്നു. മെറ്റാജീനോമിക് ഫലങ്ങൾ പ്രോട്ടിയോ ബാക്റ്റീരിയ. ഫിർമികുസ് ആക്ടിനോബാക്റ്റീരിയ തുടങ്ങിയ ഫൈലകളുടെ ആധിപത്യം എടുത്തു കാണിച്ചു. ഇത് സമൃദ്ധമായ സീസണൽ പ്രവണതകൾ കാണിക്കുന്നു. അസ്പെർജില്ലസ് നൈഗർ. കാൻഡിഡാ ട്രോപ്പിക്കാലിസ് എന്നിവയുൾപ്പെടെ വിവിധ സ്റ്റീഷീസുകളാണ് ഫംഗസ് സമൂഹത്തിന്റെ സവിശേഷത. അസ്കോമൈകോട്ടയാണ് പ്രധാന ഫൈലം. ഫംഗസ് വൈവിധ്യത്തിലേ സീസണൽ വ്യതിയാനങ്ങളും നിരീക്ഷിക്കപ്പെട്ടു. ബാക്റ്റീരിയ. ഫംഗസ് ഐസൊലേറ്റു കൾക്കിടയിലെ ജനിതക വ്യത്യാസം പഠനം വെളിപ്പെടുത്തി. അവയുടെ പാരീസനിതീക പൊരുത്തപ്പെടലുകളും പരിണാമപാതകളും ഉന്നിപറഞ്ഞു. ഈ കണ്ടെത്തലുകൾ ഈഡിസ് ഈജിപ്റ്റിയിലെ കൂടൽ മൈക്രോബയോമിന്റെ സൂക്ഷ്മജീവി സമ്പന്നതയും പാരീസനിതീക പ്രാധാന്യവും അടിവരയിടുന്നു. ഇത് കൊതുക്കുകളുടെ ജീവശാസ്ത്രം. രോഗകാരി സാക്രമണം. പൊതുജനാരോഗ്യത്തിനായുള്ള വെക്ടർ മാനേജ്മെന്റ് തന്ത്രങ്ങൾ എന്നിവയ്ക്കുള്ള സാധ്യതകളെ കുറിച്ചുള്ള ഉത്കൃഷ്ടത നൽകുന്നു.

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ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF BACTERIA AND FUNGI FROM THE GUT OF FOURTH INSTAR LARVAE OF *Aedes Aegypti* (LINNAEUS, 1762) COLLECTED FROM KOLE WETLANDS BY CULTURE DEPENDENT AND METAGENOMIC ANALYSIS

Abstract

This study investigates the microbial diversity within the gut of fourth instar *Aedes aegypti* larvae collected from the Kole wetlands, employing an integrative approach that combines culture-dependent methods, molecular characterization, and metagenomic analysis. A total of 15 distinct bacterial species were isolated, including *Enterobacter roggenkampii*, *Bacillus pacificus*, *Rahnella aquatilis*, and *Klebsiella quasivariicola*, with high sequence similarities confirmed through 16S rRNA sequencing. Phylogenetic analyses indicated significant genetic diversity among the bacterial genera, notably *Bacillus*, *Enterobacter*, and *Rahnella*. Metagenomic results highlighted the dominance of phyla such as Proteobacteria, Firmicutes, and Actinobacteria, exhibiting seasonal trends in abundance. The fungal community was characterized by a variety of species, including *Aspergillus niger* and *Candida tropicalis*, with Ascomycota as the predominant phylum. Seasonal variations in fungal diversity were also observed. The study revealed inter- and intraspecific genetic divergence among bacterial and fungal isolates, emphasizing their ecological adaptations and evolutionary trajectories. These findings underscore the microbial richness and ecological significance of the gut microbiome in *Aedes aegypti*, providing insights into potential implications for mosquito biology, pathogen transmission, and vector management strategies for public health.

Keywords: Mosquitoes; *Aedes aegypti*; Bacteria; Fungi; Microbes.

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CHAPTER 1

INTRODUCTION

INTRODUCTION

The mosquito vectors are known to spread various deadly diseases including dengue fever, chikungunya, zika, West Nile, yellow fever, malaria, and Japanese encephalitis. Among the various reported mosquito vectors, the mosquitoes belong to *Aedes*, *Anopheles*, and *Culex* are known to possess significant threat to human population. The recent research focusing on the eradication of mosquito borne diseases indicated that the mosquitoes belonging to *Aedes* genus are principally contributing to enhance the frequency of deadly diseases in the recent scenario (Naik, Tyagi, & Xue, 2023).

The arboviral disease-disseminating mosquitoes vectors including the *Aedes aegypti* spread the most dangerous pathogens responsible for chikungunya, zika and dengue fever. More than 129 countries are now facing the intimidating impacts of dengue fever and of which 70% of cases are focused in the Asian continent. Notably, the endemic regions of India comprises 12 states, in which Kerala and Tamil Nadu states are recorded to be most distressed with dengue cases (Acharya et al., 2024). In addition to this, the aforesaid regions are also threatened by chikungunya diseases which was spread by the *Aedes aegypti* and *Aedes albopictus*. However, the causative mosquito vector *Aedes aegypti* is restricted to the tropics and subtropics while the *Aedes albopictus* does exist in temperate regions. Clinical trials are dynamically directing over the globe, even though it may yield years before specific antiviral drugs or immunizations are made available to the general public (Gulland, 2016). Similar to the potential severe impacts of chikungunya, the yellow fever has also be noted to produce destructive impacts to the public. The primary vectors and reservoirs of the disease are fall into the category of mosquitoes belonging to the genus *Aedes*.

The primary hosts of yellow fever are monkeys, while the primary vectors and reservoirs of the disease are *Aedes* and *Haemogogus* species. Although there isn't currently a specific antiviral medication on the market, the immunization is safe, affordable, and only needs one injection (Organization, 2014).

Innovative methods to combat mosquito-borne diseases are urgently required due to the restrictions on elimination progress imposed by insecticide- and drug-resistance and the absence of vaccinations (Naik et al., 2023). In this work, we examine the microorganisms that were found in the gut of *Aedes aegypti* larvae using both the culture independent and culture dependent methods and the larvae were collected from kole wetland in thrissur district. Wetlands and disease-carrying mosquitoes coexist on a global and local scale because mosquito larval stages need water to develop. Wetland restoration are found to lowers mosquito populations by preventing or reducing floods and by creating a favourable environment for mosquitoes' natural predators (Medlock & Vaux, 2015). For example, when a 1,500-acre marsh in Massachusetts was restored as a part of the Essex County Mosquito Control Project, the mosquito population dropped by 90% and thus the local experts are aware that restoring wetlands works hand in hand with successful mosquito control. Likewise, in Indiana, the worst mosquito problems are usually found in areas with floods and forested pools. Therefore, by restoring healthy wetlands, we can actually do a lot of good for ourselves and all Indians. The environment in which organisms reside has a significant impact on and defines the microbiota within the gut, and here we have collected fourth instar larvae from kole wetlands. This area is a special place for mosquito breeding, and only a few researches have been done there.

***Aedes aegypti* and inhabited microbes**

In 2001, a study on the microbiota of *Aedes aegypti* mosquitoes was published (Luxananil, Atomi, Panyim, & Imanaka, 2001). The researchers used a culture-based approach, where they collected larvae from natural breeding sites in Thailand, isolated their guts, and plated the gut homogenates on Luria-Bertani (LB) agar. The resulting bacterial colonies were analyzed based on their appearance, Gram-staining, and standard biochemical tests (Koneman, Allen, Janda, Schreckenberger, & Winn, 1997). This initial study identified two strains of *Bacillus cereus* as particularly common and suggested their potential use in developing mosquito larvicides (Luxananil et al., 2001). Later studies used additional methods, including 16S rRNA analysis and Sanger sequencing, to confirm and expand on these findings. These studies identified *Serratia* as a predominant species, along with *Bacillus* sp. and *Bacillus subtilis* strains as part of the mosquito microbiota (Desiely Silva Gusmão et al., 2007). Over the following decades, researchers continued using culture-dependent methods with various media and isolation techniques to explore the diversity and composition of microbiota in both *Aedes aegypti* and *Aedes albopictus* mosquitoes (Desiely S Gusmão et al., 2010).

Eukaryotic organisms and microbes share a symbiotic relationship where bacteria produce essential nutrients that help their hosts absorb and digest food more effectively. While it is well-known that gut microorganisms are crucial for metabolism, health, and development in animals, the specifics of this symbiotic relationship remain unclear (Pernice, Simpson, & Ponton, 2014). Recently, insects have gained attention as model systems for studying these interactions because they typically host fewer microbial species compared to vertebrates. Research has shown that bacteria are present in insects and play specific roles in their hosts. Both culture-dependent (Demaio, Pumpuni, Kent, & Beier, 1996; Dinparast Djadid et al., 2011; Dong, Manfredini, & Dimopoulos, 2009;

Y. Wang, Gilbreath III, Kukutla, Yan, & Xu, 2011) and culture-independent methods (Chavshin et al., 2012; Osei-Poku, Mbogo, Palmer, & Jiggins, 2012; Y. Wang et al., 2011) have been used to identify gut bacteria in these studies.

The composition of bacterial communities in insects varies significantly based on diet, life stage, sex, species, and environmental factors (Merritt, Dadd, & Walker, 1992). Field studies on mosquitoes from the Culicidae family reveal that adult mosquitoes typically host bacterial communities with low diversity but significant variations between individuals and species. However, it is still unclear how these microbiomes develop, what influences their composition, and whether they are essential for mosquito survival.

The structure and physicochemical properties of insect digestive tracts vary widely, influencing the microbial communities they host. As holobionts, mosquitoes undergo significant changes in their microbiomes during their metamorphic transformation from larvae to adults. For example, *Aedes aegypti* has a complex life cycle that involves drastic changes in its environment, form, and function (H. Singh, Akhtar, & Gupta, 2024). Female mosquitoes lay eggs on moist surfaces inside water-filled containers. When water covers the eggs, larvae hatch and feed on microbes and organic particles, growing through four larval stages. After sufficient growth, the larvae metamorphose into pupae, non-feeding forms that eventually become flying adults.

Each stage of the mosquito life cycle is associated with changes in its microbiota (Alfano et al., 2019) due to shifts in habitat and feeding patterns. For example, larvae primarily feed on bacteria and plankton, which establish the initial bacterial colonization in their guts. When mosquitoes reach adulthood, their diet shifts to blood

and nectar, leading to changes in their gut microbiota. Some bacterial species thrive in the adult stage, while others decline.

Studies by (Duguma et al., 2013) showed that the bacterial communities in *Culex nigripalpus* adults varied depending on the nutrient levels in their larval habitats. Mosquitoes from high-nutrient environments had an abundance of Clostridiales, while those from low-nutrient environments were associated with Burkholderiales. Additionally, research by (Coon, Brown, & Strand, 2016b) demonstrated that larvae from different mosquito species in the same habitat had similar bacterial communities, while larvae of the same species from different habitats had distinct bacterial compositions. Comparing aquatic larvae and adult mosquitoes revealed that larval gut bacteria closely resemble the microbes in their habitats, whereas adult mosquito gut bacteria differ significantly. These findings highlight the major roles of diet, habitat, and developmental stages in shaping the gut microbiota of mosquitoes.

Furthermore, the results of additional investigations show that some bacteria carried by larvae can survive in adults (Figure1). It is fascinating to note that the *Thorsellia* genus was found in *Culex tarsalis* in both immature (early and late larval instars and pupae) and adult stages (Duguma et al., 2015). Some bacteria from the *Micrococcaceae*, *Pseudomonadaceae*, and *Staphylococcaceae* families are present in *Aedes albopictus* larvae, adult males, sugar-fed females, and blood-fed females (Yadav et al., 2016). Mosquito metamorphosis from pupa to adult would not result in total bacterial clearance, which strongly suggests that a portion of this environmental bacterial microbiota will naturally be included in the holobiont cell components (Moll, Romoser, Modrakowski, Moncayo, & Lerdthusnee, 2001). Similarities between their bacterial communities were found in a larger study that included 12 mosquito species found in

the USA, including *Aedes*, *Anopheles*, and *Culex*. *Gluconobacter*, *Propionibacterium*, and *Staphylococcus* bacterial species dominated these communities.

Thus, a portion of the microbiota in adult mosquitoes would come from the aquatic larval habitat, and the remaining portion would be reliant on food sources, such as naturally occurring plant-based sugars or blood meals for females. Bacterial diversity and composition are subsequently altered by a blood- or sugar-meal, which heightens inter-individual variances (Gimonneau et al., 2014). Particularly, blood meal modifies the makeup and organization of microbial populations, which results in a systemic change in the oxidative conditions in the gut. Both the presence of a viral infestation and how long it has been since the blood meal have an impact on the diversity and makeup of the bacterial communities in *Aedes albopictus*.

Blood meal influences bacterial composition but not structure, whereas only a small number of taxa changed substantially as a result of chikungunya infectious agent (Zouache, Michelland, FAILLOUX, Grundmann, & Mavingui, 2012). *Aedes japonicus* and *Aedes triseriatus* infected with the La Crosse virus (LACV) had higher bacterial abundance while the richness and evenness of the local fungus reduced (Muturi, Ramirez, Rooney, & Dunlap, 2016). Otherwise, *Aedes aegypti* infected with DENV has less bacterial abundance (Ramirez et al., 2012). Additionally, Rhodobacteraceae and Desulfuromonadaceae, which have been proposed as possible markers for ZIKV (L. E. Villegas et al., 2018), are affected by ZIKV infection in *Aedes aegypti*.

Blood meal and infection-related effects tend to emphasize microbiota-mosquito immunological interactions, such as immune response and redox and detoxifying enzyme metabolisms. Similar to this, a study by (Short, Mongodin, MacLeod, Talyuli, & Dimopoulos, 2017) revealed that the midgut microbial populations in *Aedes aegypti*

were impacted by the host amino acid metabolic pathway, which involves branched chain amino acid breakdown. This may help to partially explain variability in the midgut

Recent research has focused on the host- microbiota phylosymbiosis or the agreement between the host phylogeny and the divergence in the makeup of the accompanying microbiome. According to a field study done by (Novakova et al., 2017) on 11 mosquito species from Canada, the phylogeny of the hosts and the variations in the bacterial communities they are linked with are consistent. Moreover, not all closely linked host species showed this co-evolutionary tendency. In fact, *Aedes aegypti* had a more divergent microbiota from the closely related species, *Aedes atropalpus* than from the distantly related species *An. gambiae* in conventional laboratory settings (Coon, Vogel, Brown, & Strand, 2014). On the other hand, species belonging to the same subgroup of microbial species also showed similar trend in their microbial communities. *Aedes albopictus* and a cryptic species living in sympatry in Vietnam were shown to have a lot in common, according to an analysis of gut bacterial flora (Minard et al., 2017).

Those results might be accounted for subsequent host-microbe co-adaptation. To fully understand how host genotypes and local environmental factors affect the composition of the mosquito microbiome, more research is required. Additionally, horizontal gene transfers (HGTs) between *Wolbachia* and *Aedes* mosquitoes have been documented (Hou et al., 2014). As *Wolbachia* can control the transmission of some infections depending on the mosquito species, the evolution of *Wolbachia*-mosquito partnerships raises the question of the impact of bacterial-to-mosquito HGTs on the adaptive capacity of the holobiont or/and mosquito vector competence (Caragata, Dutra, & Moreira, 2016).

Functional bacterial HGTs may also have an effect on the holobiont's ability to adapt. The aforesaid circumstance has been observed in coffee-crop bug where the HGT may expand and allow the insect to persist in a new environment (Acuña et al., 2012). The co-evolution and diversification of some MSVs from the Bunyaviridae and Flaviviridae families with their mosquito hosts have also been emphasized by phylogenetic studies (Marklewitz, Zirkel, Kurth, Drosten, & Junglen, 2015). Along with the bacterial microbiota, MSVs should also be given consideration in this evolutionary setting since they may serve as biological trait-drivers that affect whether the mosquito holobiont can spread infections or not.

Role of bacteria in various physical activities and immunological response

Symbiotic relationships in insect guts play critical roles in host development and survival. Most gut bacteria assist with metabolism, enhance digestion, and protect the host from harmful pathogens. In mosquitoes, bacteria primarily inhabit the midgut, though they are occasionally found in the salivary glands, ovaries, and male accessory glands. For instance, *Asaia* species are prominent in the microbiota of *An. stephensi* and influence host growth by modulating cuticle synthesis genes (Mitraka, Stathopoulos, Siden-Kiamos, Christophides, & Louis, 2013), resembling how *Saccharomyces cerevisiae*, *Klebsiella*, and *Aeromonas* support *Cx. pipiens* development (Steyn, Roets, & Botha, 2016).

Experimental studies highlight the essential role of gut microbiota in mosquito development. Axenic larvae, raised without bacteria through antibiotic treatments, fail to survive or develop without microbial support, whereas gnotobiotic larvae (colonized with single bacteria species) grow normally (Muturi et al., 2018). For

example, *Asaia* can reverse growth delays caused by antibiotics in *An. stephensi* (Chavshin et al., 2012).

Gut bacteria may act as signaling agents by altering pH or oxygen tension, crucial for larval growth and molting. In *Aedes aegypti*, cytochrome bd oxidase reduces gut oxygen levels, stabilizing hypoxia-induced transcription factors (HIFs), which are necessary for molting and development (Coon et al., 2017; Valzania, Coon, Vogel, Brown, & Strand, 2018).

Microbial influence extends to egg production. While *Aedes aegypti* females require blood meals to produce eggs, *Aedes atropalpus* can lay eggs without blood, relying on their gut microbiota to provide nutritional reserves from larval development. Moreover, *Cx. pipiens* females prefer oviposition sites containing *Klebsiella* and *Aeromonas* (Coon et al., 2016b; Diaz-Nieto, D' Alessio, Perotti, & Beron, 2016).

Interestingly, some gut bacteria increase mosquito susceptibility to pathogens. For example, the presence of *Escherichia coli*, *Aeromonas*, and *Serratia odorifera* in *Aedes aegypti* enhances vulnerability to DENV-2, potentially by suppressing immune responses through P40-porin interactions. *S. marcescens* facilitates dengue virus entry by cleaving membrane-bound mucins, while aminopeptidases secreted by *Chromobacterium* degrade viral E proteins, reducing infection rates (Saraiva et al. 2018). Other bacterial species, such as *Proteus* sp., may have anti-dengue effects, either by boosting immunity or directly obstructing viral colonization (Diaz-Nieto et al., 2016).

Fungal symbionts also play a role in vector-pathogen interactions. For instance, the fungus *Talaromyces* (Tsp PR) in *Aedes aegypti* enhances DENV infection by

suppressing gut trypsin transcripts, reducing enzymatic activity that otherwise counters viral colonization. Similarly, *Penicillium chrysogenum* components in *An. gambiae* increase susceptibility to *Plasmodium* by modulating the mosquito's innate immune response, particularly through the overactivation of ornithine decarboxylase, which depletes protective nitric oxide pathways (Angleró-Rodríguez et al., 2016; Chavshin et al., 2012).

Further research on mosquito-microbiota coadaptation has revealed that midgut bacteria differ between mosquito species. For instance, *Pantoea stewartii* from *Aedes aegypti* demonstrated greater survival in its host compared to its performance in *An. stephensi* (Terenius et al., 2012). The reported results illustrates the interrelationship between the microbial consortia and mosquito vectors, and the strong correlation not only impacts the reproduction and development, but also influence the vector competence and the disease transmission.

Various methods for bacterial identification and characterization

The genes having 1.5 kb-long size were used for the bacterial identification while the ITS-based strategies are implemented for identification of fungal strains according to the various seasons. Also, 16S rRNA based sequencing coupled with the metagenomics were used for throughout the molecular analysis. The CR was completed with universal primers and verified using gel electrophoresis.

The metagenomic strategies allow to differentiate all the microbial population including bacteria, fungi and viruses without any culture based approaches. In addition to this, the metagenomic strategies also permits the scientists to discover novel microbial population along with their functional role. Such kind of movement can advance our understanding regarding the mosquito biology, thereby we can open new way for the

development of advanced and efficient strategies for mosquito control and diseases prevention. By unveiling the specific microbial consortia inhabited in the mosquito vectors, the scientific community can exploit the identified roles in diseases spread, pathogen transmission and susceptibility. Hence, studies investigating the microbial dynamics and significant association with the mosquito vector is principally critical and vital.(Karunaratne & Surendran, 2022). Additional techniques for identifying sequences include temperature gradient gel electrophoresis (TGGE) (Zoetendal et al. 1998), terminal-restriction fragment length polymorphism (T-RFLP) (Marsh, 1999), denaturing high-performance liquid chromatography (DHPLC) (C. Liu et al., 2010), single-stranded conformation polymorphism (SSCP) (Gong, Zhou, Plowman, Dyer, & Hickford, 2012), constant denaturing capillary electrophoresis (CDCE), and temporal temperature gradient gel electrophoresis (TTGE) (Rani, Sharma, Rajagopal, Adak, & Bhatnagar, 2009). Culture-dependent techniques cannot detect microbes that cannot grow in the lab, while culture-independent methods cannot use the identified microbes for further studies like Para transgenesis or detailed characterization. Therefore, combining both approaches is more effective for accurately identifying microorganisms. Additionally, fluorescent in situ hybridization (FISH) can be used to directly visualize bacterial symbionts without sequencing the 16S rRNA gene, by using fluorescently labeled probes. Other visualization methods include DAPI (4',6-diamidino-2-phenylindole) and green fluorescent protein (GFP), which help observe gut microbes.

Fungal diversity in mosquito

In watery habitats where mosquito larvae grow, they come into contact with various bacteria and fungi, and the environment plays a key role in shaping fungal diversity in mosquitoes. Mosquitoes interact with fungi in different ways, ranging from parasitic to

non-harmful relationships. Research has focused mainly on fungal pathogens that kill mosquitoes and their potential use as bioinsecticides to control larvae and adults. However, not all fungi harm mosquitoes.

The majority of fungi associated with mosquitoes belong to Ascomycota (73–92% in *Aedes* and *Culex* species) and Basidiomycota (8–25% in *Aedes* and *Culex* species), including yeasts and filamentous fungi (Luis et al., 2019; Thongsripong et al., 2018). Common fungal species found in mosquitoes include *Aspergillus gracilis*, *Aspergillus puulaauensis*, *Cladosporium* sp., *Phaeophleospora hymenocallidicola*, and *Penicillium* sp., which were present in over 70% of sampled mosquitoes (Bozic et al., 2017; Frants & Mertvetsova, 1986; Luis et al., 2019; Thongsripong et al., 2018). Yeasts and yeast-like fungi make up a large part of mosquito fungal communities, ranging from 19% to 47% on average and reaching as high as 84% in some *Aedes albopictus* populations.

Fungal diversity changes with mosquito development stages. In newly emerged adults, fungal diversity drops because the midgut undergoes partial sterilization during metamorphosis (Diaz-Nieto et al., 2016; Steyn et al., 2016). Female mosquitoes that feed on blood also experience reduced fungal diversity in the midgut, as certain species like *Meyerozyma* spp. become dominant (Muturi et al., 2016). Fungi not only inhabit the midgut but also invade the ventral diverticulum and reproductive organs of mosquitoes (Desiely S Gusmão et al., 2010; Desiely Silva Gusmão et al., 2007).

While vertical transmission of bacteria has been observed in mosquitoes (Damiani et al., 2010), it is still unclear whether fungi use the same mechanism. Additionally, dead female mosquitoes are often seen floating on water after laying eggs, which allows fungi associated with adult mosquitoes and larvae to colonize new environments (Steyn et al., 2016).

The microbiota, including fungi, is essential for mosquito larvae development. Axenic (microbe-free) larvae grow more slowly, taking over six extra days to develop or failing to grow past the first stage, even with plenty of sterile food (Valzania et al., 2018). Yeasts also play a role in mosquito growth by causing gut hypoxia. For example, *Saccharomyces cerevisiae* lowers oxygen levels in the gut of *Aedes aegypti* to below 5% through aerobic respiration, triggering growth and molting (Valzania et al., 2018). Hypoxia activates transcription factors (HIFs) that promote fat storage and molting (Coon et al., 2014; Valzania et al., 2018). A similar process may occur in other mosquito species like *Culex pipiens* (Diaz-Nieto et al., 2016). In adult mosquitoes, a yeast-only microbiota helps maintain survival rates (68–100%) but slightly shortens their lifespan.

Fungi can significantly affect how many eggs a female mosquito lays. For example, female *Aedes aegypti* mosquitoes infected with *Edhazardia aedis* (a type of microsporidia parasite) consume 23% less blood and lay 30% fewer eggs (Barnard, Xue, Rotstein, & Becnel, 2007). Similarly, *Anopheles gambiae* females infected with *Vavraia culicis* show a 16% decrease in eggs laid during their first egg-laying cycle and a 45% decrease by the fourth cycle, with the negative effect increasing as the mosquito ages (Lorenz & Koella, 2011).

The yeast *W. anomalus* has been found in the reproductive organs of both male and female *An. stephensi* mosquitoes, suggesting it plays a role in mosquito reproduction and might be passed down from one generation to the next (Ricci et al., 2011). The presence of *W. anomalus* in the midgut of larvae (L1-L4) and pupae, as well as adult mosquitoes reared under controlled laboratory conditions (fed only sterile food and raised without *W. anomalus* in the larval water), supports the idea that the yeast is transferred through stages of mosquito development. The fact that *W. anomalus* was

still present in adults at least ten days after emergence suggests it can survive in the midgut and get nutrients from the mosquito's diet (Ricci et al., 2011).

Most interactions between fungi and mosquitoes help both the mosquito and the fungus get nutrients and energy (Guégan, Zouache, Démichel, Minard, Tran Van, et al., 2018). Both the male and female mosquitoes usually acquire sugar (glucose, fructose, and sucrose) from the environment, they may also get the chance of acquiring the fungal population directly to the gut of mosquito vector (Barredo & DeGennaro, 2020; Desiely Silva Gusmão et al., 2007). According to Guégan et al., 2020, the fungal composition may not differ according to the gender of mosquito vector. Recent investigations reported that some of the fungal strains including *Wickerhamomyces guilliermondii* may colonize within the *Phlebotomus perniciosus*, which are known to be the predominant vector of leishmaniasis. Furthermore, the potential of the fungal strain *Wickerhamomyces anomalus* once it is inhabited in the midgut and gonads of certain mosquito vectors including *Anopheles*, to produce toxic elements with strong antimicrobial potential made them to get the name as ““killer yeasts”” as mentioned by earlier studies (Barredo & DeGennaro, 2020), (Desiely Silva Gusmão et al., 2007), (Foster, 1995; Guégan et al., 2020; Souza-Neto, Machado, Lima, Valle, & Ribolla, 2007), (Stefanini, 2018), (Guégan et al., 2020), (Esquivel, Cassone, & Piermarini, 2016), (Martin et al., 2018), (Bozic et al., 2017; Steyn et al., 2016), (Ricci et al., 2011), (Cappelli et al., 2019; Ricci et al., 2011), (Valzano et al., 2016).. Studies have shown that yeasts can reduce parasite populations (zygotes and ookinetes) by 65%. However, the later stages of the parasite's life cycle, the oocysts and sporozoites, are unaffected by these yeast toxins, likely because they are outside the midgut lumen and do not come into contact with the toxins (Cappelli et al., 2019; Valzano et al., 2016).

On the other hand, some fungi may promote the growth of pathogens in mosquitoes. For example, the filamentous fungus *Talaromyces sp.*, which commonly lives in the midgut of *Aedes aegypti* mosquitoes, enhances *Plasmodium* infection in *Anopheles gambiae* mosquitoes and supports the transmission of the dengue virus. It does this by inhibiting the mosquito's gut digestive enzymes. The fungus secretes heat-sensitive substances that suppress the activity of genes responsible for digesting blood meals, including trypsin and endo-protease genes. These fungal by-products alter the activity of trypsin, affecting the mosquito's ability to process the blood meal (Angleró-Rodríguez et al., 2017).

Modulation of immune system by fungus

Unlike adaptive immunity, which is influenced by previous encounters with pathogens, mosquitoes rely solely on innate immunity. The presence of certain non-entomopathogenic fungi including *Saccharomyces cerevisiae* and *Candida albicans*, can induce melanization within various mosquito vectors including *Culex quinquefasciatus* and *Anopheles albimanus*. The process of melanisation may develop when protein that constitutes the thioesters (TEPs) detects the aforesaid fungal population and resulting them to cover the fungi cells with the formed melanin (A. Kumar et al., 2018), (Tawidian, Rhodes, & Michel, 2019). As a result, the fungal cells die from nutritional deficiency, without being consumed by immune cells (Da Silva, De Albuquerque, De Araujo, Peixoto, & Hurd, 2000; Hernández-Martínez, Lanz, Rodríguez, González-Ceron, & Tsutsumi, 2002). Fungi may therefore affect a mosquito's ability to transmit diseases by either stimulating or suppressing the immune system. For example, microsporidia like *Vavraia culicis*, which don't harm mosquitoes, can prevent the development of *Plasmodium berghei* (a malaria-causing parasite)

in *Anopheles gambiae* mosquitoes. This happens because the fungi trigger immune reactions like melanisation.

Studies have shown that mosquitoes infected with microsporidia produce significantly fewer oocysts (58%) compared to uninfected mosquitoes (81%) (Bargielowski and Koella 2009). Similarly, another type of microsporidia, *Microsporidia MB*, found in the midgut and ovaries of *Anopheles arabiensis*, affects the development of *Plasmodium falciparum* (another malaria parasite). Certain fungi can't have the potential to produce toxic elements while they can activate the immune system of mosquito population as reported by Cappelli et al., 2019.

The potential of some of the fungal strains to produce reactive oxygen species or cyanide to stop the proliferation of virus or other diseases causing infectious agents was reported in earlier observations by (Cirimotich et al., 2011; Herren et al., 2020; Ramirez et al., 2014) (Angleró-Rodríguez et al., 2016).

Fungi's effect on mosquito behaviour and the volatile components they produce

To locate their food sources (vertebrate hosts and/or nectar), mate, and find places to lay their eggs, mosquitoes use a variety of signals, such as visual, humidity, and olfactory cues (Ko, 2022). Chemical signals that influence mosquito behaviour, such as feeding, mating, and oviposition, primarily include carbon dioxide (CO₂) and volatile organic compounds (VOCs). While some of these chemicals are directly emitted by plants and vertebrate hosts, others are produced by organisms like fungi, which colonize nectar or the skin of humans and animals.

Specifically, yeasts involved in fermentation produce CO₂ and other volatile secondary metabolites, which attract various insects, including mosquitoes (Aldridge et al., 2016;

Becher et al., 2018; Madden et al., 2018; Smallegange et al., 2010). It has been demonstrated that, alongside VOCs, the CO₂ produced by yeasts during fermentation of different carbon sources attracts mosquitoes much more strongly than industrial CO₂ or octenol (a fungal aromatic molecule) on its own (Jerry, Mohammed, & Mohammed, 2017; Mweresa et al., 2014; Smallegange et al., 2010).

However, mosquitoes can be either attracted to or repelled by VOCs, depending on the mosquito species, the specific VOCs present, and their concentrations. For example, sucrose and molasses are more attractive to mosquitoes than honey, even though the fermentation of honey by yeasts leads to higher levels of VOCs, including appealing chemicals like hexanoic acid or phenylethyl alcohol. This suggests that the absence of certain VOCs that repel mosquitoes could enhance their attractiveness to mosquitoes (Aldridge et al., 2016). Some entomopathogenic species, such as *Beauveria bassiana* and *Metarhizium anisopliae*, which produce fungal spores, attract *Anopheles stephensi* females by releasing unidentified VOCs (George, Jenkins, Blanford, Thomas, & Baker, 2013).

Gravid (egg-laden) female mosquitoes use both physical signals and semiochemicals released by larvae, eggs, and/or microorganisms to determine the suitability of potential oviposition sites. For instance, gravid *Aedes aegypti* females prefer to lay their eggs in locations with their own species' eggs and in water that contains larvae that are neither hungry nor infested with harmful parasites.

The yeast *Candida pseudoglebosa*, which commonly inhabits *Aedes aegypti* mosquitoes, has been shown to attract gravid females to breeding sites with this yeast in the water (Reeves, 2004). In contrast, gravid *Culex pipiens* females do not seem to be

attracted to breeding sites by *Saccharomyces cerevisiae*, a yeast that is not part of the mosquito gut mycobiota (Diaz-Nieto et al., 2016).

Impact on larval and adult feeding behavior

As previously established, CO₂ and VOC emissions from plant mycobiota, particularly yeasts that inhabit nectar, attract mosquitoes (Barredo & DeGennaro, 2020; Peach et al., 2021). These fungal volatile chemicals enable yeasts to be dispersed during insect foraging, signalling the presence of sugar sources (Becher et al., 2018; Madden et al., 2018). This behaviour helps explain why many phytopathogenic fungi and yeasts that occupy floral nectar are also found in the mosquito mycobiota (Barnard et al., 2007; Luis et al., 2019; Thongsripong et al., 2018).

Infected gravid females are more likely to feed on blood more frequently. For instance, *Edhazardia aedis* significantly reduces both the number of eggs laid and the amount of blood consumed by *Aedes aegypti* females (Barnard et al., 2007). As mentioned earlier, yeasts can facilitate larval development in *Aedes aegypti* mosquitoes by promoting food uptake and energy accumulation. Interestingly, *Anopheles* mosquito larvae have been shown to respond to the presence of yeasts, which are known to be potentially behaviourally active odorants. In contrast to the insect repellent DEET, *Saccharomyces cerevisiae* increases rest periods while decreasing average larval movement speeds and body rotations (C. Liu et al., 2010; Xia et al., 2008).

Entamopathogenic fungi inside the gut

Only fungi that can be produced in vitro at a reasonable cost and have the potential to kill larval stages reliably should be considered for use against mosquitoes. In addition to a single application, repeated use is necessary, and the fungi must exhibit residual

activity and persistence. They should also retain their activity during storage (long shelf life) and be safe for humans and other non-target organisms. Furthermore, their application should be effective across a wide range of salinities, temperatures, relative humidity's, and breeding sites with varying water quality. Despite human intervention, entomopathogenic fungi have been shown to significantly impact insect populations worldwide. This suggests that modifications, such as enhancing inoculum potential, could improve the effectiveness of fungi in vector control (Roberts, 1965).

Fungi such as *Lagenidium*, *Culicinomyces*, and *Coelomyces* are crucial for mosquito control strategies. Each of these fungi has one or more beneficial characteristics for controlling mosquitoes, but none possesses all the required qualities for cost-effective and widely applicable eradication. While they are effective at killing mosquitoes, *Coelomyces* spp. have been found to cause epizootics, and their production is limited to in vivo methods, with species having constrained host ranges. *Lagenidium giganteum* is considered one of the best fungi for controlling mosquito larvae, although it is only effective in stagnant waters. A major advantage of *Lagenidium* is that it requires only one treatment per season, which differentiates it from *Bacillus thuringiensis israelensis* (Bti). *Lagenidium* has also been developed for commercial use as a mosquito repellent (Khetan, 2000).

Numerous fungi have been identified as entomopathogenic and suitable for use in mosquito control. While non-target species are largely unharmed, some mosquitoes show susceptibility to *Leptolegnia chapmanii* Seymour (Pelizza, Scorsetti, & Tranchida, 2013). These fungi typically follow a similar mode of action. Conidia, the asexual spores responsible for infection, begin to germinate upon contacting a compatible host's cuticle, triggering enzyme activity that facilitates recognition between the pathogen and

host. The fungus then penetrates the circulatory system through the exoskeleton's cuticle, forming penetration pegs, appressoria, and germ tubes (R. A. Samson, Evans, & Latgé, 2013).

In entomophthoralean fungi, hyphal bodies spread throughout the insect's body, consuming nutrients until the host dies. Some fungi also produce spherical protoplasts to evade detection by the host's immune system (Arora, Ajello, & Mukerji, 1991). Once the host insect dies, sporulation or conidiogenesis typically occurs on the outside of the body. However, in fungi like *Metarhizium anisopliae* var. *acridum*, sporulation starts inside the body, with spores emerging through the ventral surface or mouth parts. These spores may be dispersed by wind or other insects present (Christine Roy, Dewit, & Aubert, 2001; Hemmati, McCARTNEY, & DEADMAN, 2001). The fungi produce resting spores that remain dormant in the soil during unfavourable conditions (Wilding & Brady, 1984).

Examples of entomopathogenic fungi used for pest eradication include *Entomophthora maimaiga* for controlling *Lymantria dispar* (Hajek, Humber, & Elkinton, 1995), *Zoophthora radicans* against spotted alfalfa aphid (Kenneth & Olmert, 1975), *Therioaphis trifolii* f. *maculate*, *Verticillium lecanii* against aphids on chrysanthemums (Hall, 1981), *Beauveria bassiana* for insect pests in North America (Poprawski, Carruthers, Speese III, Vacek, & Wendel, 1997), *Metarhizium anisopliae* for locusts and grasshoppers in Africa (Shah & Goettel, 1999), *Beauveria brongniartii* for cockchafers in Europe (Keller, 1992), and *Neozygites fresenii* for *Aphis gossypii* in the USA (Pell, Macaulay, & Wilding, 1993).

When using these fungi for insect pest control, safety for humans, the environment, and non-target organisms is paramount (Mehra & Avanthi, 2024). Profitability, long-term

control, and practical application are also important considerations. The first step in these operations is the identification and characterization of the relevant molecules.

When compared to other pathogens such as bacteria, viruses, and protozoa, which exhibit a wide variety of virulence traits that enable them to be ingested and infect from the oral cavity and/or intestinal tract, the ingestion route for infection by entomopathogenic fungi is much rarer. However, recent advancements in next-generation sequencing technologies have provided insights into the genomes of major entomopathogenic fungi, uncovering numerous fungal genes that may contribute to their ability to cause oral infections (Schabel, 1976). In contrast, pathogens like *Bacillus thuringiensis*, which primarily infect their host through ingestion, contain several virulence factors within their genomes, allowing them to penetrate and kill an insect from within (Vilcinskas, 2016).

In addition to reported perspectives concerning the genomic diversity, it is well inferred that the mosquito vectors usually feed the fungi present in their surrounding habitat and wit helps them to maintain the microbial population within their gut. Also through various events like vertical transmission certain microbial population may transferred from one female mosquito to the next generation. However, no specific studies are conducted earlier to exploit the predominant role of the fungi population with special reference to the aforesaid mechanisms and events (Biswas, Joop, & Rafaluk-Mohr, 2018; Rafaluk-Mohr, Wagner, & Joop, 2018), (Steyn et al., 2016), (Damiani et al., 2010), (Steyn et al., 2016), (Correa, Matusovsky, Brackney, & Steven, 2018), (Valzania et al., 2018), (Stefanini, 2018), (Souza et al., 2019), (Valzania et al., 2018), (Diaz-Nieto et al., 2016), (Souza et al., 2019), (Barnard et al., 2007), (Lorenz & Koella, 2011).

The fungal strains predominantly impacts the fecundity of certain female mosquito vectors including the *Aedes aegypti* (D. R. Barnard et al., 2007). Similarly, Lorenz & Koella (2011) reported that the egg laying potential of *Anopheles gambiae* females was reduced to 16% when they exposed with the *Vavraia culicis*. This detrimental effect of microsporidia on egg-laying intensifies with the mosquito's age. Additionally, the yeast *Wickerhamomyces anomalus* has been detected in the reproductive organs of both male and female *An. stephensi* mosquitoes, suggesting that these organisms may play a role in mosquito reproduction and that vertical transmission is likely (Ricci et al., 2011).

The discovery of *W. anomalus* in the midgut of pre-adult stages (larvae L1-L4 and pupae) and in adults under laboratory conditions, where mosquitoes were reared on sterile food without *W. anomalus* in their larval breeding water, further supports the hypothesis of transstadial transfer. The persistence of *W. anomalus* in adult mosquitoes for up to ten days after emergence highlights its ability to survive in the midgut and derive nutrients from the mosquito diet (Ricci et al., 2011).

Trophic interactions between fungi and mosquitoes often result in a mutual benefit for both parties in terms of acquiring nutrients and energy. Differences observed within the microbial population of mosquito vectors including the *Aedes albopictus* are primarily influenced by the sex of the vector, specifically concerning the mechanism allied with the fructose. For instance the *Malassezia* genus and *Cyberlindnera* genus has been reported to metabolise the fructose in an aggressive manner male and female mosquito vectors respectively. Moreover, the *Aspergillus* and *Cladosporium* has been witnessd to be actively engaged in various mechanisms in male population as reported by previous studies (Guégan et al., 2020), (Barredo & DeGennaro, 2020). (Foster, 1995), (Guégan et al., 2020; Souza-Neto et al., 2007). (Stefanini, 2018), (Guégan et al., 2020). (Malassigné, Valiente Moro, & Luis, 2020), (Esquivel et al., 2016), (Martin et al.,

2018). (Ricci et al., 2011), (Valzano et al., 2016), (Cappelli et al., 2019), (Cappelli et al., 2019).

On the other hand, certain fungi may play a role in promoting the development of mosquito pathogens. For example, the filamentous fungus *Talaromyces* sp., which commonly inhabits the midgut of *Aedes aegypti*, has been shown to enhance *Plasmodium* infection in *Anopheles gambiae* mosquitoes. This fungus also contributes to the transmission of the Dengue virus by inhibiting the activity of digestive enzymes in the mosquito's gut. *Talaromyces* secretes heat-sensitive metabolites and proteins that suppress the expression of various genes involved in the digestion of blood meals, particularly trypsin and endo-proteases. These fungal by-products disrupt the normal activity of trypsin, which may impair the mosquito's ability to digest blood effectively, potentially promoting pathogen survival and enhancing transmission (Angleró-Rodríguez et al., 2017).

How they acquire and transmit these microbes.

The physicochemical properties of insect digestive tracts exhibit considerable variation, and these characteristics profoundly influence the composition and diversity of microbial communities present. Microorganisms can be transferred through different life cycle phases in two main ways: as beneficial symbionts or as harmful pathogens. These microorganisms rely on two modes of transmission for successful colonization of their hosts: vertical transmission (parental mode of transfer) and horizontal transmission (non-parental transfer). Both types of transmission are essential for the survival and establishment of microorganisms within the host. Vertical transmission can occur through two primary methods: transovarian transfer and trans ovum transfer. Transovarian transfer, which takes place during the development of the eggs, is more

likely to be successful compared to trans ovum transfer, which occurs when microorganisms attach to the egg's surface.

Numerous bacteria, including *Wolbachia*, *Asaia*, *Serratia*, *Rickettsia*, *Spiroplasma*, *Buchnera*, *Hamiltonella*, and *Elizabethkingia*, demonstrate vertical transmission. In addition, several viruses, such as *Dengue virus* (DENV), *West Nile virus* (WNV), *Japanese encephalitis virus* (JEV), *Chikungunya virus* (CHIKV), and *St. Louis encephalitis virus*, also exhibit vertical transmission. While vertical transmission is important, the primary mode of pathogen transfer is typically horizontal transmission, which does not rely on parental involvement (Fine, 1975; Leake, 1992).

Paratransgenesis

The paratransgenesis contributes a new strategy to manage the drastic spread of mosquito vectors followed by the prevention of extensive spread of mosquito borne diseases. The paratransgenesis usually consists various major steps in the following way; 1) isolation and identification of specific microbial population, (2) identification of effector proteins with the potentials of preventing the disease instigating infectious agents, (3) release of the identified elements towards the host.

One of the major approach is to manipulate the microbial consortia an effective antagonist against the various pathogens including Chikungunya virus, *Plasmodium* and *Dengue virus* (Wilke & Marrelli, 2015), (Beard et al., 2001), (Chang et al., 2003; Rao et al., 2005), (Valiente Moro, Tran, Nantenaina Raharimalala, Ravelonandro, & Mavingui, 2013). The Paratransgenesis based approaches can extensively offers an effective and innovative way towards the mosquito vector control by microbial consortia manipulation.

The success of paratransgenesis based approaches primarily depends on unveiling the diversity of microbial population and how the particular microbial population communicate with the infectious agents and host. The *Aedes aegypti* larvae collected from the Kole wetlands, Thrissur, Kerala, India provided a strong environment for the microbial diversity along with wide distribution of both bacterial and fungal population. By investigating the genomic variation between and within of both bacterial and fungal population, the scientific community can unveil the specific interactions formed by the microbial consortia (Favia et al., 2008), (Gladieux et al., 2014; Stelkens, 2024), (J. Wang, Gao, & Aksoy, 2023) (Alves et al., 2018), (Alves et al., 2018), (Wilke & Marrelli, 2015).

Studies targeting the genomic difference within and between the microbial population can point out how the microbial population vary across diverse populations of mosquito vectors and it allowing the development of an efficient mosquito vector strategies in a region specific manner. Also, the genomic diversity of both the bacterial and fungal population not only significant for controlling the mosquito vector population or the infectious agents but also for reporting novel microbial strains that may have not explored for diseases control earlier. With the aid of advanced strategies including metagenomic analysis, the scientific community can report novel microbial population having unique characteristic features (Alves et al., 2018).

The primary objective of this study is focused on the isolation, characterization and identification of the specific bacterial as well as the fungal population inhabited within the *Aedes aegypti* fourth instar larvae gut collected from the Kole wetlands, Trissur, Kerala, India. This study have employed both culture dependent approaches and advanced molecular strategies including 16 s sequencing and metagenomic analysis to unveil the extensive diversity of the microbial population within the *Aedes aegypti*

fourth instar larvae. The next strand of this investigation have focused on the extensive genetic divergence within and between both the bacterial and fungal populations. The findings of the intra- and inter specific genetic divergence of both bacterial and fungal populations can provide significant information concerning the adaptative nature of the reported microbes according to the varying environmental setting. Altogether the objectives of the present study augment our understanding concerning the microbial consortia allied with *Aedes aegypti* mosquito vectors and their significant implications towards the development of advanced and efficient strategies for mosquito vector control.

CHAPTER 2

REVIEW OF LITERATURE

REVIEW OF LITERATURE

The mosquito vectors fall under the *Aedes* genus are witnessed to be the public health concern in earlier observations as they instigate severe diseases including the Zika, chikungunya, dengue and other arboviruses as reported by Obtel et al., (1995). The principal way to combat the spread of diseases spread by the mosquito vector under the *Aedes* genus is targeting the larval population since there exists no specific vaccines for many of the infectious disease transmitted by *Aedes* mosquito vectors. Recent studies have focusing on the role of microbial consortia with special reference to interaction with hosts to develop an efficient and promising tool for the control of mosquito borne diseases (Obtel et al., 1995).

Mosquito Gut Physiology

The midgut of mosquito vector usually harbours various predominant functions as reviewed by Hecker (1977). It switches its functions from other to process the blood meal and sugar for nutrient acquisition together with energy for reproduction and survival when it serve as a digestive organ. Simultaneously, it provides selective habitat for the microbial population thereby forms stable structure between the immune responses towards microbial tolerance. In addition to this the role of midgut in disease transmission along with the potential to serve as a filter for pathogens has also been is reported in earlier studies (Franz, Kantor, Passarelli, & Clem, 2015; Smith, Vega-Rodríguez, & Jacobs-Lorena, 2014).. (Hecker, 1977), (Franz, Kantor, Passarelli, & Clem, 2015; Smith, Vega-Rodríguez, & Jacobs-Lorena, 2014).

A study by (A. G. Ramirez et al., 2025) inferred the predominant role of chymotrypsin (AaCHYMO) in the *Aedes aegypti* midgut and they concluded that the area remain unexplored. According to their major findings, the AaCHYMO possessed critical and

unique role during the digestion of blood meal. Furthermore, in addition to the aforesaid findings, the results from the knockdown experiments revealed a diminished activity of chymotrypsin without affecting the fecundity of the mosquito vector. This circumstance indicates the unique interaction allied with the midgut proteases.

Bacteria isolation from *aedes aegypti* gut

The microbiota of *Aedes aegypti* was first studied in 2001 (Luxananil et al., 2001). In this research, a culture-based method was used. The researchers collected larvae from natural breeding sites in Thailand, extracted their guts, and plated the gut homogenates onto Luria-Bertani (LB) agar. They identified the resulting bacterial colonies based on Gram-staining, biochemical tests, and colony morphology (Koneman et al., 1997). Among the bacteria isolated, two strains of *Bacillus cereus* were found to be common. Since these bacteria were consistently associated with *Aedes aegypti*, the researchers suggested they could be useful for developing mosquito larvicidal systems (Luxananil et al., 2001).

Later, another study focused on identifying bacterial symbionts of *Aedes aegypti* using a similar approach (Desiely Silva Gusmão et al., 2007). Alongside morphological and biochemical methods, the researchers extracted bacterial DNA and sequenced the 16S rRNA gene using the Sanger method to identify the bacteria.

Microbiota Across Mosquito Life Stages

Mosquito vectors usually interact with the connected microbial population according to their life cycle stages. During the larval stages, the mosquito vectors acquire the microbial consortia directly from breeding sites through feeding the water. The microbial consortia of mosquito larvae consists Operational Taxonomic Units (OTUs)

and this has been verified for both the *Aedes aegypti* and *Aedes albopictus* as reported by earlier studies (Coon et al., 2016b), (Dada et al., 2014; Ponnusamy et al., 2008), (Coon et al., 2016b), (Dickson et al., 2017), (Lindh, Borg-Karlson, & Faye, 2008), (Coon et al., 2016b), (Kaufman, Walker, Smith, Merritt, & Klug, 1999), (Benzon & Apperson, 1988), (Coon et al., 2016b; Osei-Poku et al., 2012; Valiente Moro et al., 2013; Zouache et al., 2011). The findings of earlier studies indicates that the microbial consortia within the larval population may vary according to the locations, seasons and species (Coon et al., 2016b). This inference illustrates the significance of studying the surrounding environment that alter the profile of microbial consortia within the mosquito vector. As reported by Dickson et al., (2017), distinct microbial consortia has been exists in the *Aedes aegypti* inhabited in the forest and residential habitat. The composition of the microbiota changes significantly as an organism transitions from a larva to an adult (Thiery, Nicolas, Rippka, & Tandeau de Marsac, 1991; Vázquez-Martínez et al., 2002). During metamorphosis, adult insects eliminate most of the bacteria present in their larval midgut. For instance, *Aedes aegypti* removes more than 90% of its bacterial species (Moll et al., 2001; Xia et al., 2008), a process observed in other insects as well (Y. Wang et al., 2011) . However, some bacteria are carried over between life stages, as seen in the arboviral vector *Culicoides imicola* (Coon et al., 2014; Moll et al., 2001). The gut microbiota, which directly influences mosquito vector biology, has been the focus of most studies (Dharne, Patole, & Shouche, 2006; Mancini et al., 2018).

Both *Anopheles* and *Aedes* mosquito species host microorganisms that inhabit various organs, such as the salivary glands and reproductive tissue. According to Mancini et al., (2018) the Proteobacteria has been reported as the predominant microbe within the gut, salivary glands, ovaries and testes in both *Aedes aegypti* and *Aedes albopictus*.

However, differences has been noted for the distribution of microbial consortia across the tissues. (Mancini et al., 2018; Punita Sharma et al., 2014; Tchioffo et al., 2016).

In the salivary glands of female *Aedes albopictus*, Gammaproteobacteria dominate, whereas *Aedes aegypti*'s salivary gland microbiota is more diverse, including genera such as *Serratia*, *Escherichia-Shigella*, *Pantoea*, *Acetobacter*, *Sphingomonas*, *Burkholderia*, and *Cupriavidus*. According to the findings of Dickson et al., 2018; Dickson et al., 2017; Mancini et al., 2018, it is uncertain that whether the diversity of microbial population within the *Aedes* mosquitoes are more diverse than other mosquito vectors. They also mentioned that the following factors may significantly impacts the composition of microbial population within each mosquito vector genera; age of insect, number of generation, genetic history and surrounding environmental conditions (Dickson et al., 2018; Dickson et al., 2017; Mancini et al., 2018).

Factors Influencing Mosquito Microbiota

The various factors allied with the environment may possess predominant role in modulating the microbial consortia inhabiting the mosquito vector. The temperature, chemistry of water followed by other habitat associated critical factors may significantly impacts the composition of microbial population within the mosquito vectors. For instance as reported by Hörtnagl, Pérez, Zeder, & Sommaruga (2010); Onchuru et al., (2016) the elevated temperatures in their habitat supports the proliferation of Betaproteobacteria. Simultaneously the increased temperature may enhance the population density of *Anopheles* mosquito vector compared to the *Aedes*. In addition to this, anthropogenic impacts may also have substantial significance in microbiota proliferation since anthropogenic activities usually alter the geochemical characteristic features of the habitat and breeding sites. The important elements such as

phosphorus and ammonium from the fertilizers may also promote the growth of bacterial population as witnessed in earlier observations by Merritt et al., (1992); Muturi, Ramirez, et al., (2016). (Zhao et al., 2025) validated the prominent influence of microbial consortia reside the surroundings of mosquito vector habitat towards the population dynamics of mosquito vectors, specifically from the urban ecosystems. By investigating the population diversity of the microbial consortia with special reference to the changing breeding sites and water quality, their findings establishes a study association between the productivity and composition of microbial population. (Hörtnagl, Pérez, Zeder, & Sommaruga, 2010; Onchuru et al., 2016), (Merritt et al., 1992; Muturi et al., 2016), (Minard, Mavingui, & Moro, 2013; Zhao et al., 2025) (Coon et al., 2014; Oliveira et al., 2011; Y. Wang et al., 2011; Yadav et al., 2016). (Terenius et al., 2012; Y. Wang et al., 2011), (Coon et al., 2014). (Muturi, Dunlap, Ramirez, Rooney, & Kim, 2019), (da Fonseca Meireles et al., 2025). Specific unique factors associated with the host including the sex, diet and development can also alter the type and intensity of microbial population and the aforesaid perspectives was verified by (Minard, Mavingui, & Moro, 2013). In agreement with this earlier observations (Coon et al., 2014; Oliveira et al., 2011; Y. Wang et al., 2011; Yadav et al., 2016) unveiled that the blood feed female mosquitoes inhabited across various habitats and acquire a plethora of microbial population. After the blood meal, the diversity of the microbial consortia often diminishes according to the season, while the diversity of mosquitoes fall under certain genera including *Delftia* and *Chryseobacterium* may increases in a significant manner. The process of blood feeding in other mosquito vector like *Anopheles gambiae* may generate slightly different result, precisely, oxidative stress, which is primarily allied with the digestion of feed blood (Terenius et al., 2012; Y. Wang et al., 2011). As per the findings of previous studies

(Muturi, Dunlap, Ramirez, Rooney, & Kim, 2019), the source of blood meal also produced substantial impact towards the composition of microbial consortia. For instance, in *Aedes aegypti*, the composition, dissolution and diversity of microbial population such as *Chryseobacterium*, *Elizabethkingia*, *Serratia* and *Leucobacter* significantly impacted by the following sources; human blood, chicken blood and rabbit blood. A study by (da Fonseca Meireles et al., 2025) offers significant information concerning the pattern of blood feeding in adult mosquitoes with special inference on the microbial population shares the same habitat with the mosquito vector.

Using 16S rRNA amplicon sequencing, the study revealed distinct bacterial assemblages in water, larval, and adult midgut samples, with marked compositional shifts based on developmental stage and feeding status. While Proteobacteria predominated in adult guts and water samples, Firmicutes were dominant in larvae, reflecting stage-specific microbial filtering processes. Notably, only a fraction of bacterial taxa overlapped between environments and host stages, suggesting selective acquisition and retention mechanisms during mosquito development and after blood ingestion.

These findings underscore the complexity of gut microbiota structuring in mosquitoes, shaped by both environmental exposure during larval life and physiological changes following blood meals. The patterns observed are broadly relevant to *Aedes aegypti*, reinforcing the hypothesis that gut microbiota is not merely passively inherited but shaped by ecological, developmental, and dietary factors. Implementing dynamic interactions towards the investigations concerning *Aedes aegypti* midgut microbial consortia can results novel insights into microbiome manipulation strategies for vector control.

Sugar-based diets influence microbiota differently. Bacteria like *Pseudomonas* spp. and unclassified *Acetobacteraceae*, often linked to sugar-feeding insects, are abundant in sugar-fed mosquitoes (Crotti et al., 2016; Muturi et al., 2019). Unlike blood, which is digested in the midgut, sugar meals are stored in the crop, where microorganisms like *Serratia* and *Pichia* sp. are found and may transfer to the midgut with food (Desiely S Gusmão et al., 2010; Desiely Silva Gusmão et al., 2007). For *Anopheles gambiae*, *Elizabethkingia* spp. are prevalent in sugar-fed females but decrease after blood feeding (David, Santos, Vicente, & Maciel-de-Freitas, 2016).

Sexual differences in feeding behavior further shape microbiota composition. Male mosquitoes primarily feed on sugar, while females alternate between blood and sugar, leading to variations in their microbiomes (Minard et al., 2013; Zouache et al., 2011). In *Anopheles* species, these differences might also result from the unique tropism of endosymbionts towards male and female reproductive organs (Segata et al., 2016).

Microbiota composition also changes as mosquitoes age, likely due to competition among symbionts for sugar resources or changes in nutrient availability and immune responses (Dong et al., 2009; Montagna et al., 2015; Terenius et al., 2012). Interactions between bacteria and other microorganisms, including viruses, also influence microbiota structure. For instance, specific bacterial families like *Rhodobacteraceae* and *Desulfuromonadaceae* are biomarkers for Zika virus (ZIKV) infection in *Aedes aegypti* (L. E. Villegas et al., 2018). Similarly, artificially introduced *Wolbachia* in *Aedes aegypti* interacts with other microbiota members without significantly altering the overall microbial community (L. E. Villegas et al., 2018).

Residual antibiotics in human blood change the gut flora of *Anopheles gambiae* females, increasing their susceptibility to *Plasmodium* infection (Gendrin et al., 2015).

In *Aedes albopictus*, antibiotics administered to larvae modify the bacterial composition, reducing certain taxa while promoting *Wolbachia* and *Dysgonomonas* spp. (Guégan, Zouache, Démichel, Minard, Tran Van, et al., 2018). *Dysgonomonas* spp., known to aid in vitamin B12 production in termites, are prevalent in *Aedes albopictus*, though their role in mosquitoes remains unclear (Husseneder, Berestecky, & Grace, 2009; Minard et al., 2015).

The study of midgut bacteria in mosquitoes, specifically *Anopheles punctipennis* (Say), *Culex pipiens* (L.), and *Aedes aegypti* (L.), focuses on the position of these bacteria in relation to meconial peritrophic membranes (MPMs) and how bacterial numbers change throughout midgut metamorphosis. The peritrophic membrane, or matrix (PM), is a noncellular, chito-protein layer that surrounds consumed food in the midgut of most insects. This membrane may act as a barrier to food-borne pathogens, isolating the food bolus from the midgut epithelium.

After the adult mosquitoes emerge, few to no microorganisms are found in the midgut of *Aedes*, *Anopheles*, and most *Culex* mosquitoes. However, a small number of bacteria were found in the lumen or within the MPMs/meconia of newly emerged adult mosquitoes. In a few *Culex* specimens, low amounts of bacteria were found in the lumen, with much higher numbers in the MPMs/meconia. Bacterial counts were highest during the fourth instar stage in all three species, decreased after the larvae passed their final feces, rose in the immature pupae, and increased even further in the mature pupae.

The microbial consortia in these mosquitoes are directly linked to their blood-feeding behavior. For instance, *Aedes aegypti* mosquitoes are known for their hematophagy, feeding on blood meals that can increase their weight by 2-3 times. Hemoglobin, the primary protein in blood, is digested in the mosquito's gut, releasing large amounts of

heme. When this heme is unbound from proteins, it can have pro-oxidant and cytotoxic effects (Jeney et al., 2002; Ryter & Tyrrell, 2000). Female *Aedes aegypti* mosquitoes, when fed sugar, constantly produce reactive oxygen species (ROS); however, after a blood meal, the production of ROS rapidly decreases through a mechanism involving heme-mediated protein kinase C (PKC) activation. This change occurs alongside an increase in gut bacterial levels, suggesting that ROS plays a role in regulating midgut bacteria (Oliveira et al., 2011).

Influence of microbiota on reproduction and development

The microbial consortia may form vital role in the maturation and growth of the mosquito vectors. A study by (Chouaia et al., 2012) done rescue experiments where treated rifampicin against *Anopheles stephensi* and provided rifampicin resistant *Asaia* bacteria and observed for the development of larvae with special reference to preventing delays. They verified that presence of eukaryotes within the *Aedes aegypti* larvae is vital to maintain its life since the microbial population may induce an hypoxic signal within the stomach of mosquito vectors as reported by (Coon et al., 2017; Valzania et al., 2018). However, recent study justifies that the mosquito vectors without the aforesaid microbiome can still continue to develop and reach adult and the major role of the microbial population is might me the supply of essential nutrients for the larval development.

The findings of each studies have been varied according to the treatment and conditions. For instance, when the midgut microbial consortia was completely removed, (A. Sharma, Dhayal, Singh, Adak, & Bhatnagar, 2013) observed diminished life span of *Anopheles stephensi* and (McMeniman, Lane et al. 2009) observed reduced longevity in *Aedes aegypti* due to the infection instigated by the *Wolbachia* (Chouaia et al., 2012).

(Coon et al., 2014; Valzania et al., 2018). (Coon et al., 2017; Valzania et al., 2018). (Correa et al., 2018; Valzania et al., 2018). (Sharma, Dhayal, Singh, Adak, & Bhatnagar, 2013), (Contreras et al., 2019).

The microbiome can also influence the preoviposition and reproductive behaviour of mosquitoes. The composition and quantity of the microbiota can vary, making male *Anopheles stephensi* mosquitoes that are transgenic and resistant to *Plasmodium* more attractive to wild-type females (Pike et al., 2017). For *Culex pipiens*, bacterial strains such as *Klebsiella* sp. and *Aeromonas* sp. promote oviposition (Diaz-Nieto et al., 2016). Similarly, *Candida pseudoglebosa*-infected *Aedes aegypti* larvae increase the attractiveness of oviposition sites (Reeves, 2004). Antibiotic treatments, however, reduce egg production in *Aedes aegypti* (Correa et al., 2018). Adding specific commensal bacteria, such as *Paenibacillus*, *Chryseobacterium*, *Sphingobacterium*, *Aquitalea*, or *Comamonas*, to germ-free *Aedes aegypti* can restore their fecundity. However, only *Comamonas* was able to restore egg production to levels similar to conventionally reared females; the other microbes were less effective in supporting egg production in *Aedes atropalpus* (Coon et al., 2016b).

Wolbachia, a bacteria that infects many arthropods, also influences mosquito populations through a process known as cytoplasmic incompatibility (CI). Infected male mosquitoes, when mating with uninfected females, result in embryonic lethality, while mating infected males with infected females leads to viable offspring (Flores & O'Neill, 2018). The molecular mechanisms behind CI are still not fully understood. For CI to occur, the actin cytoskeleton interacts with the mosquito-borne *Wolbachia* (wPip strain) Type IV Effector WD0830 (Sheehan, Martin, Lesser, Isberg, & Newton, 2016).

The deubiquitylating enzyme (DUB) CidB and its partner CidA from *Wolbachia* (wPip strain) play a role in the CI process (Beckmann, Ronau, & Hochstrasser, 2017). In mosquitoes infected with *Wolbachia* (wPip strain), a *cin* operon encoding a nuclease, CinB, and a second protein, CinA, also appear to be involved in CI (Chen, Ronau, Beckmann, & Hochstrasser, 2019), (LePage et al., 2017).

Vectorial Capacity and the Microbiome

The vectorial capacity was influenced by several significant factors including vector density, bite rate, vector competence followed by the incubation period of the respective pathogen. The aforesaid factors are recognized to be the fundamental elements deciding how each mosquito vectors transmit the diseases in a drastic way. Recent investigations have illustrated the complexity allied with the mosquito-pathogen link and such studies have emphasized the prominent role of microbial consortia in impacting the vectorial capacity (Simões, Caragata, & Dimopoulos, 2018).

The microbial consortia inhabited in the mosquito vector also modulate vectorial capacity by influencing various biological mechanisms including immune responses, pathogen replication as well as the interactions between the mosquito vector host and the microbes. The findings from earlier studies (J. L. Ramirez et al., 2012) clearly indicates that the microbial consortia can directly interfere the infectious agents through biofilm formation, replication, transmission and lysis (Simões, Caragata, & Dimopoulos, 2018),

Microbiota and Vector Competence

The vector competence usually referred as the potential of the mosquito vector to acquire, maintain and transmit the infectious agents to the specific organisms. As

reviewed in earlier sections, the microbial consortia have possessed significant role in impacting the immune responses of mosquito vectors thereby the vector competence may have altered. According to (J. L. Ramirez et al., 2012) certain microbial population have the potential to diminish the susceptibility of mosquito vector against the infections and associated processes including DENV-2 replication (Ramirez et al., 2012), (Apte-Deshpande, Paingankar, Gokhale, & Deobagkar, 2012; Carissimo et al., 2015).

Implications for Vector Control

Unveiling the predominant role of the microbial consortia towards the vectorial capacity of mosquito vectors directs new way for the development of diseases control and prevention strategies. As mentioned earlier, by manipulating the specific microbial population within the mosquito vectors, it is easier to alter the vector competence thereby diminish the rate of transmission or prevent the infection together. The aforesaid perspectives and targets could achieved through various methods including genetic modification of the microbiome or introduction of specific bacteria towards the drastic vector population (Gnambani, Bilgo, Sanou, Dabiré, & Diabaté, 2020), (Kozlova et al., 2021) (Farine, Cortot, & Ferveur, 2014) (Fischer et al., 2017; Leitão-Gonçalves et al., 2017; C. Wang & Wang, 2017).

There exists strong evidence that arboviruses are primarily influenced by the microbial consortia. For example, as per the findings of (Zouache et al., 2012), the population diversity of the microbial population fall under the family Enterobacteriaceae was dramatically increased in *Aedes albopictus* during the CHIKV infection while the population diversity of *Wolbachia* and *Blattabacterium* diminished. It unclear that

whether the aforesaid pattern are directly impacted by the CHIKV infection in *Aedes aegypti* as suggested by McFarlane et al., (2014).

Both bacterial pathogens and symbionts in mosquitoes depend on specific nutrients from their hosts. For example, the internalization of the mosquito lipoprotein lipophorin by *Plasmodium* oocysts suggests a metabolic need for lipids from the vectors (Atella, Bittencourt-Cunha et al., 2009). Similarly, effective protein digestion by DENV and WNV viruses requires intracellular membrane remodelling (Martin et al., 2018). DENV also affects lipid content and homeostasis and regulates genes involved in lipid trafficking in both vertebrate and invertebrate models (Heaton et al., 2010; Jupatanakul, Sim, & Dimopoulos, 2014; Perera et al., 2012), which alters host membrane trafficking. Inhibition of DENV replication by chemically targeting the fatty acid synthase enzyme highlights the importance of lipids in the virus's life cycle (Jupatanakul et al., 2014).

Symbionts can sequester such nutrients, potentially preventing pathogen growth and infection. For instance, *Wolbachia*, the most common intracellular symbiont in insects, may sequester cholesterol and other lipids within insect cells, limiting DENV, CHIKV, and *Plasmodium* infections (Moreira et al., 2009; Sinkins, 2013), (Caragata et al., 2013).

Fungal- bacterial link instigated mosquito death

The insect gut microbiota possess prominent role in regulating the various interactions allied with the intestinal pathogens and the host. For example, the gut microbial inhabitants also helps the mosquito vectors to control the infections through down-regulating the antimicrobial peptides. Previous studies have clearly validated the aforesaid link between the fungal population and mosquito vectors (Wei et al., 2017) (Schrack & Vainstein, 2010), (Ruiz-Sanchez, Lange, & Orchard, 2010).

Moreover, *Beauveria bassiana*'s toxin oosporein promotes fungal infection by suppressing polyphenol oxidase activity and reducing the expression of antifungal peptide genes in the insect's fat body (Feng, Shang, Cen, & Wang, 2015). Oosporein also downregulates the expression of dual oxidase (Duox) in the midgut, which leads to reduced reactive oxygen species (ROS) production. Since Duox-dependent ROS production plays a vital role in gut immunity and the regulation of gut-associated bacteria, this disruption could weaken the insect's defense mechanisms (S. Kumar, Molina-Cruz, Gupta, Rodrigues, & Barillas-Mury, 2010). The activation of Duox-dependent immunity in *Drosophila* helps to identify and control opportunistic pathogenic microorganisms (Bae, Choi, & Lee, 2010).

When Duox expression is suppressed in the gut, *S. marcescens*, an opportunistic pathogen, may overpower other commensal bacteria, further enhancing fungal pathogenicity (Wei et al., 2017). The reported observations offers significant information concerning the mechanisms of fungal toxicity within the insect groups (Wei et al., 2017). High-throughput sequencing of the microbiome showed that antibiotic treatment significantly decreased *Asaia* in both mosquito species. Interestingly, introducing an *Asaia* mutant resistant to antibiotics completely stopped *Wolbachia* transmission and reduced mortality caused by blood meals in treated mosquitoes. These findings suggest that the local mosquito microbiome plays a crucial role in preventing *Wolbachia* transmission (Hughes et al., 2014).

In vertebrates, the gut microbiota also contributes to the invasion of intestinal pathogens. For example, the gut commensal microbiota can indirectly promote viral infections by encouraging the proliferation of target cells or directly by activating immunosuppressive cytokines (Kane et al., 2011; Kuss et al., 2011; Wilks & Golovkina, 2012).

Other microbes in mosquitoes

Mosquitoes are known to carry two types of viruses: insect-specific viruses (ISVs), which can only replicate in insects, and arboviruses, which infect humans and animals (Öhlund, Lundén, & Blomström, 2019). Earlier investigations have unveiled that various pathogenic bacterium including the *Bacillus thuringiensis* produced toxic impacts towards the lepidopterans (Broderick, Raffa, & Handelsman, 2006). Furthermore, the organisms fall under *Clostridia* and *Serratia* inhabited inside the host augmented the intensity of death rate of *Spodoptera littoralis* by invading the body through specific toxin-instigated epithelial lesions and it was investigated by (Caccia, Di Lelio et al. 2016).

The effectiveness of this approach would depend on the host specificity of the MSVs being used, which determines how specific the target mosquito species would be. This method would be similar to current vector control strategies, such as insecticides or treatments with fungi and bacteria, which aim to reduce the overall mosquito population and thereby limit arbovirus transmission. For example, when applied to manmade or natural ponds, densovirus successfully reduced mosquito population density (Johnson & Rasgon, 2018). However, this method could face challenges such as issues with target specificity or the evolution of resistance in mosquitoes. A potential solution to these challenges could involve using MSVs that are more specific, do not harm the environment, and are resistant to evolution. Such MSVs could harm mosquitoes only during the later stages of their life or when they are infected with an additional arbovirus, thus minimizing disruption to the ecosystem. AgDV, another densovirus, is an example of a virus that shows late-life pesticide activity. Unlike other densoviruses, AgDV does not reproduce significantly in mosquito larvae or pupae, but it does in older adult mosquitoes.

MSVs often cause chronic infections in mosquitoes with minimal effects on the host, which could be advantageous in a strategy where the simultaneous infection of mosquitoes with both MSVs and arboviruses is required to harm the insect. This balance might be disrupted by the presence of more arbovirus infections, as they could consume more nutrients, occupy more space, or provoke an immune response, which could ultimately be harmful to the mosquito. The arthropod borne viruses are primarily instigating various diseases towards the human population through the mosquito bites as reported by (Öhlund, Lundén, & Blomström, 2019).

The conventional strategies focusing on the detection of viruses are usually targeting the specific diseases while the bacteria can be easily detected with the aid of simple molecular strategies including the ribosomal gene sequencing. Although, with the emergence of advanced strategies in the current scenario, our understanding concerning the viral ecology together with the environment has been greatly diversified.

Mosquito-specific viruses (MSVs) with the potential to diminish and impact the fitness, survival, and fertility can be exploited for controlling various mosquito vectors, specifically those that transmit the arboviruses, regardless of taxonomic characteristic features. In addition to controlling mosquitoes directly, MSVs can also be used to prevent arbovirus infection and transmission without harming the mosquito vector. As discussed earlier, some MSVs interfere with or restrict arbovirus infection, supporting the idea that MSVs could be used in this manner.

This strategy would be more environmentally friendly because it would target arbovirus transmission rather than the mosquito population itself. The availability of MSVs that interfere with various arboviruses would be crucial for the success of this strategy. However, no MSV has yet been identified that can restrict arboviruses from all

significant arbovirus families. Most reported cases of restriction have been seen when both the MSV and arbovirus belong to the same viral family. More research is needed to understand how MSVs interfere with arboviruses, including theories like superinfection exclusion, immune system induction, and competition for resources such as food and space.

Recent studies have investigated the potential of mosquito-specific viruses (MSVs) and found that they might not only diminish the fitness but also immunize the mosquito vector population. The findings of such studies (Leggewie & Schnettler, 2018; Olson & Blair, 2015) also unveiled that the aforementioned mechanism might be inherited towards the progeny or next generation since it is associated with the genetic factors and hence they always referred as “vaccination”.

However, a significant limitation of this approach is its viral specificity. These MSVs would likely only be effective for a single arbovirus species in a given region, making them less useful in areas with multiple arboviruses. To broaden the effectiveness, different arboviruses would need to be incorporated into MSVs, potentially through mixing virus components or creating multiple dsRNA molecules targeting different viruses. In addition to viruses, mosquitoes are host to various microorganisms, including bacteria, fungi, and protozoa. Protozoan infections have been less studied but can also affect mosquito populations. For example, *Lambornella stegomyiae* was reported to infect mosquito larvae in 1921, and other species of ciliated protozoa, like *Tetrahymena pyriformis*, *Lambornella clarki*, and *Chilodonella uncinata*, have been identified as being virulent to mosquitoes. These protozoa invade the mosquito's haemocoel, impacting tissues like the gut epithelium, fat bodies, and muscles. Protozoa, particularly during the larval stages, can multiply within the mosquito, eventually killing it by consuming the host's tissues.

No discussion of mosquito microbiota would be complete without mentioning *Plasmodium*, the protozoan responsible for malaria. *Plasmodium* species are transmitted by *Anopheles* mosquitoes and require the mosquito to serve as a definitive host, where sexual reproduction of the parasite occurs. There are five species of *Plasmodium* that cause malaria in humans: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*. While *P. knowlesi* is primarily maintained in macaque monkeys and causes zoonotic malaria in Southeast Asia, the other four species are specific to humans. *Anopheles* mosquitoes transmit *Plasmodium* by biting an infected human and then injecting the parasite into another host during a second blood meal. *P. falciparum* is the most deadly species, especially in young children and pregnant women. Over 30 species of *Anopheles* mosquitoes can spread malaria, but only certain species are capable of supporting the development of *Plasmodium* parasites, and this varies by parasite species. *Plasmodium* has a remarkable ability to adapt genetically, allowing it to rapidly develop resistance to treatments like antimalarials and alter its host specificity, which poses a significant challenge to malaria control (Leitão-Gonçalves et al., 2017).

Mosquito and kole wetlands

Wetlands are undoubtedly important ecosystems, a fact supported by numerous studies and resources spanning many years. These ecosystems have been the focus of considerable attention in various conferences, books, and scholarly articles. The Kole Wetlands of Kerala, a designated Ramsar wetland ecosystem, provide highly favourable environmental conditions for mosquito breeding because of their extensive seasonal flooding, shallow water bodies, dense aquatic vegetation, organic-rich sediments, and fluctuating hydrological regimes. The wetlands remain inundated for nearly six months annually, creating numerous temporary and permanent aquatic habitats suitable for mosquito larval development. The seasonal flooding pattern of the Kole ecosystem

increases the availability of stagnant and semi-stagnant water pools, which serve as ideal breeding sites for several mosquito species. Previous studies conducted in the Kole wetlands have documented a rich mosquito fauna, indicating that the wetland functions as an important reservoir for mosquito populations throughout different seasons. For *Aedes aegypti*, the Kole Wetlands may function as an important ecological interface where natural wetland habitats coexist with nearby human settlements and agricultural landscapes. Such heterogeneous environments facilitate exposure to diverse microbial communities, organic substrates, and breeding sites, contributing to both mosquito proliferation and microbiome diversity. These conditions make the Kole Wetlands a valuable natural system for studying mosquito–microbe interactions, seasonal microbiome shifts, and vector ecology.

Vector control and genomic inferences

Wiemers, Graf, Addo, Arck, & Diemert, (2025) reviewed the impact of climate change towards the transmission of vector-borne disease. They emphasize how the elevated temperature across the globe have supported the rapid distribution of mosquito vectors towards newer provinces where the diseases has not been reported earlier. Their observation agreed the extensive discussion regarding the ecology of mosquito vectors and detailing how the climate shift affects the distribution of mosquito vectors in sensitive habitats. These observations highlights the need to integrate the microbial consortia based research with climate shift for investigating the implications concerning the development of efficient approaches for mosquito control (Chandra & Bhattacharjee, 2024). This work reinforces the significance of integrated vector management and public health education, particularly in the context of changing ecological and socio-economic conditions that continue to drive the spread of mosquito-borne diseases.

Unfortunately, these diseases are increasing due to several factors: changes in how we deal with public health, global warming, mosquitoes becoming resistant to insecticides, the diseases themselves becoming resistant to drugs, and shifts in how people live and the structure of society. For a disease to spread, three things need to come together: the disease itself, the insect that carries it, and the human it infects. These diseases disproportionately affect developing countries in tropical regions.

In the early 1900s, the diseases spread by insects were controlled by simply controlling the insect populations. These successes led to high hopes that we could even eliminate these diseases entirely using insecticides and drugs against the diseases themselves. However, this optimism didn't last long (Gubler, 1998). Insects started becoming resistant to insecticides, some insecticides harmed the environment, and there weren't enough resources or the ability to carry out these control plans effectively (Abraham, CHA, & JACOBS-LORENA, 2007). It's clear that our current methods to fight insect-borne diseases are not enough, and we need to find new ways to combat them.

Mosquitoes are recognized as significant vectors for numerous infectious diseases, including those caused by parasitic and viral agents such as lymphatic filariasis, chikungunya, and Zika. These diseases pose substantial health risks to both humans and animals (Weaver, Charlier, Vasilakis, & Lecuit, 2018). Emerging evidence suggests that mosquitoes may also transmit bacterial pathogens, such as *Rickettsia felis*. The global burden of mosquito-borne diseases (MBDs) is substantial. Malaria alone, according to the World Health Organization, accounted for an estimated 229 million cases in 2019, resulting in 409,000 deaths. The majority of these cases and associated fatalities occur within the African region, primarily concentrated in sub-Saharan Africa (Tolle, 2009). While North African countries generally experience lower MBD prevalence, climate change poses a significant threat. Modeling studies predict an

increased transmission risk of various MBDs in these regions (Tolle, 2009). Furthermore, the emergence of indigenous mosquito species capable of transmitting pathogens in temperate zones presents a growing concern (Blagrove et al., 2017).

Mosquito-borne viruses are influenced by a complex mix of factors, including human immunity, mosquito abundance, and environmental conditions. This makes controlling outbreaks challenging, as each situation requires a tailored approach. Since vaccines and effective treatments are often lacking, prevention strategies primarily focus on personal protection against mosquito bites and controlling mosquito populations.

Virus genomics plays a crucial role in improving our understanding and response to outbreaks. By analyzing the virus's genetic data, scientists can identify the specific viruses causing outbreaks, which aids in developing better diagnostic tests. Furthermore, advanced genomic techniques can track the virus's spread within communities, pinpoint outbreak origins, and identify factors driving transmission (Nebbak, Almeras, Parola, & Bitam, 2022). This information empowers public health officials to target control efforts more effectively. Evolutionary biology provides valuable insights into virus spread and evolution during outbreaks.

Outbreaks and Genomic perspectives

Across the globe, over 3,500 different mosquitoes are reported so far (Foster & Walker, 2019). Majority of these mosquitoes are recognized as the vector for a plethora of infectious diseases with the help of microbial consortia inhabited in their gut. Recent studies are now focusing on how the microbial consortia has been reached inside the mosquitoes and what are the major predominant microbial consortia reside there. Frequent research on these aspects unveiled that the microbes produced predominant

role in mosquito vectors and there by the disease transmission happens (Foster & Walker, 2019).

Mosquito Gut Microbiota: A Review

Research on the gut bacteria of mosquitoes (Culicidae family) has become increasingly important in recent years (Gao, Cui, Wang, Jacobs-Lorena, & Wang, 2020). Mosquitoes start their lives in water, where they spend their early stages as larvae once they grow up, they become adults and live on land. Larvae mainly eat decaying matter, tiny single-celled organisms, and small creatures. Adult mosquitoes of both sexes typically feed on sugary liquids from plants (H. Liu et al., 2024). However, adult female mosquitoes also need to feed on blood from animals to produce eggs. This blood-feeding can unfortunately spread diseases between animals. Researchers have long known that mosquitoes have tiny living things called microbes inside their gut. But only recently have we started to understand these microbes and how they affect mosquitoes.

Research shows that these gut microbes can both help and hinder a mosquito's ability to transmit diseases. The article (H. Liu et al., 2024) have discussed how mosquitoes get these gut microbes, what kinds of microbes live there, and how these microbes influence the development of mosquito larvae and the health of adult mosquitoes.

Mosquitoes primarily acquire their gut microbiota from the environment, with larvae hatching devoid of extracellular microbes. The composition of larval gut microbiota mirrors the surrounding aquatic environment, and the high variability observed across individuals suggests environmental acquisition rather than inheritance (H. Liu et al., 2024).

Although vertebrate blood typically contains few microbes, its ingestion can significantly impact the gut microbiota through changes in redox conditions or host metabolism (Jaffar, Ahmad, & Lu, 2022). Furthermore, infections with various vector-borne pathogens can also alter the composition of the gut microbiota, likely through complex and yet to be fully understood mechanisms (Ishigami, Jang, Itoh, & Kikuchi, 2023).

Composition and functional roles of the gut microbiota in mosquitoes

Strand, in 2018 primarily learn about the types of microbes living in a mosquito's gut by studying their genetic material. Most of these microbes are bacteria that thrive in the presence of oxygen or can survive with or without oxygen. They belong to four main groups: Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria.

These groups are also common in other insects that get their gut microbes from their environment, suggesting that their presence in mosquitoes might be due to their abundance in the insect's surroundings. Some of these bacteria have been successfully grown in the laboratory. In addition to bacteria, other tiny organisms like fungi, algae, and parasites have also been found in the guts of some mosquito species.

Earlier studies have reported that the larvae of *Aedes aegypti* with naturally occurring bacteria usually have reduced level of oxygen compared to the larvae lacking the microbial population (Coon et al., 2017). This circumstance indicates that the bacteria within the mosquito vector may develops reduced oxygen environment and this low setting may accelerates the growth of the same.

Based on the various research article reviewed here, it was perceived that the impact of altering habitat, diet and other environmental factors towards the microbial consortia

inhabited inside the mosquito vector remains not investigated and the same inference was reported by (Strand, 2018). To address the aforesaid research gap, research should be carried on this direction and the scientific community can determine the specific and exact function and role of each microbial population within the mosquito vector.

The impacts of insecticides on the mosquito microbiota have been extensively investigated across various mosquito species, including *Aedes aegypti*, *Aedes albopictus*, *Anopheles albimanus*, *Anopheles arabiensis*, *Anopheles coluzzi*, *Anopheles gambiae sensu stricto*, *Anopheles stephensi*, *Culex pipiens*, and *Culex quinquefasciatus* (Strand, 2018). Conversely, the abundance of *Bacteroides faecichinchillae* was significantly decreased in the midguts of resistant *Aedes aegypti*, while *Akkermansia muciniphila* was observed to be more abundant in insecticide-susceptible mosquitoes. Furthermore, the specific changes in the microbial community structure and the predominant species or genera vary significantly among different mosquito species and even within the same species across different study sites.

Now the scientific community have focused to find out the answer for how the mosquito vectors manage the fungal infection? By investigating the types and patterns of fungi inhabiting inside the mosquito vector, the scientific community can develop efficient approach for mosquito control (P Tawidian et al., 2019).

The Aforesaid research article primarily focused on gathering the information concerning the predominant fungi that have the potential to kill the mosquito vectors. Previous studies also reported the potential of certain fungi like Entomophthorales to kill the mosquito vectors. However, the short life span of spores followed by the difficulty in cultivating under laboratory conditions made them unsuitable for the mosquito vector control programs (Scholte et al., 2004; P Tawidian et al., 2019).

A balanced microbial ecology is suggested by the coexistence of dominant and low-abundance species found in this study. Through metabolic complementarity and niche specialisation, low-abundance bacterial groupings like Actinobacteriota, Bacteroidota, and Campylobacterota may support community stability. Similarly, fungal colonies can maintain microbial homeostasis in the gut environment by influencing bacterial colonisation patterns and taking part in nutrient cycling. According to (Wei et al. 2024), despite their low abundance, uncommon microbial species frequently make a disproportionate contribution to ecosystem resilience and functional stability. Prior research has shown that immunological activation mediated by microbiota influences vulnerability to pathogens such *Plasmodium* parasites, dengue virus, and chikungunya virus. Native gut bacteria affect mosquito immune responses and can lessen pathogen development in the stomach, according to (Dong et al. 2009) and (Ramirez et al. 2012). Through immunological activation, competitive exclusion, or the synthesis of antimicrobial compounds, some bacterial species prevent the growth of pathogens. Changes in the environment may have an impact on vector competence by changing the microbial makeup. This is in line with the findings of (Muturi et al. 2018), who found that environmental factors greatly influence mosquito microbiota and, consequently, mosquito-pathogen interactions. Larval mosquitoes live in nutrient-rich watery environments where they continuously consume microorganisms from their surroundings. These microorganisms aid larvae in using a variety of nutrition sources and adjusting to shifting environmental circumstances. Research by Duguma and his colleagues in 2015 and Muturi and his colleagues in 2018, shown that the microbial composition of mosquitoes is mostly determined by the features of breeding habitats.

The present study focused to investigate the genetic divergence within and between the bacterial and fungal population inhabited in the *Aedes aegypti* vectors mosquitoes, that

are collected from the Kole wetlands of Thrissur, Kerala, India. Investigating the genomic diversity between and within both the bacterial and fungal strains can provide significant information concerning their ecological role followed by the pathogen transmission. The reported findings of the present study can be used for the development of efficient strategies for mosquito control. Also, earlier studies according to the aforesaid direction in *Aedes aegypti* found scanty in Kerala, India. Hence the current study focused to fill this research gap by offering significant information concerning the extensive diversity of both the bacterial and fungal strains allied with the *Aedes aegypti* in the selected study site in a regionwide manner. In addition to this none have piloted a study concerning the isolation and identification of both bacterial and fungal population inhabited in the *Aedes aegypti* with advanced approaches including the culture dependent strategies and metagenomics.

OBJECTIVES

OBJECTIVES

1. To Isolate, characterize and identify bacterial inhabitants from gut of fourth instar *Aedes aegypti* larva collected from kole wetlands by culture dependent method and metagenomic analysis.
2. To isolate, characterize and identify fungi from fourth instar *Aedes aegypti* larva collected from kole wetlands by culture dependent and metagenomic analysis.
3. To find out the inter and intraspecific genetic divergence between bacterial and fungal isolates from the fourth instar *Aedes aegypti* larvae collected from kole wetlands.

The objectives are discussed in separate chapters as follows:

❖ Chapter 3:

To Isolate, characterize and identify bacterial inhabitants from gut of fourth instar *Aedes aegypti* larva collected from kole wetlands by culture dependent method and metagenomic analysis.

❖ Chapter 4:

To isolate, characterize and identify fungi from fourth instar *Aedes aegypti* larva collected from kole wetlands by culture dependent and metagenomic analysis.

❖ Chapter 5:

To find out the inter and intraspecific genetic divergence between bacterial and fungal isolates from the fourth instar *Aedes aegypti* larvae collected from kole wetlands.

CHAPTER 3

**TO ISOLATE, CHARACTERIZE AND IDENTIFY BACTERIAL
INHABITANTS FROM GUT OF FOURTH INSTAR *AEDES*
AEGYPTI LARVA COLLECTED FROM KOLE WETLANDS
BY CULTURE DEPENDENT METHOD AND METAGENOMIC
ANALYSIS.**

INTRODUCTION

The *Aedes aegypti* mosquitoes are recognized to be the principal vector of many diseases including Zika, yellow fever, and chikungunya and it instigates severe health concerns across the globe as reported by (Leta et al., 2018). The complex and strong link between the mosquito vector and the microbial consortia including the fungal and bacterial strains possessed vital role in various mechanism allied with the behaviour, development and transmission capacity of mosquito vectors (Ricci et al., 2012). Such microbial consortia usually harbours the various parts of the mosquito vector including the gut, where they may impact the immunity, acquisition of nutrients and hoist potential to spread the diseases. Many of the earlier studies have directly investigated the ecological role while the research targeting the microbial consortia within the gut of mosquito vectors still scanty.

The microbes inhabiting the mosquito vectors can have both detrimental and advantageous effects (Guégan, Zouache, Démichel, Minard, Tran Van, et al., 2018; Wei et al., 2017). Unveiling the prominent role of microbial consortia inhabiting the *Aedes aegypti* larvae is not only for basic information on its ecological function but also for mosquito control approaches targeting the microbial population.

No earlier studies have completed the isolation and identification of both bacterial and fungal population inhabited in the *Aedes aegypti* with advanced approaches including the culture dependent strategies and metagenomics from the current study site. The wetlands were opted as the study site for the present investigation since the Wetlands usually offers a strong base with standing water for mosquito vectors. The Kole wetlands of Thrissur, Kerala, India also known to exhibit extensive distribution of

biodiversity along with distinct and unique aquatic ecosystems that sustains the life of various organisms and plants.

The microbial populations of wetland ecosystems are prominently impacted by various environmental factors including the organic matter availability, quality of water followed by the variations across the seasons (Faulwetter et al., 2009). According to (Scolari, Casiraghi, & Bonizzoni, 2019), the potential role of microbial population in mosquito vector is multifaceted in *Aedes aegypti*, since they verified the role of microbes in nutrient acquisition, digestion, immunity and resistance towards pathogens. From this point of view, to get deeper understanding, here the present implemented both culture-dependent strategies and advanced approaches including metagenomic analysis.

MATERIALS AND METHODS

Study Area

The Kole Wetlands of Thrissur district of Kerala, India was chosen for the current study site (10.52°N, 76.21°E). The total wetland area was divided into different sampling zones based on geographical location, habitat characteristics, and accessibility. Zone I – Puthenchira Kole region, Zone II – Mala–Annamanada Kole region, and Zone III –Puzhakal Kole region. Within each zone, potential mosquito breeding habitats such as shallow stagnant water collections, marshy areas, and vegetation-associated water bodies were identified. Sampling sites were randomly selected from each zone to obtain representative larval populations. A total of nine sampling sites were selected, with three sites from each zone. Mosquito larvae were collected using a standard larval dipper from different breeding habitats. Approximately 10–15 fourth instar mosquito larvae were collected from each sampling site. Three collections (replicates) were made from each zone. Thus, a total of approximately 90–135 fourth instar larvae were collected during the study period. The collected larvae were transferred into sterile containers containing habitat water and transported to the laboratory for further processing. Larvae collected from each sampling zone were pooled together and used for culture-dependent and metagenomic analyses. The larvae of *Aedes aegypti* at regular interval from June 2019 to May 2020 was collected across the three seasons such as Premonsoon, monsoon and post monsoon with respective code as AAMB, AAMA and AAMC.

Culture-Dependent Isolation of Bacteria from the Gut

Mosquito larvae were surface sterilized with 70% ethanol and rinsed twice with 250 µl phosphate-buffered saline (PBS) (Walters et al., 2016). Larvae were collected during

the pre-monsoon, monsoon, and post-monsoon seasons. The midguts of fourth instar *Aedes aegypti* larvae were dissected under sterile conditions using a binocular dissecting microscope. The midguts were placed in 2 ml microcentrifuge tubes containing 500 µl PBS, homogenized, and transferred to 10 ml of nutrient broth. The samples were incubated at 28-37°C for 24-48 hours. A control was set up without the dissected gut.

Bacteria Isolation from Plate Cultures

The bacterial cultures were serially diluted in sterile distilled water, and 100 µl of each dilution was plated onto sterile agar plates. These were incubated at 28-37°C for 24-48 hours. The number of colony-forming units (CFUs) was counted, and morphologically distinct colonies were selected, subcultured on nutrient agar to obtain pure bacterial cultures (Pereira et al., 2011).

Molecular Identification Using 16S rRNA Sequencing

Genomic DNA was isolated using the NucleoSpin® Tissue Kit (Macherey-Nagel) according to the manufacturer's instructions (Chandy, Swamy, & Sen, 2024). A 1.5 ml culture was treated with 180 µl T1 buffer and 25 µl proteinase K, and incubated at 56°C until the cells were lysed. After adding 5 µl of RNase A, the mixture was incubated for 5 minutes at room temperature, followed by incubation at 70°C with 200 µl B3 buffer. After adding 210 µl of 100% ethanol, the sample was processed using the NucleoSpin® Tissue column. The DNA was eluted in 50 µl BE buffer.

Agarose Gel Electrophoresis for DNA Quality and Quantity

The quality of the isolated DNA was checked by loading 5 µl of DNA mixed with 1 µl of 6X gel-loading buffer onto a 0.8% agarose gel in 0.5X TBE buffer containing 0.5

µg/ml ethidium bromide. Electrophoresis was performed at 75 V until the bromophenol blue dye reached the bottom of the gel. The gel was visualized under UV light, and images were captured using a gel documentation system.

PCR Amplification of 16S rRNA

PCR amplification was performed in a reaction mixture containing 5 µl of 2X Phire master mix, 4 µl distilled water, and 0.25 µl of forward (5'CAGGCCTA ACACATGCAAGTC) and reverse primers (GGGCGGWGTGTACAAGGC). The PCR cycling conditions included an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 60 seconds. A final extension was performed at 72°C for 7 minutes. The amplified product was visualized on a 1% agarose gel containing ethidium bromide under UV light.

16S rRNA Sequencing

Sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) in a PCR thermal cycler (GeneAmp PCR System 9700). The sequence quality was checked using Sequence Scanner Software v1. Sequences were aligned and edited using Geneious Pro v5.1.

Phylogenetic Analysis

BLAST (Basic Local Alignment Search Tool) was used for sequence comparison and alignment against the GenBank database.

Metagenomic Analysis

Bacterial community profiling was performed by high – through put 16S rRNA gene amplicon sequencing targeting the V3 – V4 hypervariable regions.

Quality Control of Raw Reads

Metagenomic sequencing generates raw data in FASTQ format, which is first subjected to quality control using FastQC. This tool evaluates base quality scores, GC content, sequence length distribution, duplication levels, and presence of adapter contamination. Summary statistics and per-read metrics are reviewed to ensure data integrity before proceeding.

Trimming and Filtering of Reads

Reads containing low-quality bases (Phred score < 20) and residual adapter sequences are trimmed using tools like Trimmomatic or Cutadapt. Parameters such as sliding window trimming and minimum read length thresholds are applied to retain high-quality data. For paired-end reads, orphaned or unpaired sequences are removed post-trimming to ensure consistency.

Host DNA Depletion

To remove host (mosquito) DNA contamination, trimmed reads are aligned against a reference mosquito genome using Bowtie2. The resulting SAM/BAM files are processed using SAMtools, and only unmapped reads (representing microbial origin) are retained for downstream microbial analysis.

Metagenome Assembly

Unmapped high-quality microbial reads are assembled into longer contiguous sequences (contigs) using MEGAHIT and metaSPAdes, both optimized for metagenomic data. The assembly process reconstructs partial or complete microbial

genomes from the short sequencing reads, improving taxonomic and functional resolution.

Binning of Metagenome-Assembled Genomes (MAGs)

Contigs are binned into metagenome-assembled genomes using automated binning tools such as MetaBAT2, MaxBin2, or CONCOCT. Binning relies on sequence composition and coverage profiles. The quality of the resulting genome bins is evaluated using CheckM to assess completeness and contamination.

Taxonomic Classification of Assembled Reads

For taxonomic profiling, unassembled or assembled reads can be classified using Kraken2, a fast k-mer-based classifier, and MetaPhlAn3, which uses clade-specific marker genes for accurate species-level profiling. Both tools generate relative abundance tables of bacterial taxa from phylum to species. The relative abundance of bacterial taxa at the phylum, class, genus, and species levels was separately plotted to provide a clear visualization of the microbial composition in the mosquito gut. These plots highlight the distribution and variation of microbial communities across different taxonomic levels.

Taxonomic Visualization Using Krona

The output from Kraken2 or MetaPhlAn3 is converted into input compatible with KronaTools. Krona generates interactive, multi-layered radial plots that display the taxonomic composition of microbial communities in a hierarchical manner, enabling clear visualization of dominant and rare taxa.

RESULTS

Isolation and Identification of Bacterial Inhabitants

The cultured bacteria population exhibited extensive morphological characteristic features as shown in Figure 3.1, indicating the vast diversity of microbial consortia within the *Aedes aegypti* fourth instar larvae.

Among the isolated microbial strain each microbial species such as *Enterobacter roggenkampii*, *Bacillus cereus*, *Bacillus pacificus*, *Enterobacter hormaechei*, and *Stutzerimonas stutzeri* possessed their own unique characteristics. In addition to this, the following species are known to contribute towards morphological diversity; *Bacillusillus subtilis*, *Priestia aryabhattai*, *Aeromonas enteropelogenes*, *Enterobacter cloacae*, *Rahnella aquatilis*, *Rahnella aceris*, *Bacillus wiedmannii*, and *Stutzerimonas kunmingensis*, *Klebsiella quasivariicola* and *Mycolicibacterium vaccae*. The presence of these significant microbes highlights the extensive diversity of microbial population within the *Aedes aegypti* fourth instar larvae. The diverse biochemical and morphological characteristic features of the isolated microbes observed in the current study has been given in Figure3.1.

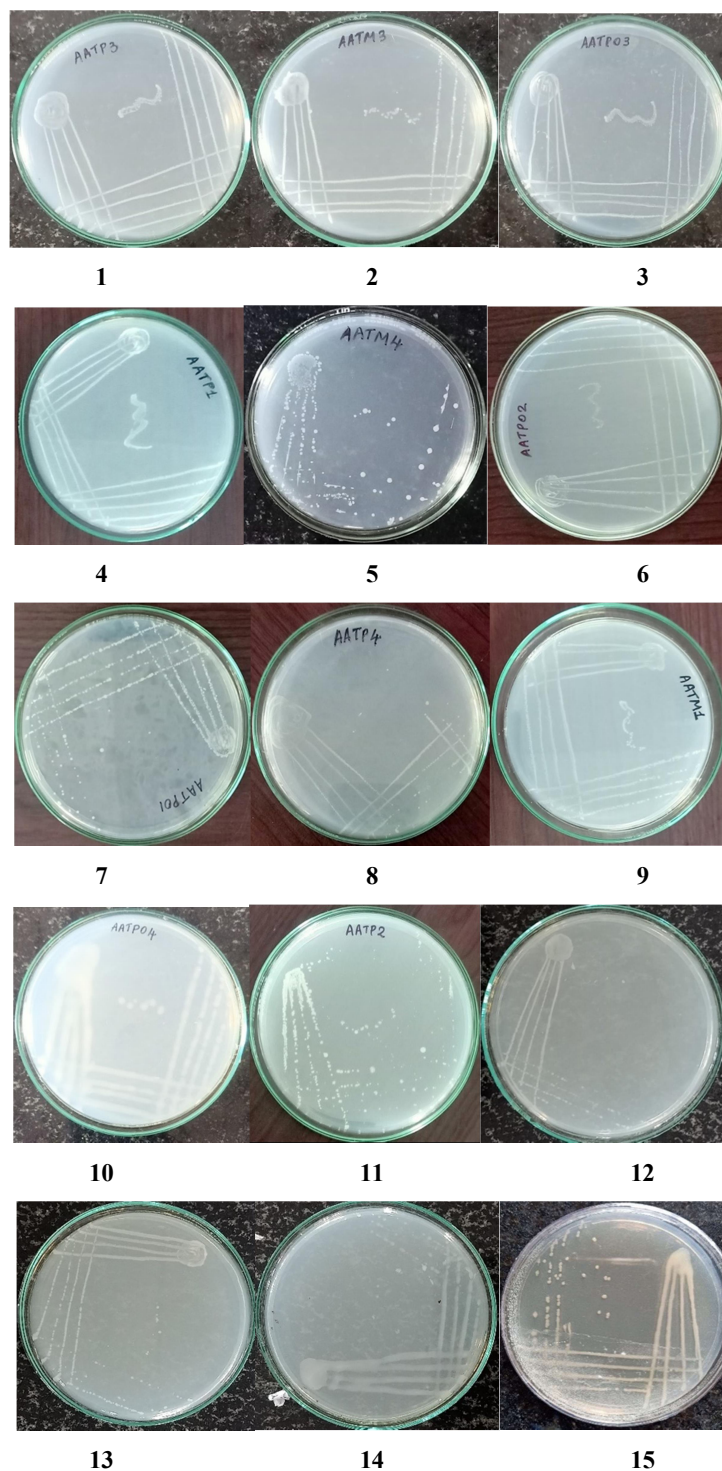


Figure 3.1 Bacteria isolated during different seasons. 1.AATP3, 2. AATM3, 3. AATPO3, 4. AATP1, 5. AATM4, 6. AATPO2, 7. AATPO1, 8. AATP4, 9. AATM1, 10. AATPO4, 11. AATP2, 12. AATP5, 13. AATP6, 14. AATM5, 15. AATM6

The fourth instar larvae of *Aedes aegypti* collected from the Kole wetlands has been witnessed to consists the 15 following bacteria; *Enterobacter roggenkampii*, *Bacillus pacificus*, *Enterobacter hormaechei*, *Bacillus cereus*, *Stutzerimonas stutzeri*, *Bacillus subtilis*, *Priestia aryabhattai*, *Aeromonas enteropelogenes*, *Enterobacter cloacae*, *Rahnella aquatilis*, *Rahnella aceris*, *Bacillus wiedmannii*, *Stutzerimonas kunmingensis*, *Klebsiella quasivariicola* and *Mycolicibacterium vaccae*. After the initial screening with culture-dependent strategies, here we implemented the molecular strategies including 16SrRNA sequencing for the identification of microbial population and the results unveiled that the microbes are fall under the following genera; *Aeromonas*, *Mycolicibacterium*, *Enterobacter*, *Rahnella*, *Bacillus*, *Klebsiella*, *Priestia* and *Stutzerimonas*.

Characterization of microbial consortia through molecular strategies

The molecular characterization using Maximum Likelihood (ML) method with the Tamura-Nei model permits to verify the phylogenetic relationships of the isolated microbial consortia inhabited in the *Aedes aegypti* larvae. The phylogenetic tree constructed for each microbe possessed high similarity towards the respective sequences retrieved from NCBI.

The phylogenetic tree for the *Bacillus* genus with 28 nucleotide sequences revealed maximum log likelihood (-6384.86). The generated phylogenetic tree for the *Bacillus* genus showed strong bootstrap support. The phylogenetic tree for the *Enterobacter* genus constituting 33 nucleotide sequences followed by 809 positions unveiled a log likelihood value of -12882.34. Both of the aforesaid trees produced significant genetic diversity within the studied genus.

The phylogenetic analysis of *Rahnella* genus with 47 nucleotide sequences followed by 804 positions produced a log likelihood of -5082.62. The diverse branching patterns indicated the evolutionary history of various microbes fall under the genus of *Rahnella*. Similar to this observation, the *Stutzerimonas* genus showed distinct genetic diversity with had a log likelihood of -4950.08. The phylogenetic analysis of *Stutzerimonas* genus consisted 635 positions and 17 nucleotide sequences.

The phylogenetic analysis of bacteria falls under the genus of *Aeromonas* with 18 nucleotide sequences followed by 769 positions produced a log likelihood of -4905.65. A found in earlier observations, here also found significant genetic diversity within the *Aeromonas* genus. The phylogenetic tree for the microbial consortia belonging to the genus *Klebsiella* depicted strong link towards the microbes under the same genus with a log likelihood of --2385.51.

Altogether, the phylogenetic analysis of the respective isolated strains in this study verified the taxonomic profiles of each strains with strong nucleotide similarities towards the known species reported across the globe. The phylogenetic tree of each microbes offers valuable insights concerning the genetic diversity and evolutionary link between the microbial inhabitants that supports the characterization and identification of each isolates.

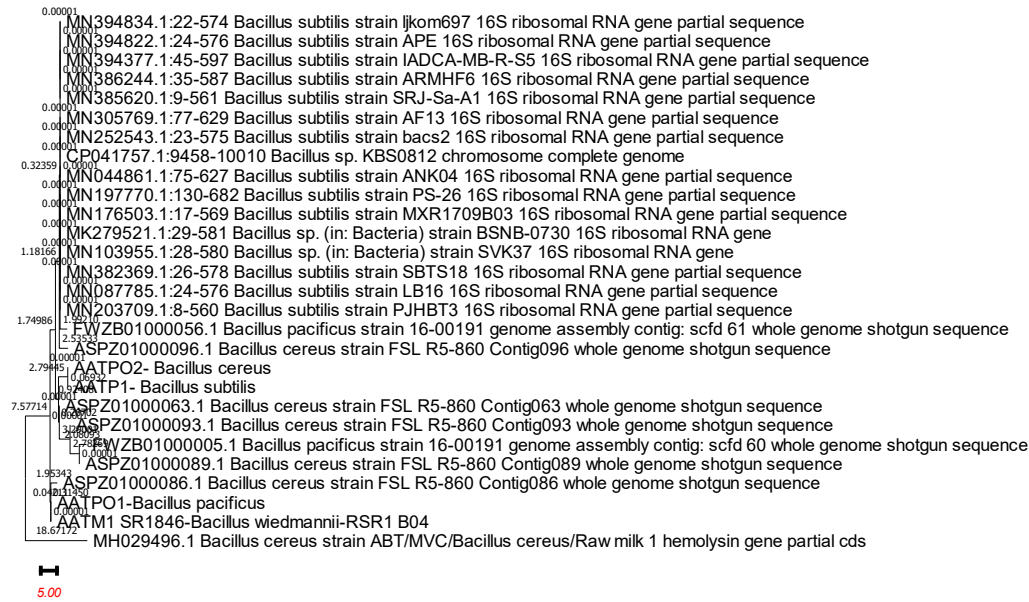


Figure 3.2 Evolutionary analysis of bacteria belongs to *Bacillus* genera by Maximum Likelihood method

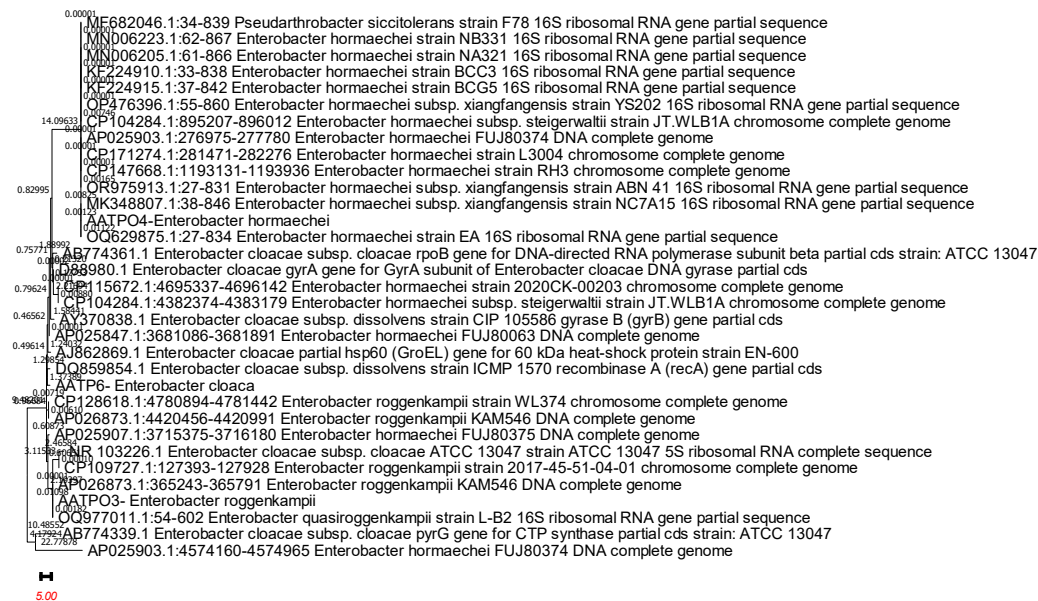


Figure 3.3 Evolutionary analysis of bacteria belongs to *Enterobacter* genera by Maximum Likelihood method

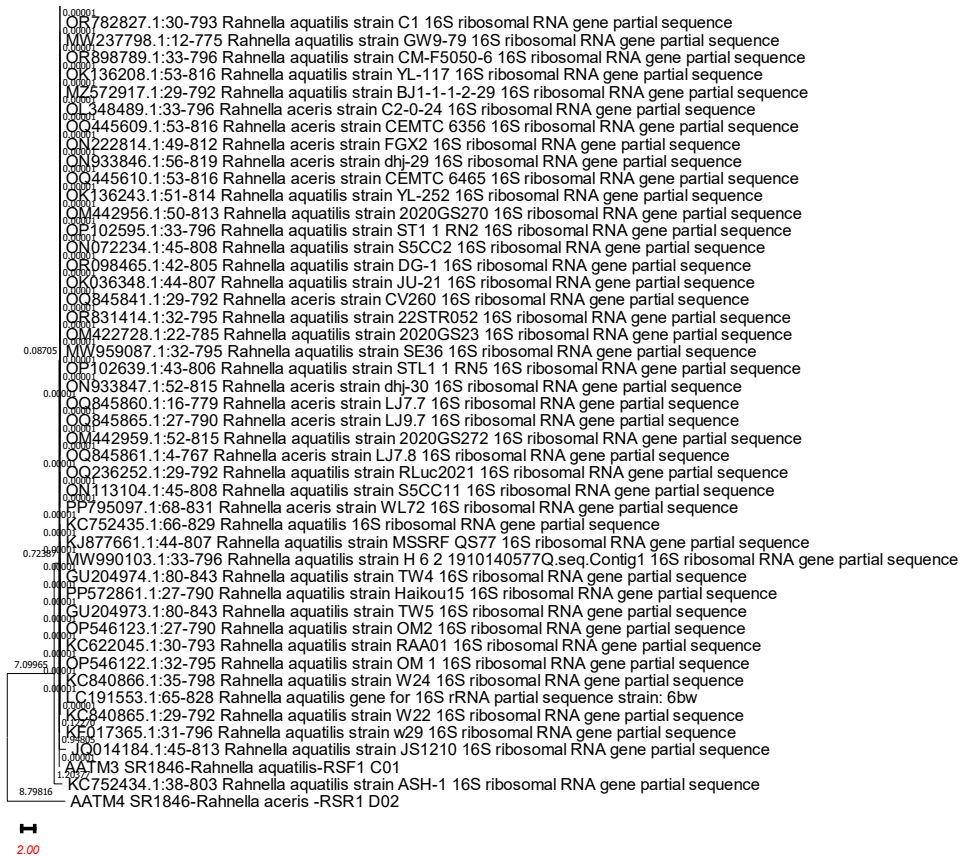


Figure 3.4 Evolutionary analysis of bacteria belongs to *Rahnella* genera by Maximum Likelihood method

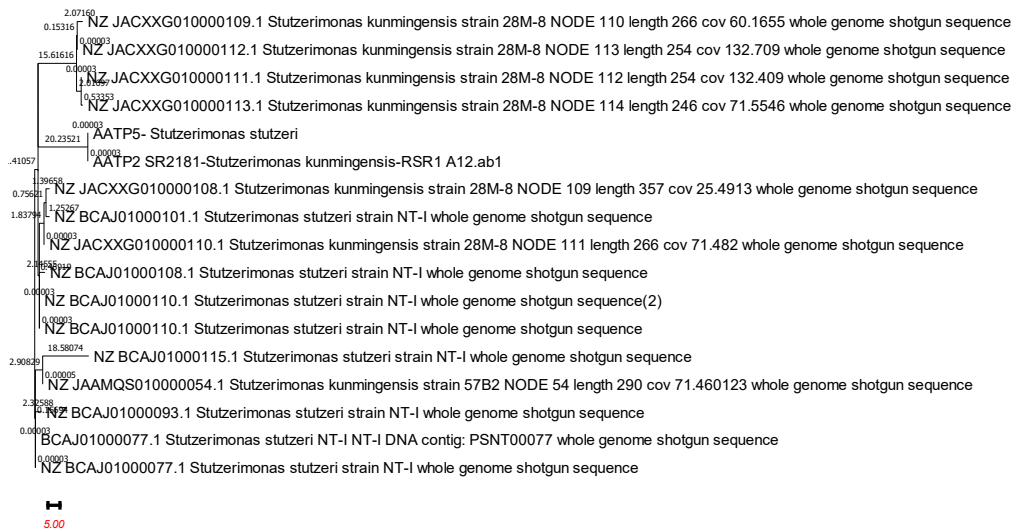


Figure 3.5 Evolutionary analysis of bacteria belongs to *Stutzerimonas* genera by Maximum Likelihood method

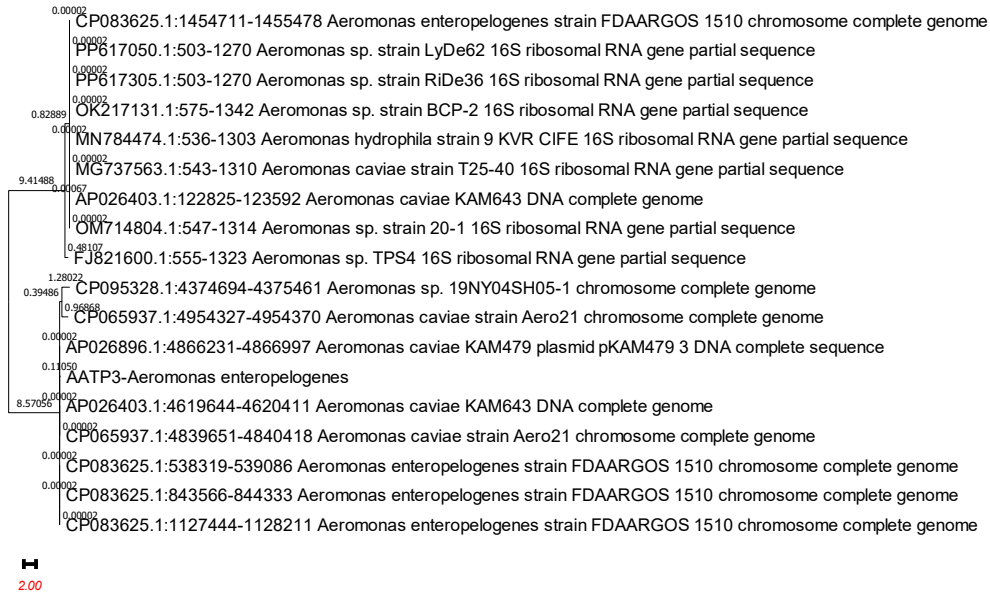


Figure 3.6 Evolutionary analysis of bacteria belongs to *Aeromonas* genera by Maximum Likelihood method

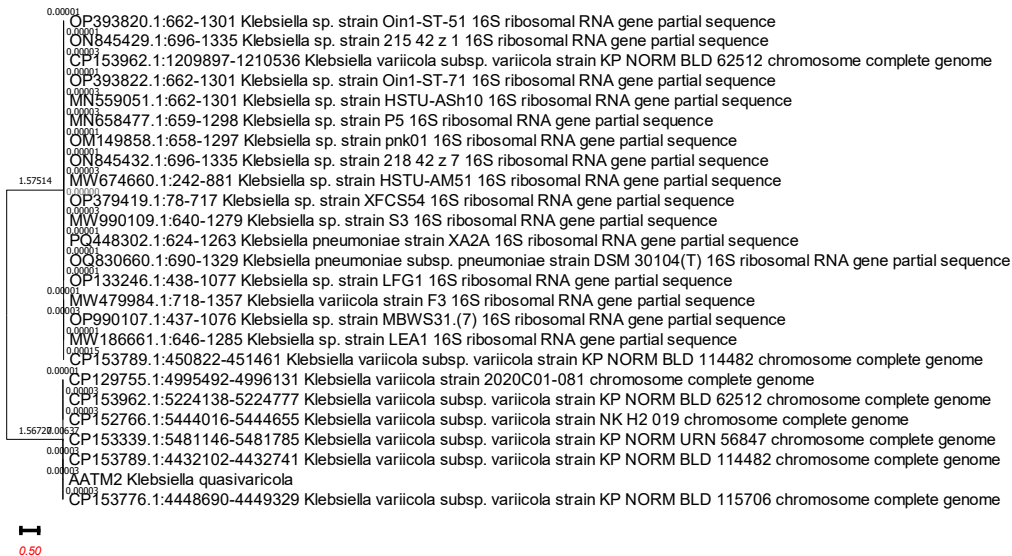


Figure 3.7 Evolutionary analysis of bacteria belongs to *Klebsiella* genera by Maximum Likelihood method

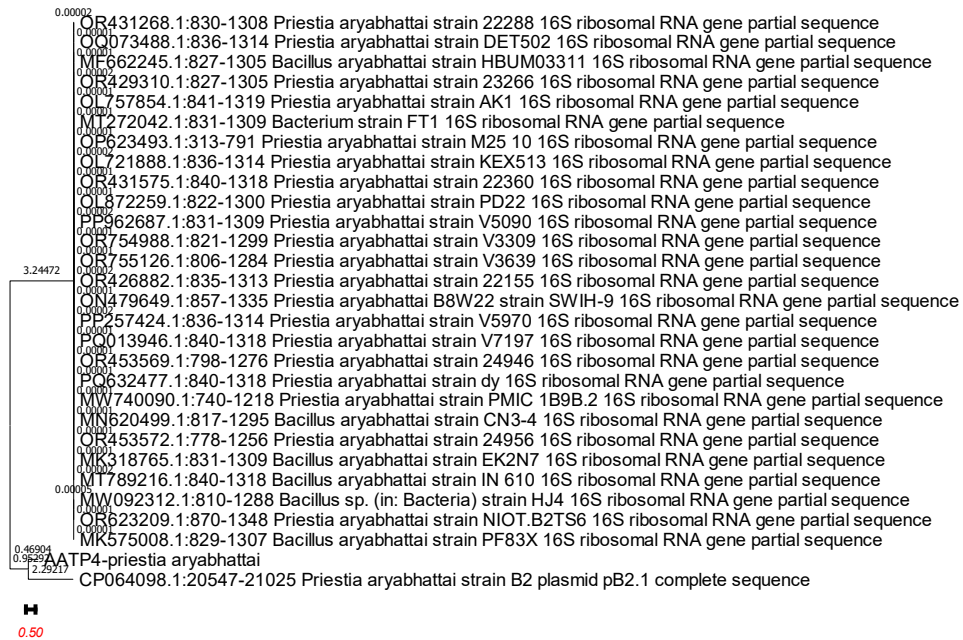


Figure 3.8 Evolutionary analysis of bacteria belongs to *Priestia* genera by Maximum Likelihood method

Nucleotide sequences of respective isolates

1. > AATP3- *Enterobacter roggenkampii*

GCTACTTTGCCGGCGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCC
TGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCG
CAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAGA
TGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAG
CTGGTCTGAGAGGATGACCAGCCACACTGGAAGTGAAGACACGGTCCAGACT
CCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATG
CACCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAG
CGGGGAGGAAGGTGCTGAGGTTAATAACCTCAGCGATTGACGTTACCCGCA
AAAAAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGT
GCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTC
AAGTCAGATGTGAGATCCCCGGGCTCAACCTGGAAACTGC

2. > AATM3-*Bacillus pacificus*

ATCCGCGATTACTAGCGATTCCAGCTTCATGTAGGCGAGTTGCAGCCTACAA
TCCGAAGTGAAGAACGGTTTTATGAGATTAGCTCCACCTCGCGGTCTTGCAGC
TCTTTGTACCGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATG
ATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTACCGGCAGTCACCTT
AGAGTGCCCAACTGAATGATGGCAACTAAGATCAAGGGTTGCGCTCGTTGC
GGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCAC
CTGTCACTCTGCTCCCGAAGGAGAAGCCCTATCTCTAGGGTTGTCAGAGGAT
GTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCC
ACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGCCTTGCGGCCGT
ACTCCCCAGGCGGAGTGCTTAATGCGTTAACTTCAGCACTAAAGGGCGGAA

ACCCTCTAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCT
AATCCTGTTTGCTCCCCACGCTTTCGCGCCTCAGTGTCAGTTACAGACCAGA
AAGTCGCCTTCGCCACTGGTGTTCCTCCATATCTCTACGCATTTACCGCTA
CACATGGAATTCCACTTTCCTCTTCTGCACTCAAGTCTCCCCAGTTTTCCAAT
GACCCTCCACGGTTGAGCCGTGGGG

3. > AATPO3-*Enterobacter hormaechei*

CTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGG
GGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAA
AGAGGGGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAG
CTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGA
GAGGATGACCAGCCACACTGGAAGTGAAGACACGGTCCAGACTCCTACGGGA
GGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGC
CGCGTTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAA
GGCGTTAAGGTTAATAACCTTGTCGATTGACGTTACCCGCAGAAGAAGCAC
CGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAA
TCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGT
GAAATCCCCGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGT
CTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATC
TGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTC
AGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAC
GCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGG
GAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGGCGCGCA

4. > AATPO1- *Bacillus cereus*

AGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACTGCCATAAGACTG
GGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCAT
GGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGT
CGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGC
CGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTC
CTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGG
AGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTT
AGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAAC
CAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGG
CAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTA
AGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAACTGG
GAGACTTGAGTGCAGAAGAGGAAAGTGGAAATTCATGTGTAGCGGTGAAAT
GCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTA
ACTGACACTGAGGCGCGAAAGCGTGGGAGCAAACAGGATTAGATACCCTG
GTAGTCCACGCCGTAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTT
AGTGCTGAAGTTAACGCATAAGCACTCCGCCTGGGGAGTACGGC

5. > AATM4- *Stutzerimonas stutzeri*

ACTAGCGATTCCGACTTCACGCAGTCGAGTTGCAGACTGCGATCCGGACTA
CGATCGGTTTTATGGGATTAGCTCCACCTCGCGGCTTGGCAACCCTTTGTAC
CGACCATTGTAGCACGTGTGTAGCCCAGGCCGTAAGGGCCATGATGACTTG
ACGTCATCCCCACCTTCCTCCGGTTTGTACCGGCAGTCTCCTTAGAGTGCC
CACCTTAACGTGCTGGTAACTAAGGACAAGGGTTGCGCTCGTTACGGGACT
TAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCACCTGTGT

CAGAGTTCCCGAAGGCACCAATCCATCTCTGGAAAGTTCTCTGCATGTCAA
GGCCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGC
TTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCC
CCAGGCGGTCGACTTAATGCGTTAGCTGCGCCACTAAGATCTCAAGGATCC
CAACGGCTAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCT
GTTTGCTCCCCACGCTTTCGCACCTCAGTGTGAGTATTAACCCAGGTGGTCG
CCTTCGCCACTGGTGTT

6. > AATP02- *Bacillus subtilis*

TGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACTGCCTGTAAGACTG
GGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCAT
GGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGG
CGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGC
CGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTC
CTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGG
AGCACGCCGCGTGAGTGATGAAGGTTTTTCGGATCGTAAAGCTCTGTTGTTA
GGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTGACGGTACCTAACC
AGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACTAGGTGGCA
AGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAG
TCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGA
ACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCG

7. > AATP01-*Priestia aryabhatai*

TCCGCGATTACTAGCGATTCCAGGCTTCATGTAGGCGAGTTGCAGCCTACAA
TCCGAACTGAGAATGGTTTTATGGGATTGGCTTGACCTCGCGGTCTTGCAGC

CCTTTGTACCATCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATG
 ATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTACCGGCAGTCACCTT
 AGAGTGCCCAACTAAATGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGC
 GGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCAC
 CTGTCACTCTGTCCCCCGAAGGGGAACGCTCTATCTCTAGAGTTGTCAGAGG
 ATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCT
 CCACCGCTTGTGCGGGCCCCCGTCAATTCCCTTTGAGTTTCAGTCTTGCGACC
 GTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAAGGGCGG
 AAACCCTCTAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTAT
 CTAATCCTGTTTGCTCCCCACGCTTTCGCGCCTCAACGTCAGTTACAGACCA
 AAAAAGCCGCCTTCGCCACTGGTGTTCTCCACATCTCTACGCATTTACCGG
 CTACACGTGGAATTCCGCTTTTCTCTTCTGCACTCAAGTTCCCCAGTTTCCAT
 GACCCTCCACGGTTGAGCCGTGGGGCTTTCC

8. > AATP4-*Aeromonas enteropelogenes*

TCTGATTTGCGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACT
 CCAATCCGGACTACGACGCGCTTTTTGGGATTCGCTCACTATCGCTAGCTTG
 CAGCCCTCTGTACGCGCCATTGTAGCACGTGTGTAGCCCTGGCCGTAAGGG
 CCATGATGACTTGACGTCATCCCCACCTTCCTCCGGTTTATCACCGGCAGTC
 TCCCTTGAGTTCCCACCATTACGTGCTGGCAACAAAGGACAGGGGTTGCGC
 TCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCAT
 GCAGCACCTGTGTTCTGATTCCCGAAGGCACTCCCGCATCTCTGCAGGATTC
 CAGACATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCA
 CATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTT
 GCGGCCGTACTCCCCAGGCGGTTCGATTTAACGCGTTAGCTCCGGAAGCCAC

GTCTCAAGGACAAGCCTCCAAATCGACATCGTTTACGGCGTGGACTACCAG
GGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAGTCTTTG
TCCAGGGGGCCGCCTTTCGCCACCGGTATTCTCCAGATCTCTACGCATTTTC
ACCGCTACACCTGGAATTCTACCCCCCTCTACAAGACTCTAGCTGGACAGTT
TTAAATGCAATTCCACGGTTGAGCCCGGGGCTTTCACATCTAACT

9. > AATM1- *Enterobacter cloaca*

CTACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAATC
CGGACTACGACATACTTTATGAGGTCCGCTTGCTCTCGCGAGGTCGCTTCTC
TTTGTATATGCCATTGTAGCACGTGTGTAGCCCTGGTCGTAAGGGCCATGAT
GACTTGACGTCATCCCCACCTTCCTCCAGTTTATCACTGGCAGTCTCCTTTGA
GTTCCCGGCCTAACCGCTGGCAACAAAGGATAAAGGGTTGCGCTCGTTGCGG
GACTTAACCCAACATTTTACAACACGAGCTGACGACAGCCATGCAGCACCT
GTCTCACAGTTCCCGAAGGCACCAATCCATCTCTGGAAAGTTCTGTGGATGT
CAAGACCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCA
CCGCTTGTGCGGGCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTA
CTCCCCAGGCGGTTCGATTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGG
GCACAACCTCCAAATCGACATCGTTTACAGCGTGGACTACCAGGGTATCTA
ATCCTGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAGTCTTTGTCCAGGG
GGCCGCCTTCGCCACCGGTATTCTCCAGATCTCTACGCATTTACCGCTAC
ACCTGGAATTCTACCCCCCTCTACAAGACTCTAGCCTGCCAGTTTCGAATG
CAGCTCCCAGGTTGAGCCCGGGGATTTACATCCGACTTGAAAGACCGCCT
GCGTGCGCTTTACGCCAGTAATTTCCGATTAA

10. > AATPO4 SR1846-*Rahnella aquatilis*

ACTTTGCCGGCGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGA
TGGAGGGGGATAACTACTGGAAACGGTGGCTAATACCGCATAACGTCGCAA
GACCAAAGTGGGGGACCTTCGGGCCTCACGCCATCGGATGTGCCCAGATGG
GATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTG
GTCTGAGAGGATGACCAGCCACACTGGAAGTGAAGACACGGTCCAGACTCCT
ACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCA
GCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCG
AGGAGGAAGGCATCATACTTAATACGTGTGGTGATTGACGTTACTCGCAGA
AGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCA
AGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAG
TCAGATGTGAAATCCCCGCGCTTAACGTGGGAACTGCATTTGAAACTGGCA
AGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGC
GTAGAGATCTGGAGGGAATACCGGTGGCGAAGGCGGGCCCCCTGGACAAA
GACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGGATTAGATACC
CTGGTAGTCCACGCTGTAAACGATGTTCGACTTTGGAGTTGTGCCCTTGAGGC
GTGG

11. > AATP2 *Rahnella aceris*

TCTACGATTACTAGCGATTCCGAACTTCATGGAGTCGAGTTGCAGACTCCAA
TCCGGACTACGACGTACTTTATGAGGTCCGCTGGCTCTCGCGAGTTTGCTTC
TCTTTGTATACGCCATTGTAGCACGTGTGTAGCCCTACTCGTAAGGGCCATG
ATGACTTGACGTCATCCCCACCTTCCTCCGGTTTATCACCGGCAGTCTCCTTT
GAGTTCCCACCCGTAGTGCTGGCAACAAAGGATAAGGGTTGCGCTCGTTGC
GGGACTTAACCCAACATTTACACAACACGAGCTGACGACAGCCATGCAGCAC

CTGTCTCACGGTTCCCGAAGGCACCAAGCCATCTCTGGCAAGTTCCGTGGAT
GTCAAGAGTAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCC
ACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGT
ACTCCCCAGGCGGTTCGACTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAG
GGCACAACCTCCAAGTCGACATCGTTTACAGCGTGGACTACCAGGGTATCT
AATCCTGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAGTCTTTGTCCAGG
GGGCCGCCTTCGCCACCGGTATTCTCCAGATCTCTACGCATTTACCGCTA
CACCTGGAATTCTACCCCCCTCTACAGACTCTAGCTTGCCAGTTTCAAATGC
AGTTCCACGTTAAGCGCGGGGATTTACATCTGACTTAACAAACCCGCCTG
CGTGCGCTTTACGCCCAGTAATTTCCG

12. > AATP5-*Bacillus wiedmannii*

ATCCGCGATTACTAGCGATTCCAGCTTCATGTAGGCGAGTTGCAGCCTACAA
TCCGAAGTGAAGAACGGTTTTATGAGATTAGCTCCACCTCGCGGTCTTGCAGC
TCTTTGTACCGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATG
ATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTACCGGCAGTCACCTT
AGAGTGCCCAACTTAATGATGGCAACTAAGATCAAGGGTTGCGCTCGTTGC
GGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCAC
CTGTCACTCTGCTCCCGAAGGAGAAGCCCTATCTCTAGGGTTTTTCAGAGGAT
GTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCC
ACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGCCTTGCGGCCGT
ACTCCCCAGGCGGAGTGCTTAATGCGTTAACTTCAGCACTAAAGGGCGGAA
ACCCTCTAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCT
AATCCTGTTTGCTCCCCACGCTTTCGCGCCTCAGTGTGAGTTACAGACCAGA
AAGTCGCCTTCGCCACTGGTGTTCTCCATATCTCTACGCATTTACCGCTA

CACATGGAATTCCACTTTCCTCTTCTGCACTCAAGTCTCCCAGTTTCCAATG
ACCCTCCACGGTTGAGCCGTGGGCTTTCACATCAGACTTAAGAAACCACT
GCGCGCGCTTTACGCCCAATAAATTC

13. > AATP6-*Stutzerimonas kunmingensis*

ACTAGCGATTCCGACTTCACGCAGTCGAGTTGCAGACTGCGATCCGGACTA
CGATCGGTTTTATGGGATTAGCTCCACCTCGCGGCTTGGCAACCCTTTGTAC
CGACCATTTGTAGCACGTGTGTAGCCCAGGCCGTAAGGGCCATGATGACTTG
ACGTCATCCCCACCTTCCTCCGGTTTGTACCGGCAGTCTCCTTAGAGTGCC
CACCTTAACGTGCTGGTAACTAAGGACAAGGGTTGCGCTCGTTACGGGACT
TAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCACCTGTGT
CAGAGTTCCCGAAGGCACCAATCCATCTCTGGAAAGTTCTCTGCATGTCAA
GGCCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGC
TTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCC
CCAGGCGGTTCGACTTAATGCGTTAGCTGCGCCACTAAGATCTCAAGGATCC
CAACGGCTAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCT
GTTTGCTCCCCACGCTTTCGCACCTCAGTGTCAGTATTAACCCAGGGGTCGC
CTTCGCCACTGGTGTT

14. > AATM5-*Klebsiella quasivariicola*

CTACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAATC
CGGACTACGACATACTTTATGAGGTCCGCTTGCTCTCGCGAGGTCGCTTCTC
TTTGTATATGCCATTGTAGCACGTGTGTAGCCCTGGTCGTAAGGGCCATGAT
GACTTGACGTCATCCCCACCTTCCTCCAGTTTATCACTGGCAGTCTCCTTTGA
GTTCCCGGCCTAACCGCTGGCAACAAAGGATAAGGGTTGCGCTCGTTGCGG

GACTTAACCCAACATTTACACAACACGAGCTGACGACAGCCATGCAGCACCT
GTCTCACAGTTCCCGAAGGCACCAATCCATCTCTGGAAAGTTCTGTGGATGT
CAAGACCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCA
CCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTA
CTCCCCAGGCGGTTCGATTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGG
GCACAACCTCCAAATCGACATCGTTTACAGCGTGGACTACCAGGGTATCTA
ATCCTGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAGTCTTTGTCCAGGG
GGCCGCCTTCGCCACCGGTATTCTCCAGATCTCTACGCATTTACCGCTAC
ACCTGGAATTCTACCCCCCTCTACAAGACTCTAGCCTGCCAGTTTCGAATG
CAGCTCCCAGGTTGAGCCCGGGGATTTACATCCGACTTGAAAGACCGCCT
GCGTGCGCTTTACGCCCAGTAATTTCCGATTAA

15. > AATM6-*Mycolicibacterium vaccae*

ACTCGAGTGGCGAACGGGTGAGTAACACGTGGGTGATCTGCCCTGCACTTT
GGGATAAGCCTGGGAAACTGGGTCTAATACCGAATACACCTTGCTGGATGC
ATGTCTGGTGGGGGAAAGCTTTTGCGGTGTGGGATGGGCCCCGCGGCCTATC
AGCTTGTTGGTGAGGTTACGGCTCACCAAGGCGACGACGGGTAGCCGGCCT
GAGAGGGTGTCCGGCCACACTGGGACTGAGATACGGCCCAGACTCCTACGG
GAGGAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGAC
GCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCGCCAGGGAC
GAAGCGCAAGTGACGGTACCTGGAGAAGAAGCACCGGCCAACTACGTGCC
AGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTACTGGGCG
TAAAGAGCTCGTAGGTGGTTTGTCGCGTTGTTCGTGAAAACCTCACAGCTTAA
CTGTGGGCGTGCGGGCGATACGGGCAGACTGGAGTACTGCAGGGGCGACTG
GAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGGTG

GCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAAGCGTGG
GGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGTA
CTAGGTGTGGGTTTCCTTCCTTGGGATCCGTGCCGTAGCTAACGCATTAAGT
ACCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGAAATTGACG
GGGGCCCGCACAAGCGGCGGAGCATGTGGATTAATTCGATGCAACGCGAA
GAACCTTACCTGGGTTTGACATGCACAGGACGCTGGTAGAGATATCAGTTC
CCTTGTGGCCTGTGTGGGGGTGGTGCATGGCTGTCGTCAGCTCGTGTCTAG
GATGTTGGGTAAAGTCCCGCAACGAGCAGTTCCCTTGTGGCCTGTGTGGGG
GTGGTGCATGGCTGTCGTCAGCTCGTGTCTAGGATGTTGGGTAAAGTCCCG
CAACGAG

Species composition revealed through metagenomic analysis

The Metagenomics analysis of the fourth instar larvae of *Aedes aegypti* revealed the presence of a plethora of bacterial strains (Figure 3.9 – 3.17; Table 3.3 -3.5) across the seasons. The number of reads of the analysed strains such as AAMA, AAMB and AAMC was detected to be 292254, 202272 and 238910 respectively; while the Q20 values of AAMA, AAMB and AAMC were reported as 99.73, 99.72, and 99.78. The higher values of Q20, specifically more than 90% represent that the gene assembly had maximum integrity along with good quality.

The season-wise distribution of the reported phylum was given in Figure 3.9. The AAMA, AAMB and AAMC strains designated in Figure 3.9 correspond to the various seasons such as monsoon, pre-monsoon, and post-monsoon respectively. According to the monsoon season and post-monsoon, the Firmicutes was found to be the majority while the pre-monsoon season detected the presence of Proteobacteria in greater

intensity. As given in Figure 3, the species abundance distribution accounted the presence of several species.

Diversity Analysis

The composition pattern of all the selected strains of microbial consortia such as AAMA, AAMB and AAMC was analysed using the Alpha diversity matrices (Table 3.1). The Shannon diversity analysis indicates that the AAMB strain ($H' = 3.11813661$) was the most abundant than the AAMA ($H' = 2.58097801$) and AAMC ($H' = 2.29624848$). It may assign that the climate change and seasonal factors are may be responsible for this variation in population diversity.

Table 3.1 Composition profile

Sample	Observed	Chao1	se.chao1	ACE	se.ACE	Shannon	Simpson	InvSimpson	Fisher
AAMA	671	873.843373	39.8991837	838.233284	14.4863412	2.58097801	0.76289486	4.21753821	98.5758471
AAMB	632	831.105263	37.8071371	844.500761	15.4236605	3.11813661	0.88626298	8.7922121	95.2304462
AAMC	603	772.166667	33.7856416	766.229948	13.8074396	2.29624848	0.71246356	3.47782007	90.3457864

Table 3.2 O for OUT

S.No	Comparison	Significant OTUs (p-value ≤ 0.05)	Significant OTUs (p-value(corrected) ≤ 0.05)
1.	AAMA_vs_AAMB	286	220
2.	AAMA_vs_AAMC	145	63
3.	AAMB_vs_AAMC	257	201

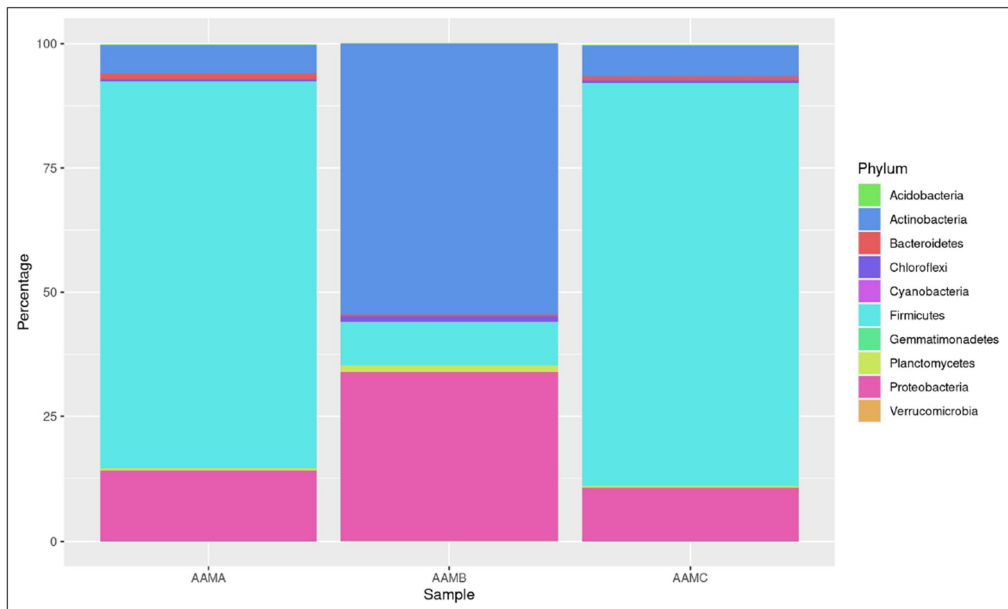


Figure 3.9 Top 10 phyla abundance distribution

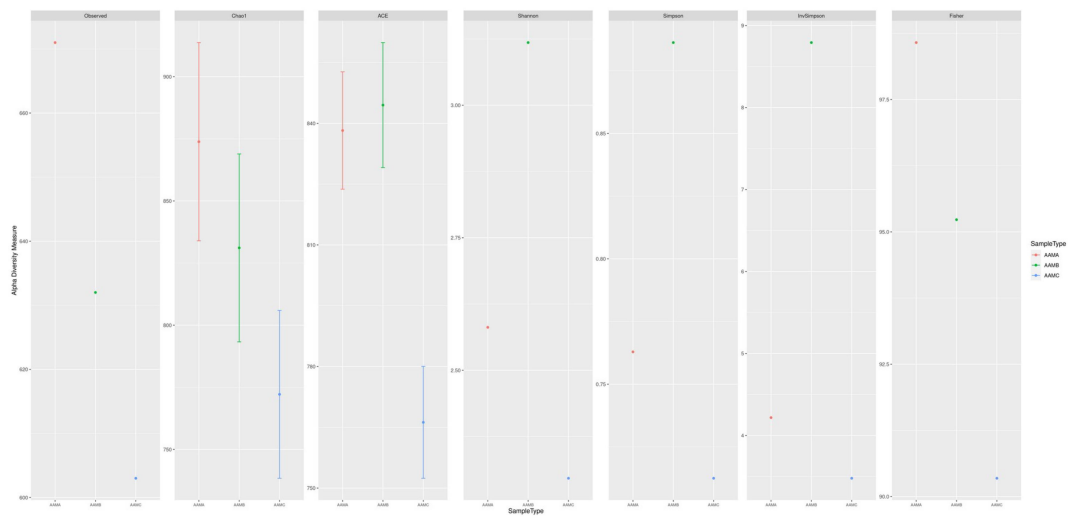


Figure 3.10 Diversity profile

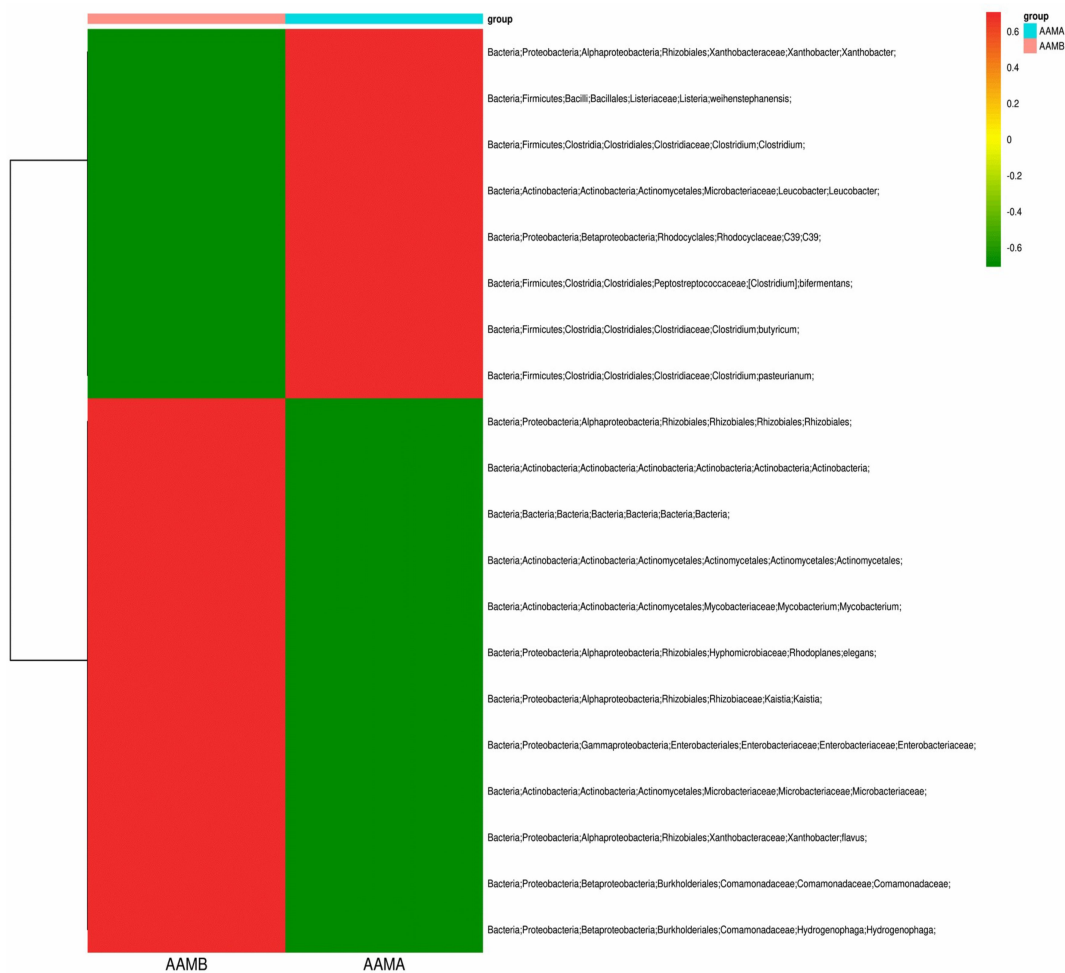


Figure 3.11 Distribution of top 20 significant OTU

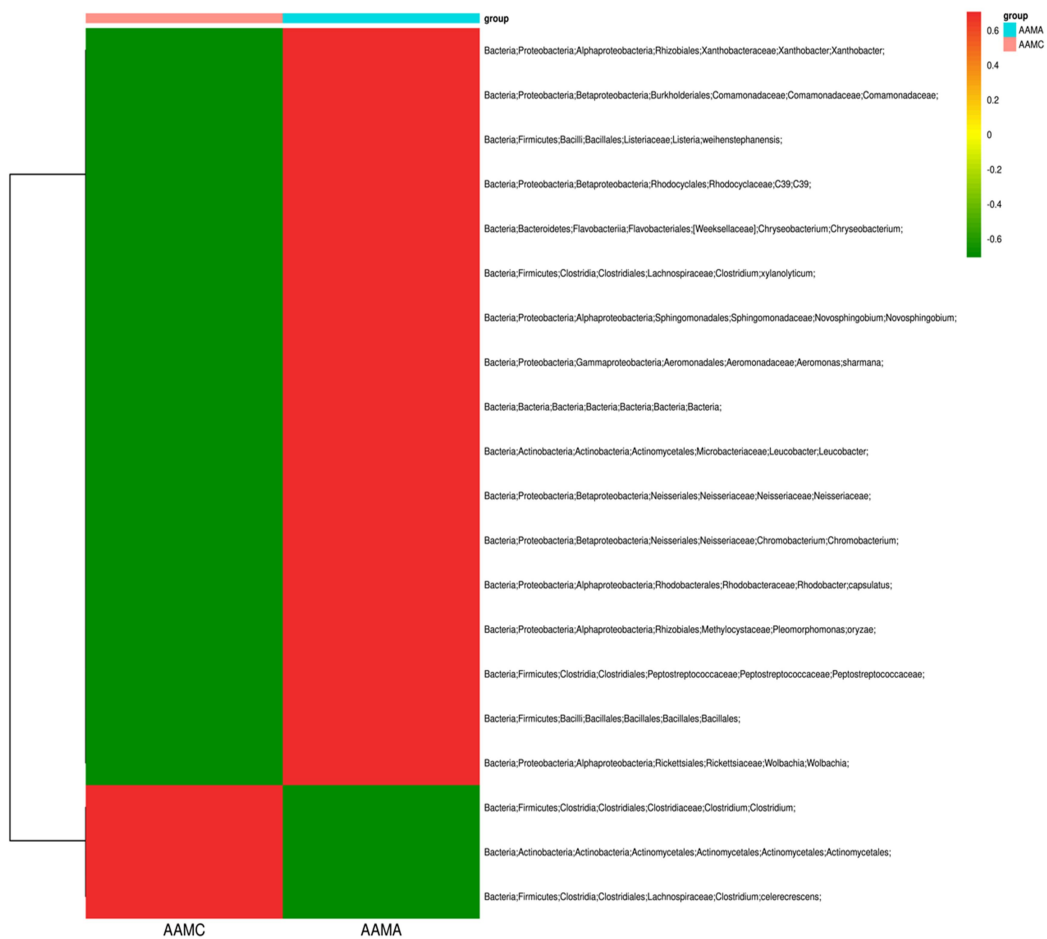


Figure 3.12 Season wise Distribution

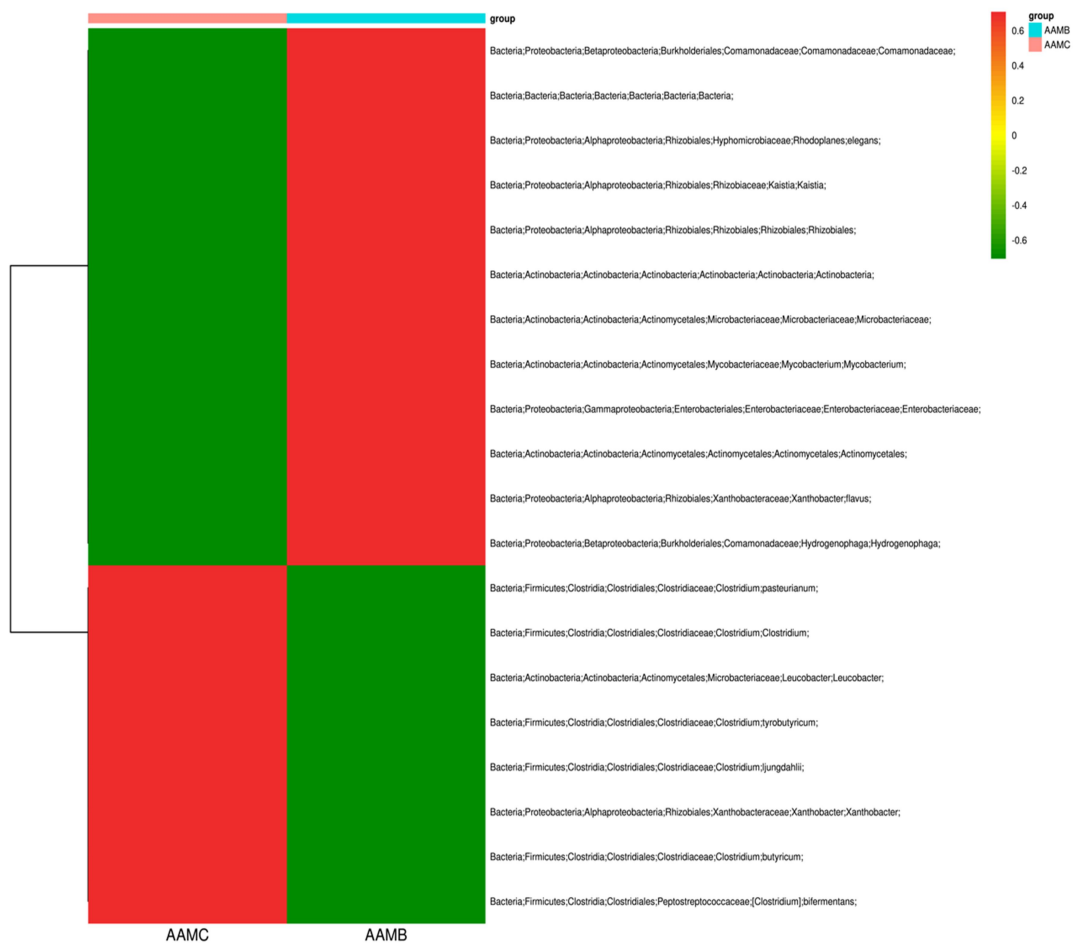


Figure 3.13 Season wise Distribution

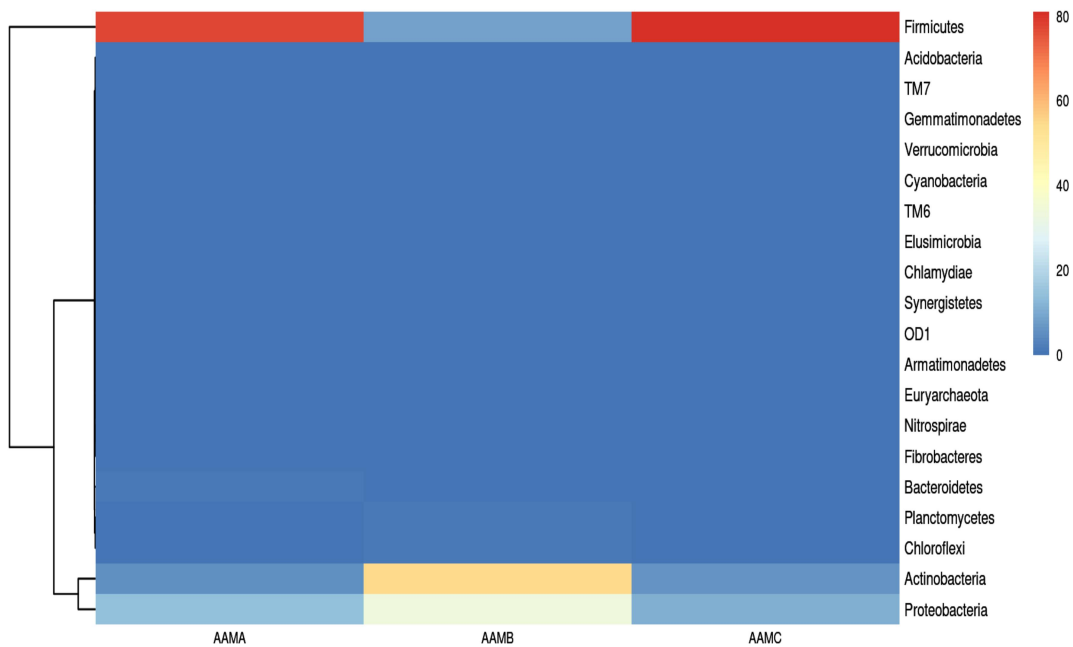


Figure 3.14 20 different phyla

Table 3.3 Microbial population according to Phylum

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Acidobacteria	2	.6	.6	.6
	Actinobacteria	84	24.0	24.0	24.6
	Bacteroidetes	18	5.1	5.1	29.7
	Chloroflexi	1	.3	.3	30.0
	Cyanobacteria	2	.6	.6	30.6
	Euryarchaeota	2	.6	.6	31.1
	Firmicutes	112	32.0	32.0	63.1
	Nitrospirae	1	.3	.3	63.4

Planctomycetes	3	.9	.9	64.3
Proteobacteria	121	34.6	34.6	98.9
Thermotogae	1	.3	.3	99.1
Verrucomicrobia	3	.9	.9	100.0
Total	350	100.0	100.0	

Table 3.4 Microbial population according to Class

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	[Saprospirae]	3	.9	.9	.9
	Acidimicrobiia	1	.3	.3	1.1
	Acidobacteriia	1	.3	.3	1.4
	Actinobacteria	78	22.3	22.3	23.7
	Alphaproteobacteria	66	18.9	18.9	42.6
	Bacilli	44	12.6	12.6	55.1
	Bacteroidia	9	2.6	2.6	57.7
	Betaproteobacteria	18	5.1	5.1	62.9
	Chloroplast	1	.3	.3	63.1
	Clostridia	61	17.4	17.4	80.6
	Coriobacteriia	4	1.1	1.1	81.7
	Deltaproteobacteria	9	2.6	2.6	84.3
	Epsilonproteobacteria	1	.3	.3	84.6
	Erysipelotrichi	7	2.0	2.0	86.6
	Flavobacteriia	3	.9	.9	87.4

Gammaproteobacteria	27	7.7	7.7	95.1
Holophagae	1	.3	.3	95.4
Ktedonobacteria	1	.3	.3	95.7
Methanobacteria	1	.3	.3	96.0
Methanomicrobia	1	.3	.3	96.3
Nitrospira	1	.3	.3	96.6
Oscillatoriothricaceae	1	.3	.3	96.9
Planctomycetia	3	.9	.9	97.7
Sphingobacteriia	3	.9	.9	98.6
Thermoleophilia	1	.3	.3	98.9
Thermotogae	1	.3	.3	99.1
Verrucomicrobiae	3	.9	.9	100.0
Total	350	100.0	100.0	

Table 3.5 Microbial population according to Order

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	[Saprospirales]	3	.9	.9	.9
	Acidimicrobiales	1	.3	.3	1.1
	Acidobacteriales	1	.3	.3	1.4
	Actinomycetales	75	21.4	21.4	22.9
	Aeromonadales	2	.6	.6	23.4
	Alteromonadales	3	.9	.9	24.3
	Bacillales	37	10.6	10.6	34.9

Bacteroidales	9	2.6	2.6	37.4
Bdellovibrionales	2	.6	.6	38.0
Bifidobacteriales	3	.9	.9	38.9
Burkholderiales	13	3.7	3.7	42.6
Campylobacterales	1	.3	.3	42.9
Caulobacterales	3	.9	.9	43.7
Chlorophyta	1	.3	.3	44.0
Chroococcales	1	.3	.3	44.3
Clostridiales	61	17.4	17.4	61.7
Coriobacteriales	4	1.1	1.1	62.9
Desulfobacterales	1	.3	.3	63.1
Enterobacteriales	5	1.4	1.4	64.6
Erysipelotrichales	7	2.0	2.0	66.6
Flavobacteriales	3	.9	.9	67.4
Gaiellales	1	.3	.3	67.7
Gemmatales	3	.9	.9	68.6
Holophagales	1	.3	.3	68.9
Ktedonobacterales	1	.3	.3	69.1
Lactobacillales	7	2.0	2.0	71.1
Legionellales	1	.3	.3	71.4
Methanobacteriales	1	.3	.3	71.7
Methanomicrobiales	1	.3	.3	72.0
Methylophilales	1	.3	.3	72.3
Myxococcales	5	1.4	1.4	73.7

Neisseriales	1	.3	.3	74.0
Nitrosomonadales	2	.6	.6	74.6
Nitrospirales	1	.3	.3	74.9
Pasteurellales	3	.9	.9	75.7
Pseudomonadales	5	1.4	1.4	77.1
Rhizobiales	30	8.6	8.6	85.7
Rhodobacterales	8	2.3	2.3	88.0
Rhodocyclales	1	.3	.3	88.3
Rhodospirillales	14	4.0	4.0	92.3
Sphingobacteriales	3	.9	.9	93.1
Sphingomonadales	11	3.1	3.1	96.3
Syntrophobacterales	1	.3	.3	96.6
Thermotogales	1	.3	.3	96.9
Verrucomicrobiales	3	.9	.9	97.7
Xanthomonadales	8	2.3	2.3	100.0
Total	350	100.0	100.0	

Species composition revealed through metagenomic analysis

Pre-monsoon

Metagenomic analysis revealed a diverse bacterial community dominated by *Stutzerimonas stutzeri* and *Bacillus cereus*, alongside Enterobacter species and the intracellular parasite *Candidatus Protochlamydia naegleriophila*. The profile included clinically relevant species like *Streptococcus sanguinis* and *Actinomyces pacaensis*, as well as environmental bacteria such as *Acinetobacter junii*, reflecting multiple microbial acquisition routes. Metagenomic analysis revealed a diverse microbial

community dominated by the following bacteria; *Stutzerimonas stutzeri*, *Candidatus Protochlamydia naegleriophila* and *Bacillus cereus*. The taxonomic profile of microbial inhabitants revealed the clinically relevant microbes such as *Streptococcus sanguinis* and *Actinomyces pacaensis*. Furthermore, the presence of environmental bacteria such as *Acinetobacter junii* indicating multiple routes of accretion. The complex ecological relation and interactions allied with the bacteria belongs to the *Bacillus* and *Oligella* genus recognized as a significant contribution towards the scientific community since it can deliver the information concerning the role of microbial consortia against the vector biology, vector competence, mosquito physiology and pathogen transmission.

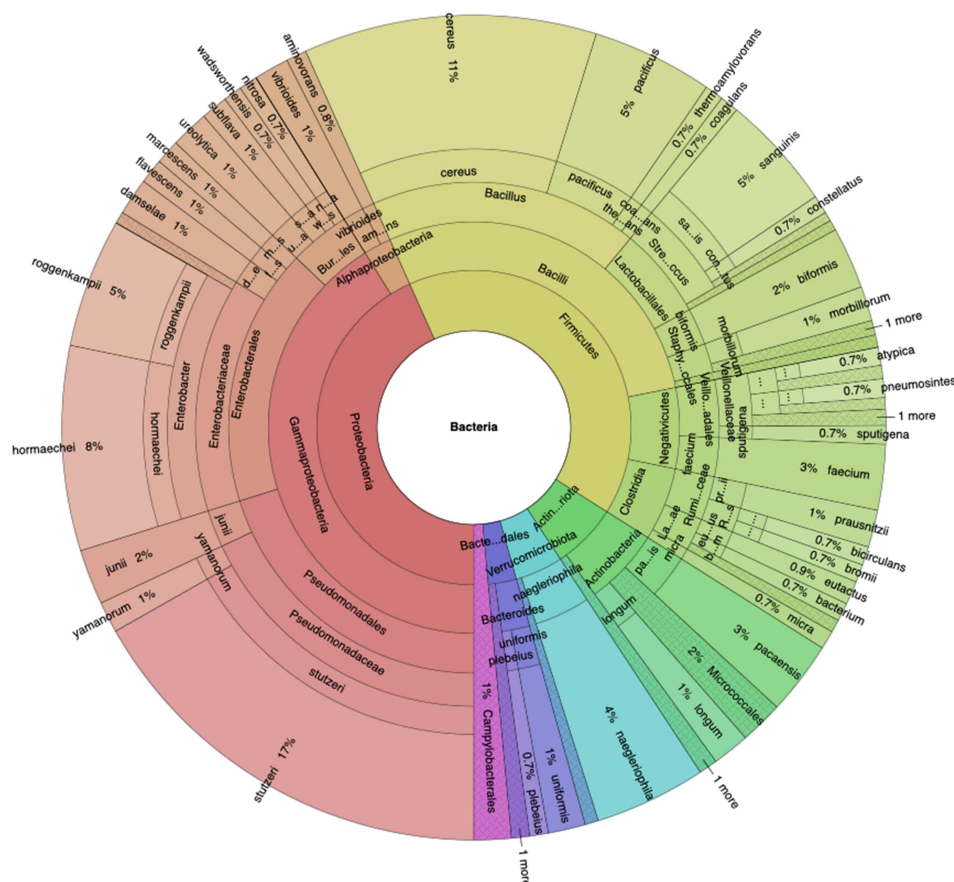


Figure 3.15 Species wise Hierarchical visualization of gut bacterial communities [Pre-monsoon] through metagenomic analysis

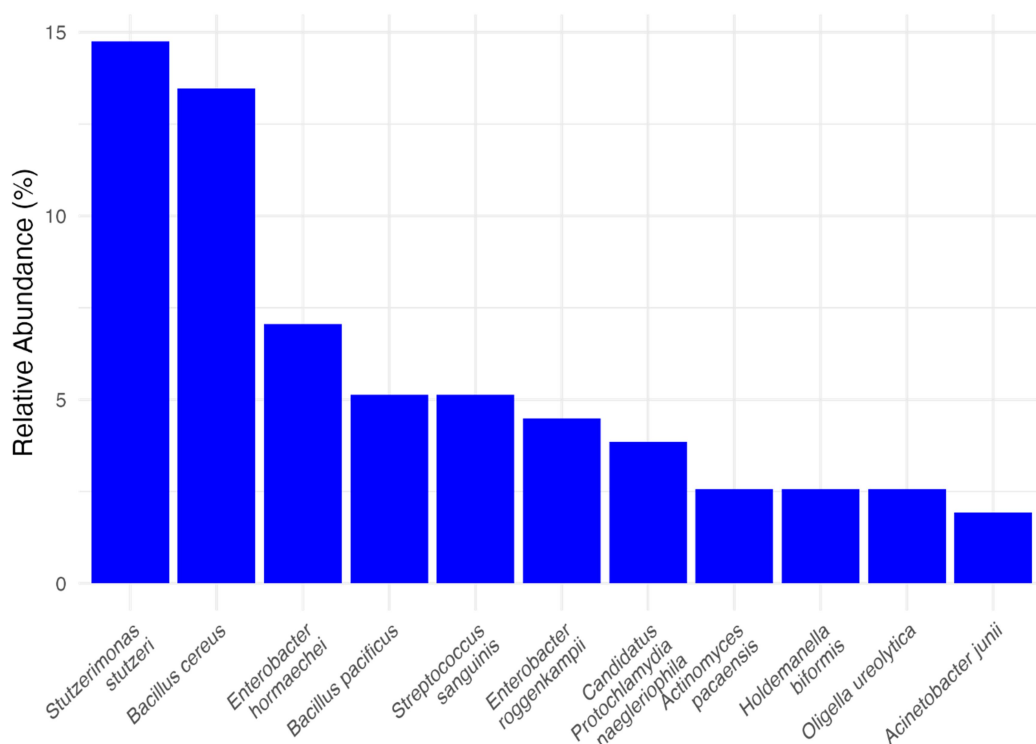


Figure 3.16 Metagenomic Profile of Top 10 Bacterial Species in Pre-Monsoon

Monsoon

The implementation of Metagenomic sequencing strategies in the present study showed distinct profile for microbial consortia according to the species level in Monsoon season. According to the Metagenomic analysis of monsoon season, *Priestia aryabhatai*, *Bacillus subtilis* and *Aeromonas enteropelogenes* was recognized to be the most predominant microbiome constituting 14.0%, 7.5% and 6.0% respectively, while the *Rahnella aquatilis*, *Enterobacter cloacae* and *Enterobacter hormaechei* was accounted to be the least dominated microbes fall under the family of Enterobacteriaceae. As observed in the earlier seasons (premonsoon), here we witnessed the distribution of *Bacillus* as well as *Priestia*, which possibly Justifying the possible dynamics of microbial uptake from the environment or other sources during the monsoon season. In addition to this, the *Gemella morbillorum*, *Ruminococcus*

bromii and *Brevibacterium casei* contributed 4.5%, 4.0% and 4.0% respectively for the whole microbial population. The metagenomic data for the monsoon season can give significant information concerning the influence and ecological interaction of microbes along with the transmission potential of the target mosquito vector, *Aedes aegypti*.

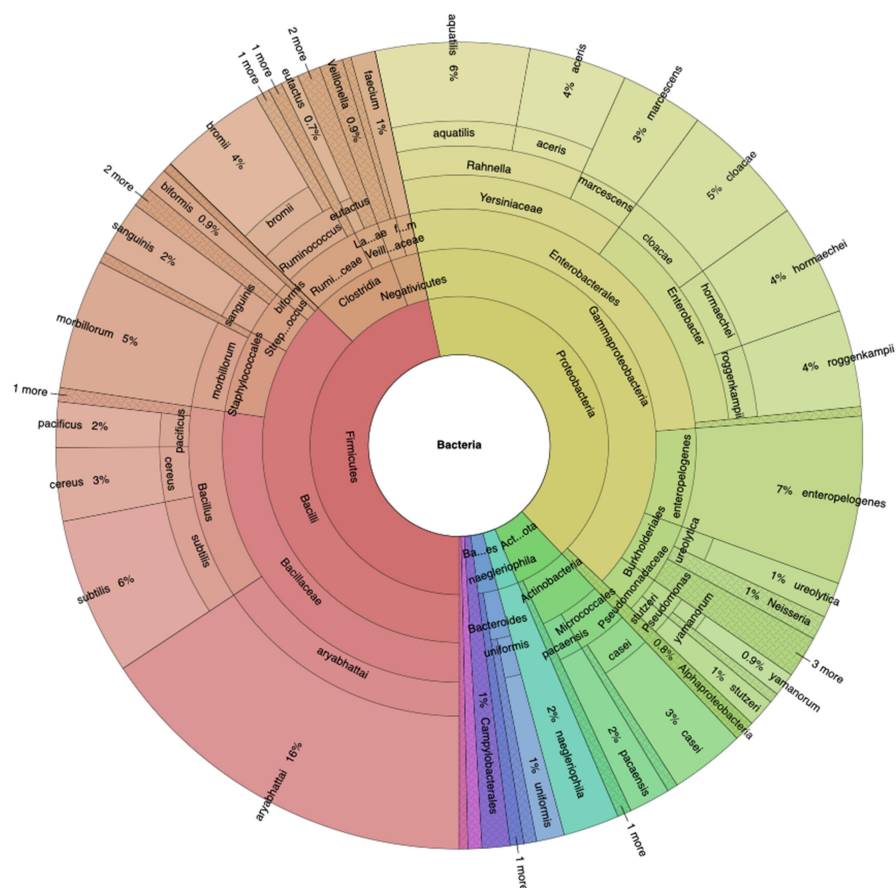


Figure 3.17 Species wise Hierarchical visualization of gut bacterial communities [Monsoon] through metagenomic analysis

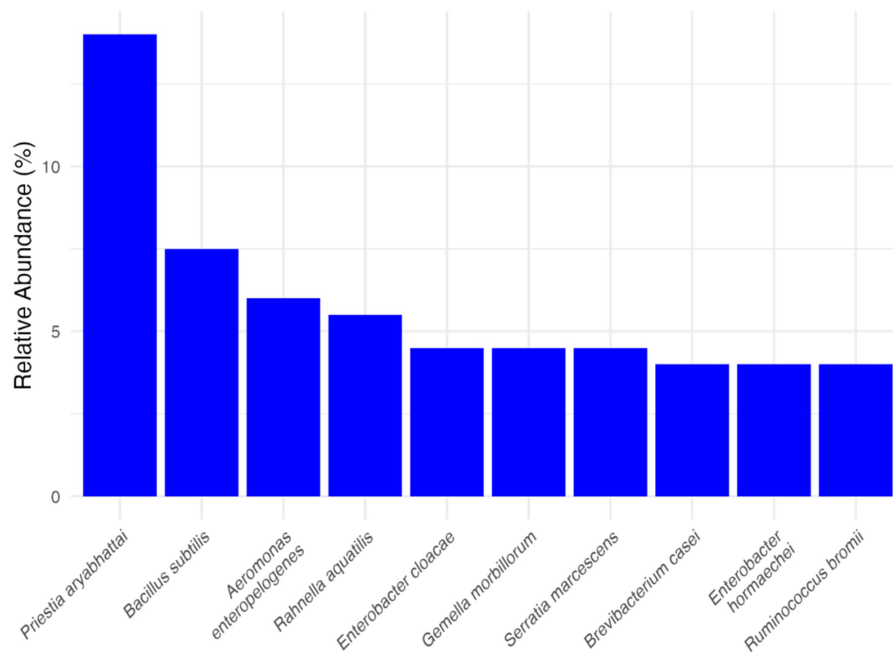


Figure 3.18 Metagenomic Profile of Top 10 Bacterial Species in Gut Microbiome

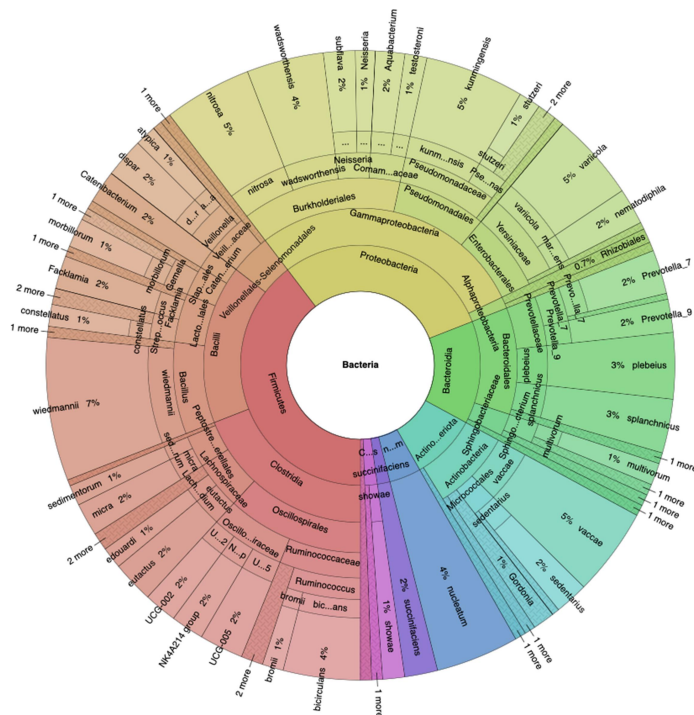


Figure 3.19 Species wise Hierarchical visualization of gut bacterial communities [Post-Monsoon] through metagenomic analysis

Post monsoon

The Metagenomic analysis for the post-monsoon season also produced distinct microbial consortia at species level. This season demonstrate a slightly different shift in microbial composition and *Bacillus wiedmannii* has been known to be the most predominant species. Furthermore the presence of *Stutzerimonas kunmingensis*, *Fusobacterium nucleatum*, *Klebsiella variicola* and *Ruminococcus bicirculans* revealing the possible adaptation to altered environmental conditions and potential exchange of microbes from the environment towards the vector.

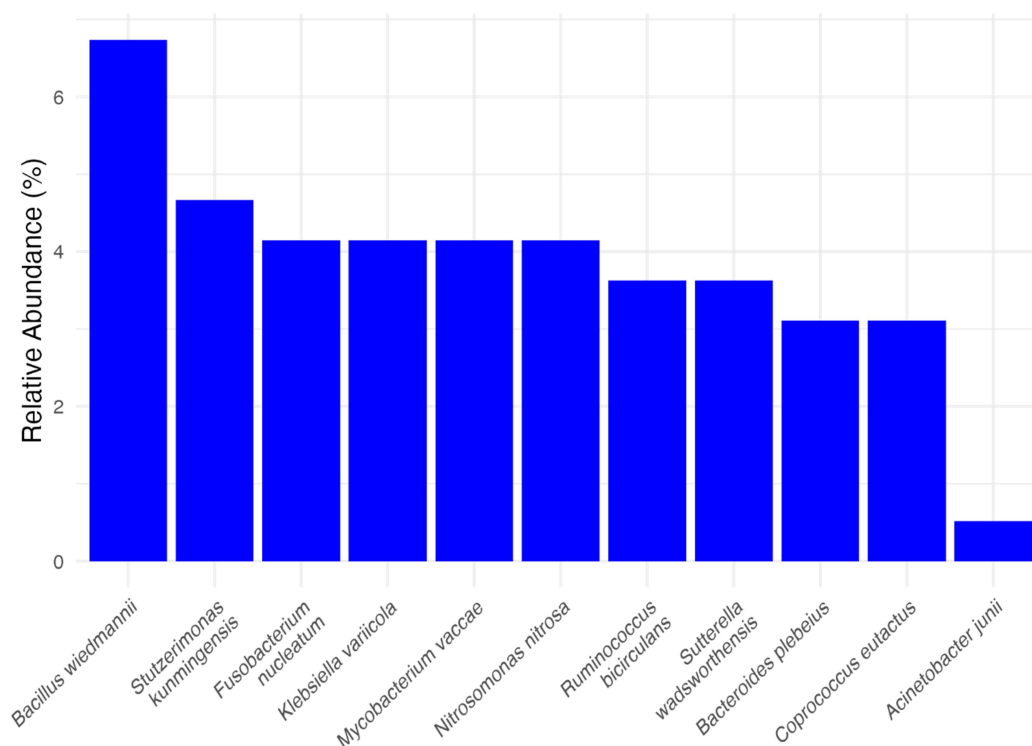


Figure 3.20 Metagenomic Profile of Top 10 Bacterial Species in Post-Monsoon Gut Microbiome

The post-monsoon gut microbiota of showed a distinct shift in composition, with *Bacillus wiedmannii* emerging as the dominant species. Gram-negative proteobacteria

such as *Stutzerimonas kunmingensis* and *Klebsiella variicola* were prominent, along with anaerobic bacteria including *Fusobacterium nucleatum* and *Ruminococcus bicirculans*, suggesting adaptation to altered environmental conditions following monsoon rains. Notable findings included the presence of *Mycobacterium vaccae* and *Nitrosomonas nitrosa*, species associated with soil and water ecosystems, reflecting potential microbial exchanges with post-monsoon breeding habitats. The gut community also contained gut-associated bacteria like *Bacteroides plebeius* and *Sutterella wadsworthensis*, indicating possible interactions with vertebrate hosts. The persistence of *Acinetobacter junii* across seasons suggests its stable colonization in the mosquito gut.

DISCUSSION

The role of microorganisms in ecosystems and the physiology of many organisms belonging to humans, plants and animals have been acknowledged in metagenomics focused studies (Xu, 2006). Because of the epidemiological significance and other various reasons associated with the transmission of deadly diseases, insects, especially mosquitoes are considered the central focus of many studies. Their ability to fight against complex environmental factors through many symbiotic microorganisms enhanced their population rate day by day. Hence, to reduce their population rate, recent studies largely focused on the profiling of microbiome settled in the various stages of the mosquito development cycle (Alfano et al., 2019; Coon et al., 2014; Duguma, Hall, Smartt, & Neufeld, 2017). In which the larval stages were accounted as the easiest and most approachable stage for microbiome profiling. The various microbial species symbiotically live in the mosquito population either directly or indirectly regulate the reproduction, growth and several physiological activities associated with the immune defence. Usually, the term “autochthonous communities” was used to designate the major microorganisms which having physiological functions. The studies focused on the profiling of mosquito midgut microbiota using metagenomics can provide sufficient information to fight against the mosquito vectors and various diseases impacts spread by the *Aedes* mosquitoes. It was obvious that the findings of this study could be considered as significant information because of the presence of microbial species belonging to the following phyla in the *Aedes* mosquitoes gut; *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Firmicutes*, *Gemmatimonadetes*, *Planctomycetes*, *Proteobacteria*, and *Verrucomicrobia*. The presence of microorganisms belonging to these phyla in high-altitude freshwater was reported by (Y. Liu, Zhang, Zhao, Zhang, & Xie, 2014). In addition to this, (Duguma et

al., 2013) characterized the microbiome associated with the *Culex* mosquito vectors with special reference to the wetland habitats. Validation of the predominant phyla in *Aedes aegypti* mosquitoes from Guadeloupe and French Guiana was accomplished through a study by (Hery et al., 2021), revealing the presence of *Proteobacteria*, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes* as the primary phyla. The findings of the present study verified the application of advanced strategies like metagenomics in mosquito control research with special reference to the microbial population exists in the *Aedes* mosquito populations of kole wetland. Duguma et al., 2013 reported that the mosquitoes usually consume the microbial consortia during the various life cycle stages according to the pre monsoon, monsoon and Post-Monsoon seasons. As inferred in the present study, Ramos-Nino et al., 2020 observed that bacteria was the most predominant microbiome constituent exists within the *Aedes aegypti*. As per the discussed observation, it was clear that *Proteobacteria* is the most dominant bacteria in *Aedes aegypti*.

The significance of *Proteobacteria* in both studies underscores its crucial involvement in the mosquito life cycle. In line with these findings, a prior investigation conducted by (Adegoke et al., 2020) revealed that *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria* stood out as the most prevalent phyla. The fact is that none have piloted as study on these aspects according to the various seasons in *Aedes aegypti* mosquito vectors. To date, no studies have been conducted to explore these aspects specifically across different seasons such as Pre-monsoon, monsoon and post-monsoon in *Aedes aegypti* mosquito vectors. Nonetheless, previous studies, including an investigation by (Parab, Jagtap, Meena, & Manohar, 2022) from the Indian region, have reported a prevalent abundance of *Firmicutes* during both the monsoon season and the post-monsoon season. In a study (Hery et al., 2021) encompassing diverse urban areas in

Guadeloupe and French Guiana, Firmicutes were identified as the dominant phylum. Likewise, (Zouache et al., 2011) successfully identified the organisms from two phyla, namely Firmicutes and *Proteobacteria*, which are acknowledged as the major findings of the current research. A study conducted by (R. Samson et al., 2023) reported that *Bacteroidetes* and *Chloroflexi* demonstrated an increase in abundance during the pre-monsoon season compared to other investigated seasons such as monsoon and post-monsoon seasons. These findings support the results of (Maestre et al., 2015) that specific factors have an impact on the composition of *Chloroflexi* and *Acidobacteria*. Upon completing the analysis at phylum level, the subsequent strand of the study detected the top 10 genera present in *Aedes aegypti* mosquito larvae.

Notably, in a study conducted by (Arévalo-Cortés et al., 2020), numerous species were found to belong to the following genera: *Parabacteroides*, *Bifidobacterium*, *Ruminococcus*, *Alistipes*, *Macellibacteroides*, *Campylobacter*, *Mucispirillum*, *Pseudomonas*, *Bacteroides*, and *Clostridium*. Among the various reported genera, the organisms belonging to the *Clostridium* are widely acknowledged as the predominant genus in the present investigation and this highlights the notable contribution of the *Clostridium* genus to the *Aedes aegypti* mosquito vectors, emphasizing its significant role. Nevertheless, as evident in this research, a study conducted by (Arévalo-Cortés et al., 2020) revealed notable variations in the abundance levels across distinct seasons.

The *Clostridium* genus mentioned above is primarily allied with the degradation of fenpropathrin (H. Wang et al., 2021) revealed that *Hydrogenophaga* exhibited the highest richness. Similarly to the results of this study, a research conducted by (Coon, Brown, & Strand, 2016a) reported that *Leucobacter* was identified as major microbial genera found in the gut of *Aedes aegypti* mosquito vectors. To date, no studies have documented the presence of *Rhodoplanes* genus microbes within the gut of *Aedes*

aegypti mosquito larvae worldwide. The previous study conducted by (Hery et al., 2021) has already established a significant association between xanthobacter and *Aedes aegypti* mosquito larvae. This underscores the importance of documenting the presence of xanthobacter in *Aedes aegypti* mosquito larvae. As previously stated, this particular strain of *Clostridium* is known for its involvement in the degradation of newly developed synthetic pyrethroids, which are commonly used to control various insects like fenprothrin. Consequently, it is probable that *Clostridium bifermentans* play a role in supporting the mosquito vectors in their ability to withstand and adapt to diverse toxic circumstances and their associated effects. Findings by (Qureshi, Chawla, Likitvivatanavong, Lee, & Gill, 2014) highlight a captivating observation that the *Clostridium bifermentans* were renowned for their capacity to elicit harmful effects on various mosquito vectors.

Notably, no previous reports have documented the presence of *Anaerospira hongkongensis* and *Listeria weihenstephanensis* in any part of the world. This absence further underscores the unique nature of the current research. In this study, another major microorganism identified was *Rhodoplanes elegans*. Similar to the previously said strains, there is a dearth of research on *Rhodoplanes elegans* specifically within the gut microbiota of *Aedes aegypti* in the current context. However, earlier studies exploring metagenomic analysis of soil have confirmed the presence of microbial species from the *Rhodoplanes* genus (N. Singh, Singh, Rai, Vamanu, & Singh, 2022), underscoring the novelty of this current investigation. In addition, the study highlighted the striking involvement of *Xanthobacter flavus* in certain insects or blowflies (Tomberlin et al., 2017), surpassing its role in vector control research. The unique aspect of the current research is further enhanced by the identification of *Xanthobacter flavus* in *Aedes aegypti* mosquito larvae using metagenomics. This detection was

conducted across different seasons, including pre-monsoon, monsoon, and post-monsoon, emphasizing the dynamic nature of *Xanthobacter flavus* presence in the mosquito larvae. The season-specific diversity analysis also illustrated the prominent diversity of microbiome in the *Aedes aegypti* mosquito larvae.

The gene profile obtained from the analysis served as the input for calculating the Simpson 1-D index and Shannon (H), which are widely recognized as the Alpha diversity metrics. (Hery et al., 2021) emphasized the reliability and integrity of employing these approaches for diversity analysis in metagenomics. The mosquito strain corresponds to the Pre-monsoon (AAMB strain ($H' = 3.11813661$)) season was noted as the most abundant than the AAMA ($H' = 2.58097801$) and AAMC ($H' = 2.29624848$).

Previous studies have primarily concentrated on either the diversity of vector mosquitoes (Chaves et al., 2011; Forrester, Coffey, & Weaver, 2014) or the distribution of microbes (Guégan, Zouache, Démichel, Minard, Van Van, et al., 2018). However, none have explored the concept of seasonal variations. The high diversity of microbiome inhabited in the *Aedes aegypti* mosquito vectors revealed through the alpha and beta diversity matrices assign that the climate change and seasonal factors are may be responsible for this variation in population diversity.

As reported by (L. E. Villegas et al., 2018), the diversity analysis based on operational taxonomic units (OUTs) was notably associated with the presence of singletons and doubletons. In this regard here the operational taxonomic units (OUTs) based diversity analysis and is depicted in Figure 3.13 -17. Altogether, the results of the present study highlight the significant influence of the microbiome on the life cycle of mosquito vectors. One significant impact of microbes on the mosquito life cycle is their

involvement in larval development through enhancing the development growth and survival rate. Similar finding were reported by (L. M. Villegas & Pimenta, 2014).

Some of the microbial consortia also possessed the capability to influence the various mechanisms allied with the reproduction in the target. Moreover, they can also impact the host-seeking potential followed by the feeding preferences and diseases transmission ability. For instance, some of the bacteria can modulate the mosquito vectors approach against the human hosts by forming certain specific efficient compounds that alter the host seeking preferences. This circumstance indicates that studying the strong link between the abovesaid microbial consortia and the mosquito vectors is vital for developing efficient approaches for mosquito control thereby mitigate the complications instigated by the mosquito borne diseases. The unique findings reported here hence can be recognized to be a strong base for future research targeting the specific mechanisms behind the interactions discussed above and to explore the same for the development of effective control strategies through the microbiota manipulations.

Species Richness and Community Composition

When compared to the pre-monsoon (632 OTUs) and post-monsoon (603 OTUs) samples, the monsoon sample (AAMA) had the highest observed richness (671 OTUs). The existence of more uncommon and unrecognised taxa in the mosquito gut microbiome during the monsoon season is also indicated by richness estimators like Chao1 (873.84) and ACE (838.23), which were likewise highest during this time. Elevated environmental humidity, more breeding sites, increased microbial proliferation in aquatic larval habitats, and increased mosquito exposure to environmental microorganisms could all contribute to this rise in richness during the monsoon. Similar

findings from earlier research on the gut microbiota of *Aedes aegypti* have shown that environmental factors have a significant impact on the acquisition of bacteria throughout larval development and adult emergence. According to studies by Coon and his colleagues in seasonal environmental changes dramatically change microbial diversity, and larval habitat water is a primary source of gut microbial colonisation.

Diversity and Evenness of Bacterial Communities

The pre-monsoon sample (AAMB) had the best bacterial diversity and evenness despite the monsoon sample having the highest richness: Shannon index = 3.118, Simpson index = 0.886, Inverse Simpson = 8.79. These numbers show that the pre-monsoon gut microbiome's bacterial taxa were more uniformly distributed, with fewer bacterial species dominating. On the other hand, the post-monsoon sample had the lowest diversity (Shannon = 2.296), while the monsoon sample had intermediate diversity (Shannon = 2.58). The reduced diversity in post-monsoon settings may indicate that dominant bacterial species have survived selectively after seasonal transition-related environmental disruptions. Similar trends have been shown in earlier studies on *Aedes aegypti* and other mosquito vectors: seasonal ecological fluctuations, nutrition availability, and environmental stress all affect the stability and dominance of microbial communities.

Seasonal Variation

The observed seasonal shifts in bacterial diversity suggest that the gut microbiome of *Aedes aegypti* is highly dynamic and environmentally responsive. Monsoon (AAMA) showed highest richness and estimated diversity which indicates extensive acquisition of environmental bacteria and favors colonization by transient and rare microbial taxa due to abundant breeding waters and organic matter accumulation. Pre-monsoon

(AAMB) had highest Shannon and Simpson diversity and it suggests a more stable and balanced microbial ecosystem which reflects optimal ecological conditions supporting coexistence of multiple bacterial taxa without dominance. Post-monsoon (AAMC) had the lowest richness and diversity which Indicates reduction in microbial heterogeneity and possible competitive exclusion by dominant taxa and it may result from environmental stabilization after monsoon flooding and nutrient fluctuations.

Research by Dada and his colleagues in 2018 demonstrated that increased environmental microbial exposure during rainy seasons significantly enhances bacterial richness in *Aedes aegypti*. Similarly, several studies reported that members of *Enterobacteriaceae*, *Bacillus*, *Pseudomonas*, and *Ruminococcaceae* fluctuate seasonally and contribute to mosquito nutrition, digestion, immunity, and vector competence.

CONCLUSION

The present study isolated, characterized and identified the predominant 15 microbial consortia inhabited in the *Aedes aegypti* mosquito larvae. Molecular characterization of these microbial population using 16S rRNA sequencing produced notable similarities towards the sequences reported across the globe. The Phylogenetic analysis of the isolated sequences with Maximum Likelihood unveiled the extensive genetic diversity towards the *Enterobacter*, *Bacillus*, and *Rahnella* genus. The metagenomic analysis justified the seasonal variation of microbial consortia within the same mosquito vector, validating that the composition of *Firmicutes*, *Actinobacteria* and *Proteobacteria* are strongly influenced by the various seasons such as pre-monsoon, monsoon, post monsoon. Precisely, while *Proteobacteria* was reported as the most predominant bacteria during monsoon in *Aedes aegypti*, *Firmicutes* was dominated within the same mosquito vector during monsoon and post-monsoon rather than pre-monsoon. In agreement with this, we have reported significant differences between the various strains (AAMA, AAMB, and AAMC) of the same species through the Shannon and Simpson indices analysis. Altogether, here we investigated the richness of microbial population inhabited in the *Aedes aegypti* mosquito larvae according to the various seasons and ecological inferences.

CHAPTER 4

**TO ISOLATE, CHARACTERIZE AND IDENTIFY FUNGI FROM
FOURTH INSTAR *AEDES AEGYPTI* LARVA COLLECTED FROM
KOLE WETLANDS BY CULTURE DEPENDENT AND
METAGENOMIC ANALYSIS.**

INTRODUCTION

The *Aedes aegypti* mosquitoes are primarily considered to be the major vector of many diseases such as Zika, yellow fever, and chikungunya and it instigates severe health concerns across the globe as reported by (Leta et al., 2018). The control of aforesaid diseases primarily rely on the most common vector control approaches, particularly targeting the larval population. Recent studies indicated that microbial population, especially the fungal population may critically impacts the survival and development of *Aedes* mosquito vectors (H. Ranasinghe & Amarasinghe, 2020; Dighton, 2018). Because of this reason, the research trends now has been directed towards unveiling the function and composition of fungal strains across the globe in a regions specific manner.

As reported earlier, the wetlands were opted as the study site for the present investigation since the Wetlands usually offers a strong base with standing water for mosquito vectors. The Kole wetlands of Thrissur, Kerala, India also known to exhibit extensive distribution of biodiversity along with distinct and unique aquatic ecosystems that sustains the life fungal populations.

Fungi are ubiquitous in both terrestrial and aquatic ecosystems, playing critical roles in nutrient cycling, plant-microbe interactions, and symbiotic relationships with other organisms (Bahram & Netherway, 2022). Some fungi are entomopathogenic, meaning they infect and kill insects, including mosquito larvae. These fungi produce various biochemicals that can disrupt the normal physiology of the host, leading to disease and eventual death of the larvae.

Several genera of fungi, such as *Beauveria*, *Metarhizium*, and *Lecanicillium*, are well-known entomopathogens that have been studied for their ability to infect and kill mosquito larvae (Perumal, Kannan, Pittarate, & Krutmuang, 2024). These fungi produce conidia or other spores that can penetrate the exoskeleton of the larvae, proliferating inside and eventually killing the host. Furthermore, fungi can also produce secondary metabolites with insecticidal properties, adding another layer of complexity to their interactions with mosquito larvae. Understanding the diversity of these fungi in natural environments like the Kole Wetlands could help identify new species or strains with potential for development into biocontrol agents for *Aedes aegypti*.

The fungal strains populated across the wetland ecosystems are prominently impacted by various environmental factors including the organic matter availability, quality of water followed by the variations across the seasons. According to (Scolari, Casiraghi, & Bonizzoni, 2019), the potential role of microbial population in mosquito vector is multifaceted in *Aedes aegypti*, since they verified the role of microbes in nutrient acquisition, digestion, immunity and resistance towards pathogens.

The isolation, characterization, and identification of fungi traditionally rely on culture-dependent methods. In these approaches, samples from the larval environment are cultured on selective media that promote the growth of fungi, which can then be identified based on their morphological, biochemical, and molecular characteristics. Although culture-dependent methods have been instrumental in identifying fungi in various ecosystems (H. Zheng, Qiao, Xu, & Yu, 2021), they are limited by the fact that not all fungal species can be cultured in laboratory conditions. Some fungi require specific environmental conditions or symbiotic interactions with other organisms that are difficult to replicate in the lab. From this point of view, to get deeper understanding,

here the present implemented both culture-dependent strategies and advanced approaches including metagenomic analysis.

MATERIALS AND METHODS

Study Area

The Kole Wetlands of Thrissur district of Kerala, India was chosen for the current study site (10.52°N, 76.21°E). The Kole wetlands of Thrissur district were selected as the study area for mosquito larval collection. The total wetland area was divided into different sampling zones based on geographical location, habitat characteristics, and accessibility. Zone I – Puthenchira Kole region, Zone II – Mala–Annamanada Kole region, and Zone III –Puzhakkal Kole region. Within each zone, potential mosquito breeding habitats such as shallow stagnant water collections, marshy areas, and vegetation-associated water bodies were identified. Sampling sites were randomly selected from each zone to obtain representative larval populations. A total of nine sampling sites were selected, with three sites from each zone. Mosquito larvae were collected using a standard larval dipper from different breeding habitats. Approximately 10–15 fourth instar mosquito larvae were collected from each sampling site. Three collections were made from each zone. Thus, a total of approximately 90–135 fourth instar larvae were collected during the study period. The collected larvae were transferred into sterile containers containing habitat water and transported to the laboratory for further processing. Larvae collected from each sampling zone were pooled together and used for culture-dependent and metagenomic analyses. Mosquito larvae were collected and identified using taxonomic keys during different seasons: Monsoon (AAMF), Pre-monsoon (AAPF1), and Post-monsoon (AAPOF1).

Culture-Dependent Isolation of Fungi from Mosquito Gut

In an aseptic environment, the larvae were surface sterilized using 70% ethanol. After fungal growth, 50 μ L of the fungal broth was spread onto Sabouraud Dextrose Agar (SDA) plates supplemented with 0.06 g of chloramphenicol. These plates were incubated in the dark at 25°C for 2–14 days, with regular monitoring.

DNA Barcoding Using Universal LSU Primers

DNA Isolation

DNA was extracted using the NucleoSpin® Plant II Kit (Macherey-Nagel). Approximately 100 mg of fungal tissue or mycelium was homogenized in liquid nitrogen (Chauhan, Sharma, Modgil, & Siddappa, 2018). The powdered sample was transferred to a microcentrifuge tube, and 400 μ L of buffer PL1 was added, followed by vortexing for 1 minute. RNase A (10 μ L) was added, and the mixture was incubated at 65°C for 10 minutes. The lysate was filtered through a Nucleospin filter and centrifuged. The flow-through was collected, and subsequent washing steps were performed with buffer PC, PW1, and PW2. After drying the silica membrane, 50 μ L of buffer PE was added to elute the DNA. The final DNA solution was stored at 4°C.

DNA Quality Check

The quality of the isolated DNA was verified using agarose gel electrophoresis. A 1 μ L aliquot of 6X gel loading buffer was mixed with 5 μ L of the DNA sample and loaded onto a 0.8% agarose gel. The gel was run at 75V in 0.5X TBE buffer and stained with ethidium bromide. DNA was visualized under UV light using a Gel Documentation System.

PCR Analysis

PCR amplification was performed using the following reaction mixture:

- 5 μ L 2X Phire Master Mix

- 4 μL distilled water
- 0.25 μL forward primer (LROR)
- 0.25 μL reverse primer (LR7)
- 1 μL DNA template

The PCR amplification conditions were as follows:

- Initial denaturation at 98°C for 30 seconds
- Denaturation at 98°C for 5 seconds, annealing at 54°C for 10 seconds, extension at 72°C for 15 seconds (40 cycles)
- Final extension at 72°C for 60 seconds
- Hold at 4°C

The PCR products were analyzed by electrophoresis on a 1.2% agarose gel, using 0.5X TBE buffer and visualized under UV light.

ExoSAP-IT Treatment

PCR products were purified using ExoSAP-IT to remove primers and dNTPs. A 5 μL sample of PCR product was mixed with 0.5 μL of ExoSAP-IT and incubated at 37°C for 15 minutes. The enzymes were inactivated by heating at 85°C for 5 minutes.

Sequencing Reaction

DNA sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit. The sequencing reaction mix contained:

- 6.6 μL distilled water
- 1.9 μL 5X sequencing buffer
- 0.3 μL forward primer

- 0.3 μ L reverse primer
- 0.2 μ L sequencing mix
- 1 μ L ExoSAP-IT-treated PCR product

Sequencing was carried out using the following cycle conditions:

- Initial denaturation at 96°C for 2 minutes
- Denaturation at 96°C for 30 seconds, annealing at 50°C for 40 seconds, extension at 60°C for 4 minutes (30 cycles)
- Hold at 4°C
- Post-Sequencing Clean-Up

After sequencing, the reaction products were cleaned up using sodium acetate and ethanol precipitation. The samples were incubated at room temperature for 30 minutes and centrifuged at 3700 rpm for 30 minutes. After washing with 70% ethanol, the samples were air-dried and sequenced on an ABI 3500 DNA Analyzer.

Sequence Analysis

The sequencing quality was checked using Sequence Scanner Software. The sequences were aligned and edited using Geneious Pro (v5.1).

Phylogenetic Analysis

BLAST searches were performed in GenBank to identify the closest matches to the obtained sequences.

Fungal Metagenomic Analysis:

Fungal community profiling was performed using high throughput sequencing of the Internal Transcribed Spacer (ITS) region, a universal DNA barcode for fungi.

Quality Control of Raw Reads

Raw sequencing data in FASTQ format is subjected to quality control using FastQC. This step assesses various quality parameters such as base quality scores, GC content, sequence length distribution, duplication levels, and the presence of adapter contamination. Summary statistics and per-read metrics are carefully reviewed to ensure data integrity before further processing.

Trimming and Filtering of Reads

To eliminate low-quality bases (Phred score < 20) and adapter sequences, trimming is performed using tools like Trimmomatic. Parameters such as sliding window trimming and minimum read length thresholds are applied to retain only high-quality data. For paired-end reads, orphaned or unpaired sequences are removed after trimming, ensuring consistency in the dataset.

Host DNA Depletion

To remove host (mosquito) DNA contamination, the trimmed reads are aligned against a reference mosquito genome using Bowtie2. The alignment results in SAM/BAM files, and only unmapped reads (representing microbial origin) are retained for further microbial analysis.

Metagenome Assembly

Unmapped high-quality microbial reads are assembled into longer contiguous sequences (contigs) using MEGAHIT. These assemblers are optimized for

metagenomic data and reconstruct partial or complete microbial genomes, enhancing both taxonomic and functional resolution.

Binning of Metagenome-Assembled Genomes (MAGs)

Contigs are grouped into metagenome-assembled genomes using automated binning tools like MetaBAT2,. Binning relies on sequence composition and coverage profiles. The resulting genome bins are evaluated using CheckM to assess their completeness and contamination.

Taxonomic Classification of Assembled Reads

For taxonomic profiling, assembled or unassembled reads are classified using Kraken2, a fast k-mer-based classifier, and MetaPhlAn3, which leverages clade-specific marker genes for accurate species-level profiling. These tools generate relative abundance tables of fungal taxa across all hierarchical levels, from phylum to species. The relative abundance of taxa at the phylum, class, genus, and species levels is visualized through separate plots, providing a detailed view of the microbial composition.

Fungal Taxonomic Visualization Using Krona

The output from Kraken2 or MetaPhlAn3 is converted into a format compatible with KronaTools. Krona generates interactive, multi-layered radial plots that display the taxonomic composition of microbial communities in a hierarchical manner, enabling clear visualization of dominant and rare fungal taxa.

RESULTS

Identification of fungal strains

The initial strand of the present study used the applicability of culture dependent strategies to unveil the presence of various fungal strains inhabited in the *Aedes aegypti* mosquito vector. Under controlled environment along with specific selected media, this study have examined and differentiated the various fungal isolates. Moreover, the subsequent implementation of molecular strategies allowed us to confirm the identity of the isolated fungi strain. The following are the most predominant fungal strain reported in the current study; *Trichoderma erinaceum*, *Mucor varisporus*, *Aspergillus niger*, *Candida tropicalis*, *Penicillium ochrochloron*, *Wickerhamomyces anomalus*, *Mucor irregularis* and *Aspergillus tubingensis*. The reported findings of the present study unveil the extensive abundance of the fungal community inhabiting the *Aedes aegypti*, justifying the significant ecological interactions formed between the mosquito vector host and the fungal strains. The fungal species identified included *Aspergillus niger*, *Candida tropicalis*, *Wickerhamomyces anomalus*, *Mucor varisporus*, *Penicillium ochrochloron*, *Mucor irregularis*, *Aspergillus tubingensis*, and *Trichoderma erinaceum*. Morphological differences among the identified fungal species were evident and are presented in Figure 4.1.

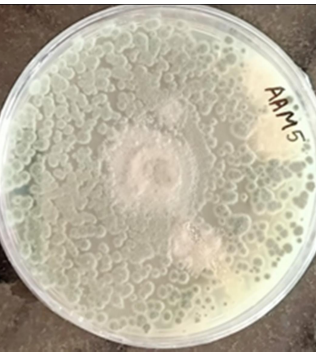
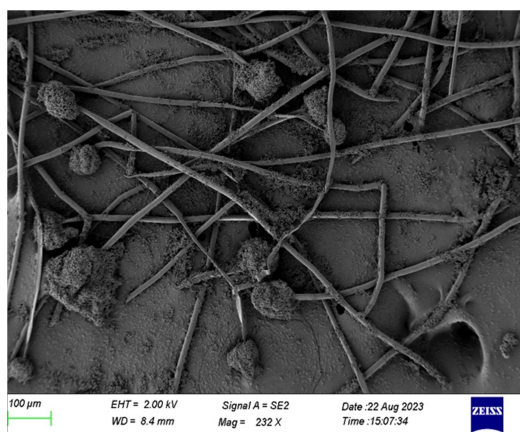
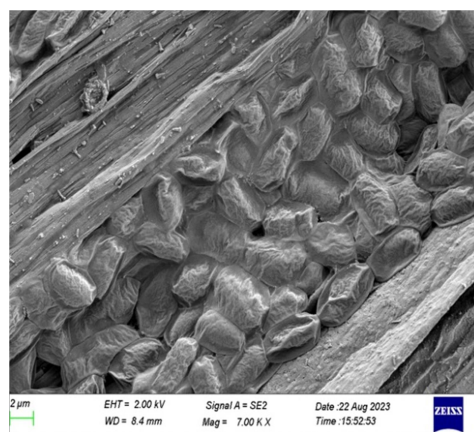
		
AABF1 <i>Aspergillus niger</i> contig An03c0110	AAYF1 <i>Candida tropicalis</i> strain MYA-3404	AADF1 <i>Wickerhamomyces</i> <i>anomalus</i>
		
AATPF3 <i>Mucor varisporus</i>	AATPF1 <i>Trichoderma erinaceum</i>	AAMF5 <i>Penicillium ochrochloron</i>
		
	AAMF6 <i>Mucor irregularis</i>	

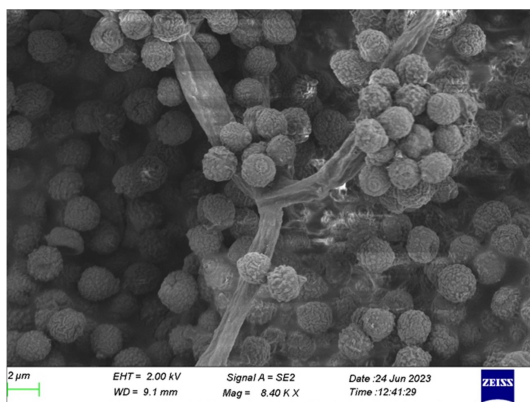
Figure 4.1. Morphological differences among the identified fungal species



Aspergillus niger



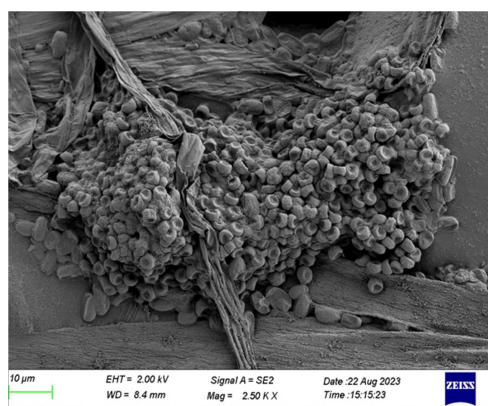
Wickerhamomyces anomalus



Penicillium ochrochloron

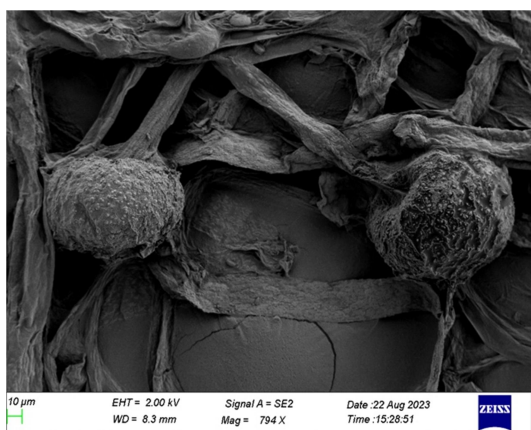


Mucor irregularis

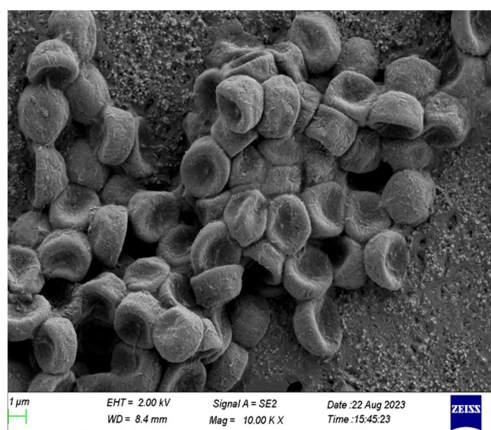


Trichoderma erinaceum

Figure 4.2 SEM analysis of the isolated fungal strains



Mucor varisporus



Candida tropicalis

Figure 4.2 Cont. SEM analysis of the isolated fungal strains

Molecular identification

Identification of Fungal Species

As reported earlier the this study have isolated and identified the predominant fungal strains inhabited in the *Aedes aegypti* mosquito vector. With the aid of molecular strategies and Scanning Electron Microscopy, the following fungal strains were successfully characterized from the *Aedes aegypti* mosquito vector; *Aspergillus niger* (AABF1-SR3114-BI-LSUR_D04), *Candida tropicalis* (AAYF1-SR3114-YI-LSUF_G03), *Wickerhamomyces anomalus* (AADF1- SR3114-01-LSUF_C05-), *Mucor varisporus* (AATPF3-SR3699-P3-LSUR_A02), *Penicillium ochrochloron* (AAMF5--SR3699-M5-LSUR_E02-), *Mucor irregularis* (AAMF6-SR3699-M6-LSUF_D01-), *Aspergillus tubingensis* (AATPF1-SR3699-P1-CAITSR_F06), *Trichoderma erinaceum* (AATPF2- SR3699-P2-LSUF_A09). The identification of these fungal species highlights a rich microbial diversity associated with *Aedes aegypti*, which may have significant ecological and functional implications, including interactions with the mosquito host and its environment.

1. *Aspergillus* Genus

The phylogenetic analysis through the Maximum Likelihood method produced a log likelihood of -4068.66 for the generated tree. The analysis comprised 30 nucleotide sequences followed by 812 positions in the final data set. As per the substitutions per site, the branch lengths were determined. The results from this strand exhibited prominent variation within the *Aspergillus* genus.

2. *Mucor* Genus

The phylogenetic analysis through the Maximum Likelihood method produced a log likelihood of - 2637.45 for the generated tree. The analysis comprised 23 nucleotide sequences followed by 797 positions in the final data set. As per the substitutions per site, the branch lengths were determined. The results from this strand exhibited prominent variation within the *Mucor* genus.

3. *Penicillium* Genus

The phylogenetic analysis through the Maximum Likelihood method produced a log likelihood of -5787.05 for the generated tree. The analysis comprised 18 nucleotide sequences followed by 699 positions in the final data set. As per the substitutions per site, the branch lengths were determined. The results from this strand exhibited prominent variation within the *Penicillium* genus.

4. *Candida* Genus

The phylogenetic analysis through the Maximum Likelihood method produced a log likelihood of -4008.31 for the generated tree. The analysis comprised 17 nucleotide sequences followed by 824 positions in the final data set. As per the substitutions per

site, the branch lengths were determined. The results from this strand exhibited prominent variation within the *Candida* genus.

5. *Trichoderma* Genus

The phylogenetic analysis through the Maximum Likelihood method produced a log likelihood of - 2668.06 for the generated tree. The analysis comprised 19 nucleotide sequences followed by 772 positions in the final data set. As per the substitutions per site, the branch lengths were determined. The results from this strand exhibited prominent variation within the *Trichoderma* genus.

6. *Wickerhamomyces* Genus

The phylogenetic analysis through the Maximum Likelihood method produced a log likelihood of -1980.51 for the generated tree. The analysis comprised 12 nucleotide sequences followed by 815 positions in the final data set. As per the substitutions per site, the branch lengths were determined. The results from this strand exhibited prominent variation within the *Wickerhamomyces* genus.

The molecular characterization of fungi inhabited within the *Aedes aegypti* offers critical insights towards the microbial ecology of the prominent mosquito vector *Aedes aegypti*, indicating the ecological significance of the present study. The identified fungi could possess significant role in transmission of infectious agents through the *Aedes aegypti* and its surrounding environment. The genomic variations observed in the current study highlights the complexity of the characterized fungi, unveiling direct way for the further research targeting the mosquito hosts, interactions and their environment. Hence, understanding the diverse dynamics allied with the microbial consortia and the mosquito vectors can contribute significantly for the development of

advanced and efficient strategies for the control of such mosquito vectors and infectious agenesi s, primarily for the disease management.

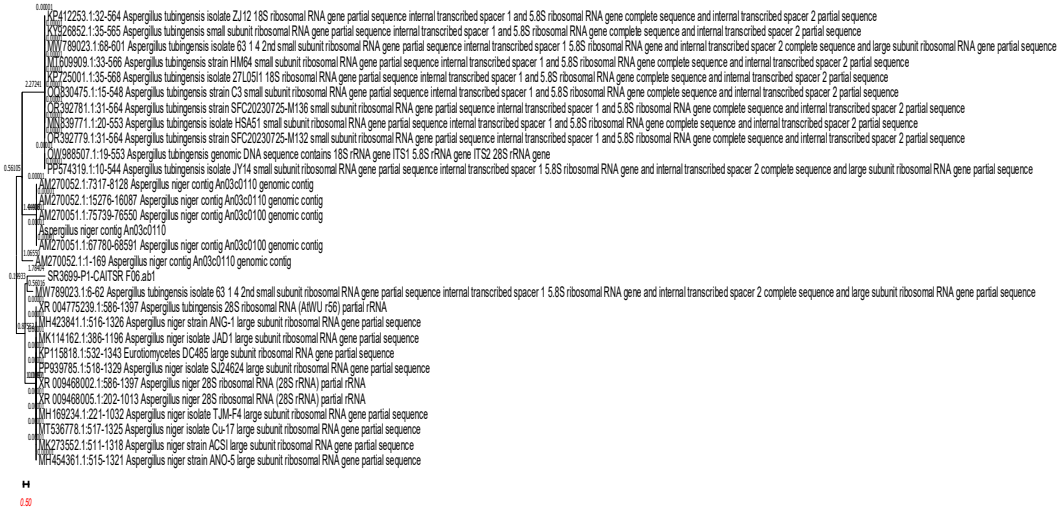


Figure 4.3 Evolutionary analysis of fungi belongs to *Aspergillus* genus by Maximum Likelihood method

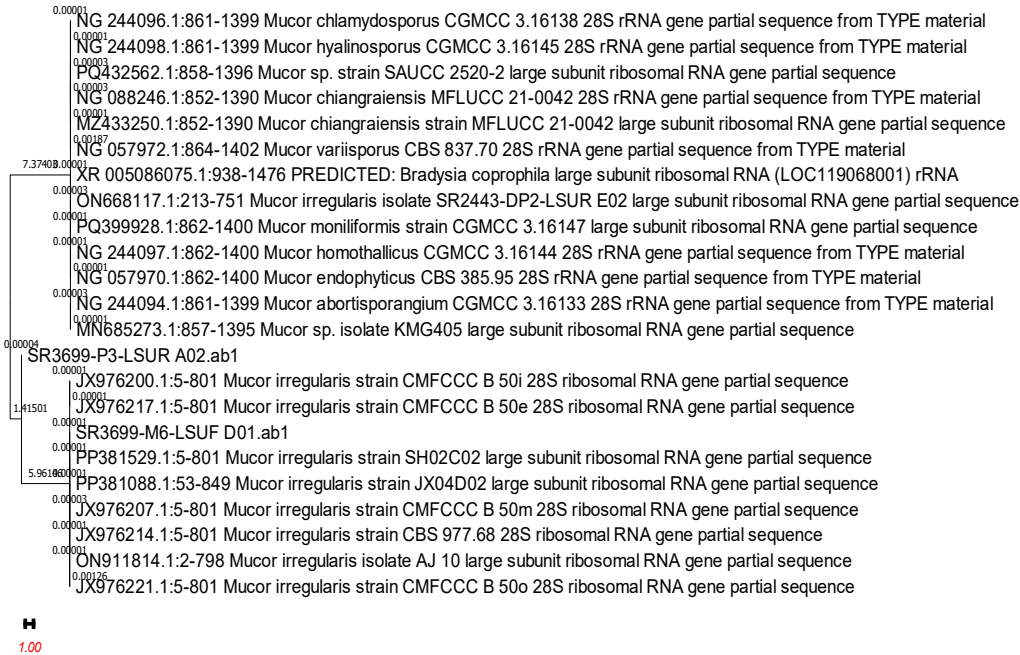


Figure 4.4 Evolutionary analysis of fungi belongs to *Mucor* genus by Maximum Likelihood method

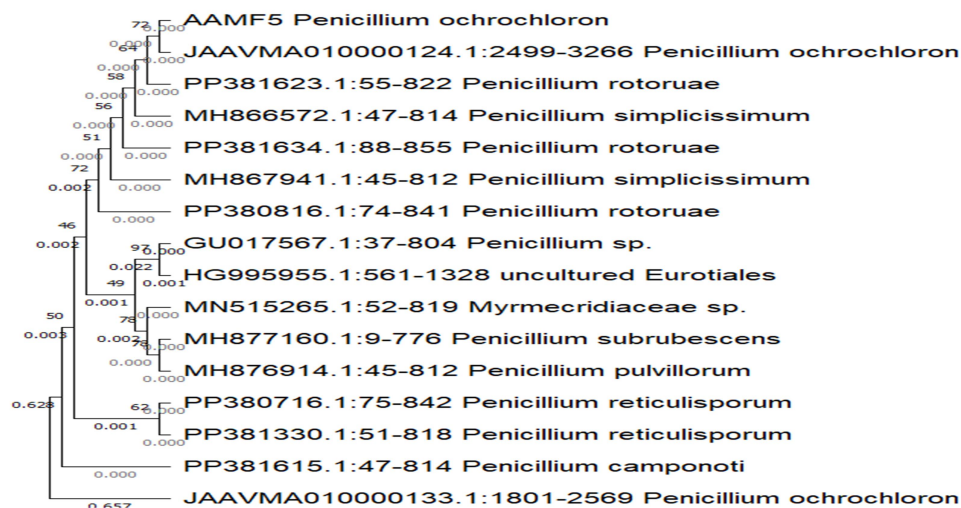


Figure 4.5 Evolutionary analysis of fungi belongs to *Penicillium* genus by Maximum Likelihood method

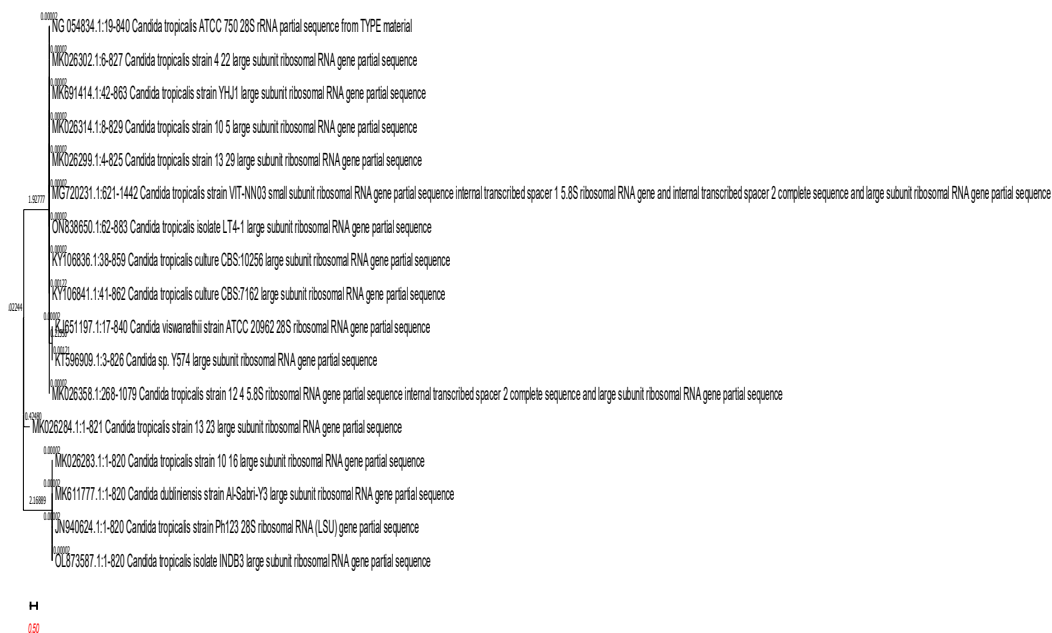


Figure 4.6 Evolutionary analysis of fungi belongs to *Candida* genus by Maximum Likelihood method

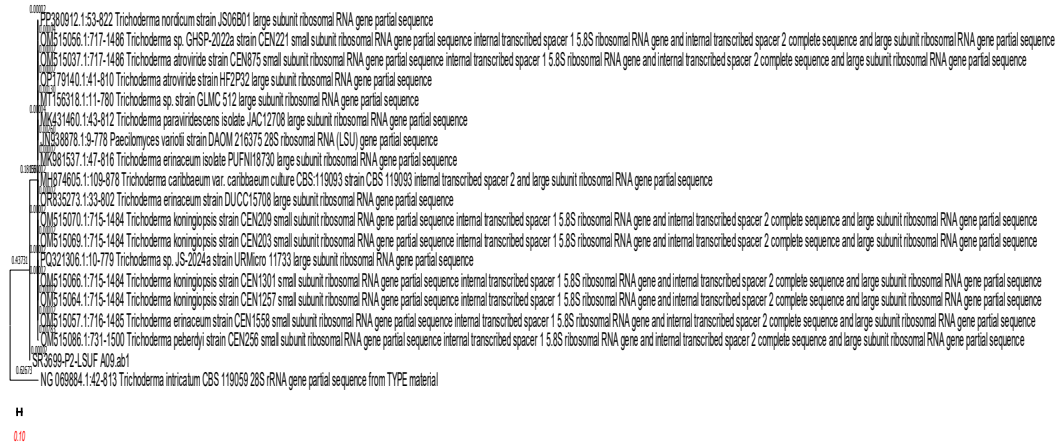


Figure 4.7 Evolutionary analysis of fungi belongs to *Trichoderma* genus by Maximum Likelihood method

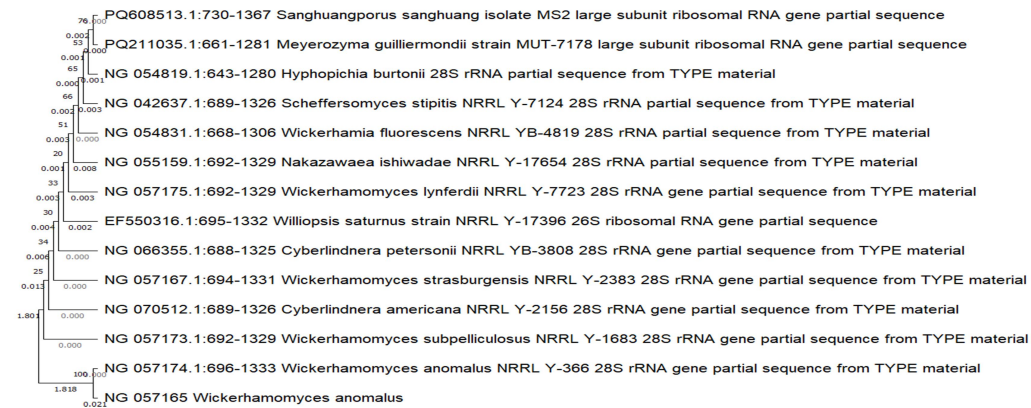


Figure 4.8 Evolutionary analysis of fungi belongs to *Wickerhamomyces* genus by Maximum Likelihood method

Isolated nucleotide sequences

1.AABF1 >SR3114-BI-LSUR_D04.ab1

Aspergillus niger contig An03c0110

AAGGCTTCGTCACGGACCCCCACGCCTGCCTACTCGTCGGGGCATCGTTTCTACCCC
GACGGCGAGGTATGGGTAGCACGCTTGAGCGCCATCCATTTTCAGGGCTAGTTCAT
TCGGCCCGTGAGTTGTTACACACTCCTTAGCGGATTCCGACTTCCATGGCCACCGTC
GGGCTGTCTAGATGAACTAACACCTTTTGTGGTGTCTGATGAGCGTGCATTCCGGC
ACCTTAACCTCGCGTTCGGTTCATCCCGCATCGCCAGTTCTGCTTACCAAAAATGGC
CCACTAGTAACGCTCCATTCTAATGCCACGTTCAACTAAGCAACAAGGGCTTCTT
ACATATTTAAAGTTTGAGAATAGGTTAAGGTTGTTTCAACCCCAATGCCTCTAATCA
TTCGCTTTACCTCATAAACTGATTTTGC GTTACTGCTATCCTGAGGGAAACTTCGG
CAGGAACCAGCTACTAGATGGTTCGATTAGTCTTTCGCCCCTATACCCAAATTTGAC
GATCGATTTGCACGTCAGAACCGCTGCGAGCCTCCACCAGAGTTTCCTCTGGCTTC
GCCCTATTCAGGCATAGTTCACCATCTTTCGGGTCCCCACAGCTACGCTCTTACTCA
AATCCATCCGAAGACATCAGGATCGGTTCGATGGTGCGCCCCGCAAGGGGGCTCCC
ACCTCCGTTCGCTTTCCTGCGCGCACGGGTTTGACACCCGAACACTCGCGTAGAT
GTTAGACTCCTTGGTCCGTGTTTCAAGACGGGTCGTTTACGACCATTATGCCAGCGT
CCGTGCCGAAGCGCGTTCCTC

2.AAYF1 *Candida tropicalis* strain MYA-3404

>SR3114-YI-LSUF_G03.ab1

TTGCCTTAGTAGCGGCGAGTGAAGCGGCAAAAGCTCAAATTTGAAATCTGGCTCTT
TCAGAGTCCGAGTTGTAATTTGAAGAAGGTATCTTTGGGTCTGGCTCTTGTCTATGT
TTCTTGGAACAGAACGTCACAGAGGGTGAGAATCCCGTGCGATGAGATGATCCAG
GCCTATGTAAAGTTCCTTCGAAGAGTCGAGTTGTTTGGAATGCAGCTCTAAGTGG
GTGGTAAATTCCATCTAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTA
CAGTGATGGAAAGATGAAAAGAACTTTGAAAAGAGAGTGAAAAAGTACGTGAAAT

TGTTGAAAGGGAAGGGCTTGAGATCAGACTTGGTATTTTGTATGTTACTTCTTCGGG
GGTGGCCTCTACAGTTTATCGGGCCAGCATCAGTTTGGGCGGTAGGAGAATTGCGT
TGGAATGTGGCACGGCTTCGGTTGTGTGTTATAGCCTTCGTCGATACTGCCAGCCTA
GACTGAGGACTGCGGTTTATACCTAGGATGTTGGCATAATGATCTTAAGTCGCCCCG
TCTTGAAACACGGACCAAGGAGTCTAACGTCTATGCGAGTGTTTGGGTGTAAAACC
CGTACGCGTAATGAAAGTGAACGTAGGTGGGGGCCCCGTATGGGTGCACCATCGAC
CGATCCTGATGTCTTCGGATGGATTTGAGTAAGAGCATAGCTGTTGGGACCCGAAA
GATGGTGAACATATGCCTGAATAGGGTGAAGCCAGAGGAAACTCTGGTGGAGGCTC
GTAGCGGGTTCTGACGTGCAAATCGATCGTCGAATTTGGGT

3. AAD1- >SR3114-01-LSUF_C05.ab1

Wickerhamomyces anomalus

GCTTCGTCACTGACCTCCACGCCTGCCTACTCGTCAGGGCATCATATCAACCCTGAC
GGCGAAGTATAAGTAACACGCTTGAGCGCCATCCATTTTCAGGGCTAGTTCATTTCG
GCCGGTGAGTTGTTACACACTCCTTAGCGGATTCCGACTTCCATGGCCACCGTCCG
GCTGTCTAGATGAACTAACACCTTTTGTGGTGTCTGATGAGCGTGTATTCCGGCACT
TTAACTTC
ACGTTTCGGTTCATCCCGCATCGCCAGTTCTGCTTACCAAAAATGGCCCACTAAAAG
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AGACATCA

4. AATPF3- *Mucor varisporus*

>SR3699-P3-LSUR_A02.ab1

CCTACTCGTTATTGCTTCGCTTTTACAATAACGGGGAAGTATGGGTAACACGCTTAA
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GTCTTTTCGCCCTATACCCAAATTTGACGATCGATTTGCACGTCAGAATCGCTACGA
GCCTCCACCGGAGTTTCCTCCGGCTTCAC

5. AAMF5 – *Penicillium ochrochloron*

>SR3699-M5-LSUR_E02.ab1

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GACCATTATGCCAGCGTCCGAGCCGAAGCGCGTTCCTCGGTCCGGGCTGGCCGC
ATGGCACCTTGCTATAAGCCGCCCCGGGGGGCGGTACATTCCAAGGGCCTTTGA
CCGGCCGCCCAAACCGACGCTGGCCCGCCGCGGGGAAGTACACCGGCACGAATG
CCGGCTGAACCCCGTGGGCGAGTCTGGTCGCAAGCGCTTCCCTTTCAACAATTTCA
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GCTATCGGTCTCCGGCCAGTATTTAGCTTTAGATGAAATTTACCACCCATTTAGA
GCTGCATTCCCAAACAACCTCGACTCGTCGAAGGAGCTTCACACGGGCGTGGGCACC
CCATCCAGACGGGATTCTCACCTCTATGACGGCCCGTTCCAGGGCACTTAGATG
GGGGCCACTCCCGAAGCATCCTCTGCAAATTACAATGCGGACCCCGAGGGGGCCA
GCTTTCAAATTTGAGCTCTTGCCGCTTCACTCGCCGTTACT

6. AAMF6- *Mucor irregularis*

>SR3699-M6-LSUF_D01.ab1

AACGGCGAGTGAAGAGGAAAGAGCTCAAAGTTGGAACCTGTTTGGCTTAGCTAAA
CCGGATTGTAAACTGTAGAAGTGTTTTCCAGACACGCCTGGTAAAAAAGTCCTTTG
GAACAGGGCATCATAGAGGGTGAGAATCCCGTCATTGGCCAGAGCTGTTGTCTTTT
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CATTGAATGTGGCCCTTCGGGGTGTTATAGCTTTGTAAAAAATACCTTGGACGGGA
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TATACTGAAACTGAC7.

AATPF2 – *Trichoderma erinaceum*

>SR3699-P2-LSUF_A09.ab1

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 ACACGGACCAAGGAGTCGTCTTCGTATGCGAGTGTTCTGGGTGTCAAAACCCCTACG
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 GTTCTCGGATGGATTTGAGTAAGAGCATACGGGGCCGGACCCGAAAGAAGGTGAA
 CTATGCCTGTATAGGGTGAAGCCAGAGGAAACTCTGGTGGAGGCTCGCA

Seasonal distribution of fungal strains revealed through Metagenomic strategies

Monsoon season

The monsoon seasons for *Aedes aegypti* was dominated by the fungal strain *Phlyctis speirea*. Moreover, *Biatora pontica* and *Ramalina* spp. constituted nearly 20% of the total population as a prevalent fungal strain, while the *Aspergilli* has been reported to occur consistently throughout the seasons including the pre-monsoon. The relative abundances relative abundances of *A. tubingensis* and *A. fumigatus* similarly augmented across the seasons. The presence of *Candida albicans* during the study period justifying the potential associations with the vertebrate hosts and the environment. Also, the seasonal shift observed across the fungal strains and their composition indicates that the studied variables could have significant influence in vector competence and mosquito biology during the disease transmission eras.

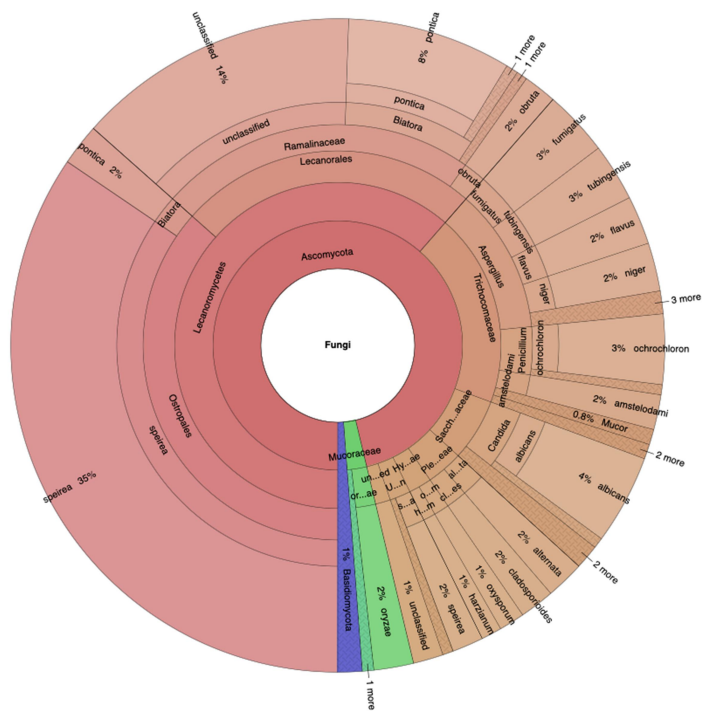


Figure. 4.9 Species specific distribution according to the monsoon season

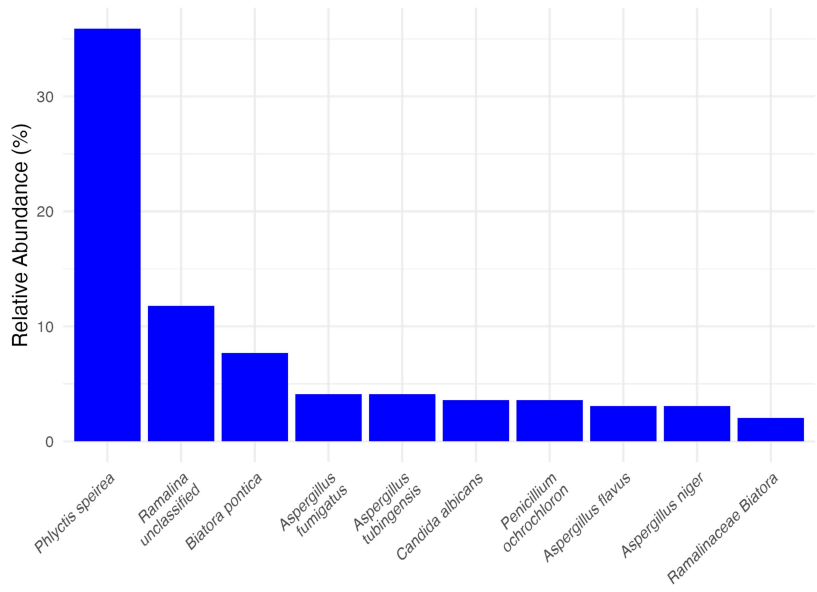


Figure. 4.10 Top ten Species distribution according to the monsoon season

Seasonal distribution of fungal strains revealed through Metagenomic strategies

Pre-monsoon season

The pre-monsoon seasons for *Aedes aegypti* was dominated by the fungal strain *Phlyctis speirea*, as found in the monsoon seasons. Similar to the earlier pattern, the fungi fall under Aspergilli was constituted major portion of the total population with *Aspergillus tubingensis* and *Aspergillus tubingensis* as the most dominant fungal strains, while *Aspergillus fumigatus* and *Aspergillus flavus* showed almost similar proportions. Furthermore, the presence of *Penicillium ochrochloron*, *Alternaria alternata*, *Rhizopus oryzae*, *Cladosporium cladosporioides* and demonstrated the possibilities of acquisition of the fungal strains from its surrounding environmental settings. The dominance of specific species fall under *Aspergillus* having the potential of drought resistance may depicting the adaptation towards the various seasons including the pre-monsoon season.

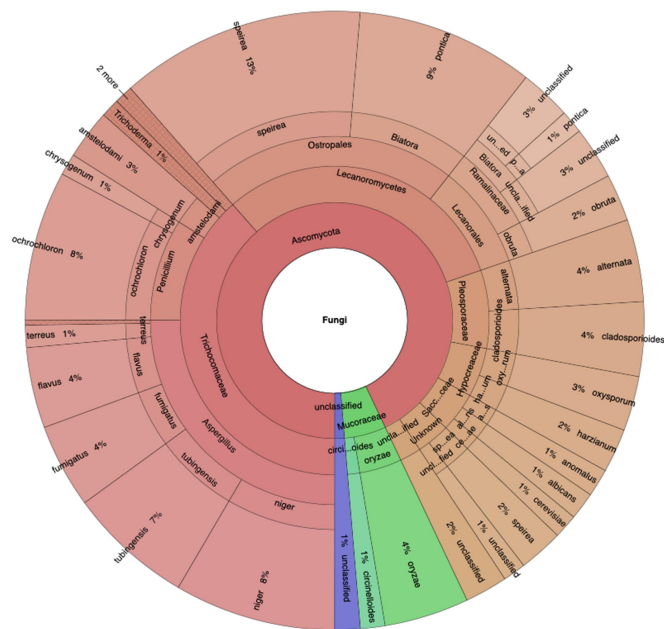


Figure 4.11 Species specific distribution according to the pre-monsoon season

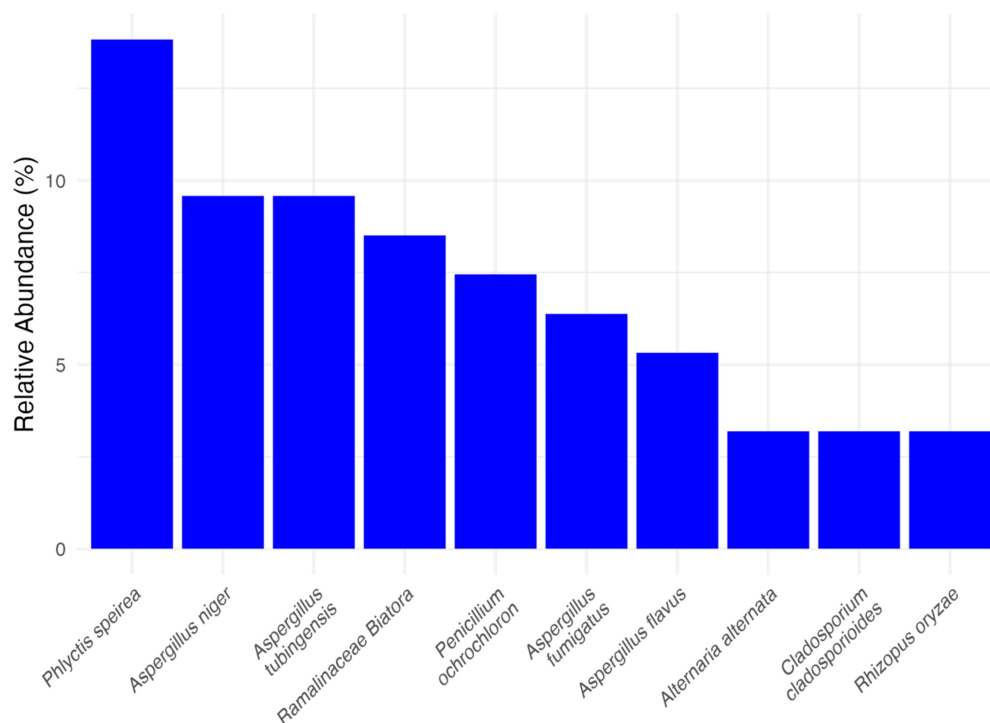


Figure 4.12 Species specific distribution according to the pre-monsoon season

Seasonal distribution of fungal strains revealed through Metagenomic strategies

Postmonsoon season

The post-monsoon seasons for *Aedes aegypti* was predominantly dominated by the fungal strain *Trichoderma erinaceum*. As found in the earlier observations and seasons, here the following *Aspergillus* spp. contributed significantly (42%) towards the whole population; *Aspergillus tubingensis*, *Aspergillus terreus*, *Aspergillus niger*, *Aspergillus fumigatus* and *Aspergillus flavus*. The presence of *Ramalina biatora*, *Penicillium ochrochloron*, *Alternaria alternata* and *Phlyctis speirea* indicating the potent acquisition of microbial consortia from the environmental setting during the post-monsoon seasons. The dominance of certain microbes fall under *Aspergillus* with the potential of drought resistance may indicate its adaptive nature towards various seasons including the pre-monsoon season.

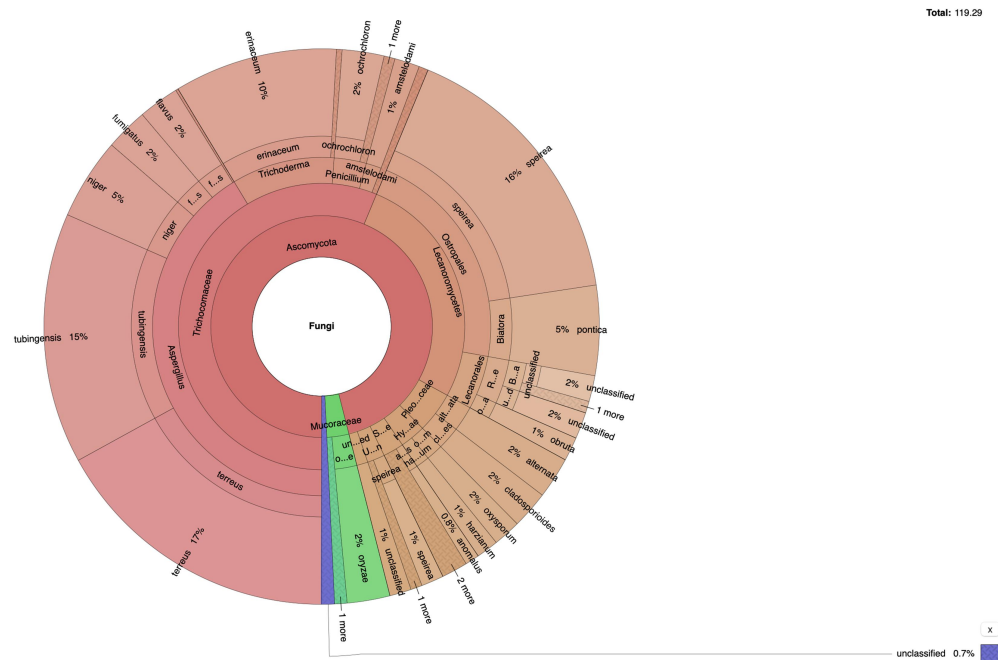


Figure 4.13 Species specific distribution according to the post-monsoon season

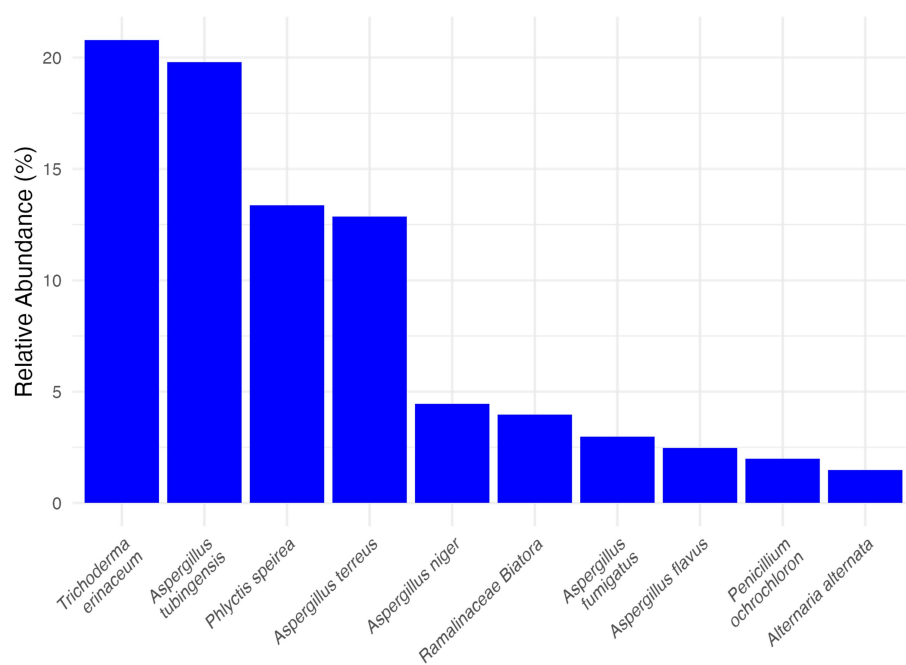


Figure 4.14 Species specific distribution according to the post-monsoon season

The table summarizes the alpha diversity (Table 4.1) indices of fungi isolated from three seasons of the *Aedes aegypti* mosquito vector, providing insights into species richness and diversity. Observed fungal species richness was highest during the AAPOFI season (1701 species), followed by AAMFI (1521 species), and AAPFI (1411 species). Chao1 and ACE estimates, which account for unseen species, confirmed this trend, with AAPOFI showing the highest estimated richness (Chao1: 1792.31, ACE: 1834.01), followed by AAMFI (Chao1: 1576.65, ACE: 1609.09) and AAPFI (Chao1: 1550.61, ACE: 1590.90).

Table 4.1 Alpha diversity indices of fungi

Sample	Observed	Chao1	se.chao1	ACE	se.ACE	Shannon	Simpson	InvSimpson	Fisher
AAMFI	152	1576.64527	11.59740204	1609.099031	31.87	18.03014187	4.730695243	0.95547588	22.45973658
AAPFI	141	1550.606061	22.37911939	1590.902402	48.02	18.68283148	4.889781116	0.967147676	30.43924686
AAPOFI	170	1792.313665	15.9671481	1834.007125	25.75	19.50511675	4.933870764	0.972983719	37.01471736

Shannon and Simpson indices, which measure species diversity, were highest in the AAPOFI season (Shannon: 19.51, Simpson: 4.93), indicating greater fungal diversity and evenness compared to the other seasons. The inverse Simpson index and Fisher's alpha also supported this pattern, with AAPOFI exhibiting the highest values (InvSimpson: 0.97, Fisher: 327.16). AAMFI exhibited the lowest diversity indices (Shannon: 18.03, Simpson: 4.73, InvSimpson: 0.95, Fisher: 288.35), suggesting relatively lower diversity compared to the other two seasons. Overall, these results highlight the seasonal variability in fungal richness and diversity associated with *Aedes aegypti*, with the AAPOFI season showing the highest diversity and richness. These findings emphasize the dynamic nature of fungal communities in response to seasonal variations in mosquito habitats.

The composition of microbial population for three seasons (Monsoon - AAMFI, Pre-Monsoon- AAPFI and Post -Monsoon- AAPOFI) according to the phylum indicated that the predominant phylum across all the three strains such as Monsoon - AAMFI, Pre-Monsoon- AAPFI and Post -Monsoon- AAPOFI is Ascomycota. Some of the following phylums contributed minimally towards the whole population; Basidiomycota, Chytridiomycota, Blastocladiomycota and Monoblepharomycota.

DISCUSSION

The results of the present study indicates the extensive diversity of fungal strains allied with the mosquito vector *Aedes aegypti*, justifying the significant role of the identified fungal strains in the target mosquito vector (Radl, 2024). The present study is recognized as one of the major investigation that combines various advanced strategies such as culture-based methods, molecular characterization and phylogenetic analysis for precise results.

As reported by S. Sharma et al., (2019), the presence of various fungal strains such as *Candida tropicalis*, *Trichoderma erinaceum* and *Aspergillus niger* indicates that the mosquito vector *Aedes aegypti* acquired the microbiome from its surrounding environmental settings. The pattern of dominance of fungi strains fall under the phylum *Ascomycota* in this study was reported in earlier observations and it justifying the adaptive potential of the phylum *Ascomycota* towards drastic environmental conditions. The prominent role of microbes under *Ascomycota* in decomposition verified in earlier studies remind that they may also engaged in nutrient cycling within the mosquito vector habitat. In addition to the aforesaid inferences, the presence of certain genera such as *Penicillium* and *Mucor* illustrates the significant role of the characterized fungi in nutrient cycling and adaptation towards threaten conditions within the mosquito vector habitat (Tobias, 2023).

The alpha diversity analysis completed in this study showed diverse richness across the various seasons because of strong possible environmental factors such as temperature and humidity followed by the habitat alterations. The elevated diversity observed during the AAPOFI season may directed towards a significant increase in interactions formed between the mosquito and the fungi.

The observed characteristic features of the reported fungal species along with the possible link need further investigation, since certain species like *Wickerhamomyces anomalus* has been previously known to produce entomopathogenic potential (Martin, 2018; Malassigné et al., 2020). The potential of *Wickerhamomyces anomalus* to produce toxic elements may recognized to be an added advantage for the mosquito vectors. Similar to this inference, the fungi fall under the genera *Candida* and *Aspergillus* already registered to produce secondary metabolites with the potential to impact the survival and pathogen transmission as reported by P Tawidian et al., (2019).

The characterization of *Trichoderma* and *Penicillium* generates specific questions concerning their ability to make antagonistic interactions with the mosquito vector *Aedes aegypti* and the allied pathogens. The ability of them to produce compounds with potent toxicity against microbiome including bacteria and virus are witnessed in earlier studies. However, the presence *Candida tropicalis* within *Aedes aegypti* instigating risks towards other organisms inhabiting the habitat animals (Cordeiro et al., 2014), indicating possible zoonotic infections.

Prominent range of genomic divergence has been observed in phylogenetic analysis. Precisely, niche specialization followed by adaptive radiation has been witnessed through the branching patterns and log likelihood values for the phylogentic trees of *Aspergillus* and *Mucor* genera. These findings directed towards the evolutionary forces that may possible reshaped the link between the mosquito vector *Aedes aegypti* and the investigated fungi groups (Girard et al., 2021). The clustering observed during the

phylogenetic analysis of diverse fungal strains from different niches suggests that the mosquito vector *Aedes aegypti* facilitates the dissemination of the studied fungal population. The genetic divergence reported in the present study within the same group of fungi suggesting its ability to fight against the drastic environmental conditions through adaptations.

Many of the earlier studies are strongly focused on the bacterial community while. The studies on the same inference towards fungi found scanty in the current scenario. The findings of the present study shed light on the principal role of the fungi with special reference to the mosquito vector *Aedes aegypti*. When we compare the fungal population against the bacteria, it was inferred that, rather than acts like endosymbionts, the fungi characterized in this study are primarily associated with the environmental elements.

The strong link between the bacterial and fungal communities could significantly impacts the various mechanisms and process allied with the vectorial capacity, physiology and immune responses of mosquito vectors (Cansado-Utrilla et al., 2021). Also, the secondary metabolites produced by the microbial consortia may form direct link with the various pathways within the mosquito metabolism. Therefore, studying these interactions could offers advantageous insights regarding the role of the chracterised microbial consortia in altering or manipulating the susceptibility.

As mentioned earlier, here we characterized and reported certain species fall under *Trichoderma* and *Wickerhamomyces* having the potential to represent as a strong candidates for the development of biocontrol approaches, hence they can surely be exploited as an alternative to synthetic elements.

The present study highlights the significance of certain fungi as a predominant constituent of mosquito microbial consortia. Moreover, the implications concerning the ecological dynamics of the mosquito vector *Aedes aegypti* along with its possible impact towards transmission of pathogen and habitat preferences has been unveiled in this investigation. The molecular characterization of various fungal taxa along with the complexity of the microbial consortia towards the mosquito vectors provides a novel way for the development of an efficient vector control strategy. Investigating the ecological roles of microbial consortia, especially the fungi, could offers diverse information to manage the microbial communities inhabited in other arthropod borne diseases vectors. By bridging the research gaps allied with the interactions of microbial consortia and mosquito vectors, specifically the *Aedes aegypti*, this study form a base for the future investigations targeting the microbial interactions to mitigate the mosquito born diseases and its impacts.

The identification of *Candida* species in both metagenomic and culture-dependent analysis is in line with earlier findings demonstrating the significance of yeasts in mosquito microbiome. It has been documented that the guts of *Aedes aegypti*, *Anopheles*, and *Culex* mosquitoes contain yeasts such *Candida tropicalis* and *Wickerhamomyces anomalus*. According to Ricci et al. (2011), *W. anomalus* can form long-lasting relationships with mosquitoes and may affect microbial interactions by generating antimicrobial chemicals. A notable finding of the present study is the dominance of *Phlyctis speirea*, *Bilatora pontica*, and unclassified Ramalinaceae members, particularly during the monsoon season. Reports of these lichen-associated fungi from *Aedes aegypti* are extremely limited or absent in the literature. Most previous studies have focused on gut-associated yeasts, environmental molds, and entomopathogenic fungi, whereas lichen-forming fungi have rarely been documented in

mosquito microbiomes. Their high abundance may reflect environmental acquisition from vegetation, tree bark, or lichen-rich habitats surrounding mosquito breeding sites. This observation suggests that the fungal diversity associated with *Aedes aegypti* may be broader than previously recognized. The high abundance of *Trichoderma erinaceum* during the post-monsoon season also differs from many earlier studies, where *Trichoderma* species were detected only at low frequencies. Species of *Trichoderma* are known for producing antifungal metabolites and may influence the structure of microbial communities through competitive interactions. Their seasonal enrichment during post-monsoon could be linked to favorable moisture conditions that promote their growth in breeding habitats.

Overall, the present findings corroborate previous studies showing that *Aedes aegypti* harbors a diverse assemblage of environmental fungi, yeasts, and saprophytes. However, the marked seasonal dominance of lichen-associated fungi (*Phlyctis speirea* and *Bilatora pontica*) and the abundance of *Trichoderma erinaceum* represent distinctive features of the current study. These results highlight the influence of seasonal environmental conditions on fungal community structure and suggest that local ecological factors may shape the mosquito mycobiome in ways not fully captured by earlier investigations.

Such seasonal fungal associations may have implications for mosquito ecology, microbial interactions, and potentially vector competence, warranting further functional studies.

Seasonal variations

The alpha diversity analysis revealed marked seasonal variations in the fungal communities associated with *Aedes aegypti*.

The AAPOFI sample recorded the highest number of observed taxa (170) and the highest richness estimates, with Chao1 (1792.31) and ACE (1834.01) values, indicating the presence of a highly diverse fungal community with many rare taxa. It also exhibited the highest diversity values, including Shannon (19.51), Simpson (4.93), Inverse Simpson (0.973), and Fisher's alpha (37.01), demonstrating greater species diversity and a more even distribution of taxa.

The AAMFI sample contained 152 observed taxa, with estimated richness values of Chao1 (1576.65) and ACE (1609.10). Although it showed considerable fungal richness, its diversity indices (Shannon = 18.03; Simpson = 4.73; Inverse Simpson = 0.955; Fisher's alpha = 22.46) were the lowest among the three samples, suggesting comparatively lower diversity and evenness.

The AAPFI sample had the lowest observed richness (141 taxa) but displayed moderate diversity, with Shannon (18.68), Simpson (4.89), Inverse Simpson (0.967), and Fisher's alpha (30.44) values. These results indicate that despite having fewer observed taxa, the fungal species were more evenly distributed than in AAMFI.

Overall, the alpha diversity analysis indicates that AAPOFI harbored the most diverse and species-rich fungal community, followed by AAPFI, while AAMFI exhibited the lowest fungal diversity among the samples. The consistently high richness estimators and diversity indices suggest that all three samples support complex fungal assemblages, with notable differences in community structure and species distribution.

These findings are broadly consistent with previous studies demonstrating that mosquito-associated microbial communities are strongly influenced by seasonal environmental conditions. Studies on *Aedes aegypti* and other mosquitoes have shown

that changes in rainfall, temperature, humidity, and breeding-site characteristics significantly affect microbial diversity and composition. For example, Gusmão et al. (2010) reported that the microbiota of *Aedes aegypti* reflects environmental microbial pools acquired during larval development and adult feeding. Similarly, Tchioffo et al. (2016) and Angleró-Rodríguez et al. (2017) observed that mosquito-associated microbial communities undergo substantial fluctuations in response to environmental conditions and habitat characteristics.

The higher fungal diversity observed during the premonsoon season may indicate increased opportunities for environmental acquisition of fungi from breeding waters, vegetation, and organic substrates. Conversely, the lower diversity during the post-monsoon period suggests that environmental conditions following heavy rainfall may favor the proliferation of a few highly adapted taxa, resulting in reduced community complexity. Similar seasonal shifts in fungal diversity have been documented in aquatic ecosystems and insect-associated microbiomes, where environmental disturbances lead to changes in species dominance and community structure.

CONCLUSION

This chapter investigated the diversity and possible ecological role of the various fungi inhabited. In the *Aedes aegypti* mosquito vector. This study have integrated both culture-dependent strategies and molecular approaches to characterize and identify the fungal species fall under the genera *Aspergillus*, *Candida* and *Trichoderma*. The phylogenetic analysis of the characterized fungal genera produced extensive genetic diversity within the same genera. The statistical approaches including Shannon diversity analysis and Simpson diversity analysis revealed maximum diversity during the studied period, justifying the seasonal variation across the strains. The direct link between the characterized fungi and mosquito vector may impacts the transmission of pathogen, mosquito biology and interaction with surrounding setting. Hence, unveiling this dynamics can provide a strong way for the development of an efficient tool for mosquito diseases control.

CHAPTER 5

**TO FIND OUT THE INTER AND INTRASPECIFIC GENETIC
DIVERGENCE BETWEEN BACTERIAL AND FUNGAL
ISOLATES FROM THE FOURTH INSTAR *AEDES AEGYPTI*
LARVAE COLLECTED FROM KOLE WETLANDS.**

INTRODUCTION

As introduced in the former strands, the *Aedes aegypti* mosquito vectors are primarily considered to be the health threatening element as it spread many diseases such as Zika, yellow fever, and chikungunya towards the human population (Leta et al., 2018). Furthermore, it is vital to unveil the predominant role of microbial population towards the *Aedes aegypti* mosquito vector since the earlier studies have witnessed the multifaceted potential of microbial populations. The microbial consortia includes both the bacteria and fungi and they can be transmitted to the mosquito vector either through the food or by means of certain elements allied with the habitat as reported by (Guégan, Zouache, Démichel, Minard, Tran Van, et al., 2018).

According to the earlier studies, none of them have investigated the inferences concerning the genetic divergence of bacteria and fungi strains with special reference to the *Aedes aegypti* mosquito vector from the current study site (Zouache et al., 2022). The microbial populations of current study site, precisely, the wetland ecosystems are prominently impacted by a plethora of environmental factors including the organic matter availability, quality of water followed by the variations across the seasons (Faulwetter et al., 2009). The potential role of microbial population including both the fungal and bacterial strains in mosquito vector is multifaceted in *Aedes aegypti*, since they have significant role in nutrient acquisition, digestion, immunity and resistance towards pathogens as verified by (Scolari, Casiraghi, & Bonizzoni, 2019),

Investigating the genomic variation within and between both the fungus and bacteria can provide significant information regarding the ecological function towards the mosquito vector population. From this point of view, here the present study focused to investigate the genetic divergence within and between the various fungal and microbial strains according to the seasons.

MATERIALS AND METHODS

Data Collection and Microbial Isolation

The current study aimed to investigate the genomic diversity flowed by functional role of microbial consortia (Bacteria and Fungi) inhabited in the *Aedes aegypti* mosquito larvae, collected from the Kole Wetland ecosystem of Thrissur, Kerala, India. Sterile conditions were maintained in the laboratory to dissect the *Aedes aegypti* mosquito larvae, which were proceeded for culture dependant analysis of fungal and bacterial strains. The following microbial population were isolated from the aforesaid steps; *Bacillus*, *Enterobacter*, *Stutzerimona*, *Aspergillus*, *Penicillium*, and *Mucor*.

DNA Extraction and Sequencing

After the isolation of specific microbial population using culture dependant manner, they were proceeded for further molecular analysis including DNA Extraction and Sequencing. The DNA of bacteria and fungal population was extracted using the QIAamp DNA Mini Kit (Qiagen) and GeneJET Kit (Thermo Fisher) respectively. NanoDrop spectrophotometer was used to determine the purity and concentration of the extracted DNA. This study have preferred (ITS) region and 16S rRNA gene for fungal strain and bacterial strain respectively. With unique and species specific primers, PCR has been completed.

Sequence Quality Control

To ensure the quality, the raw sequences were trimmed and processed. Sequences with relatively low-quality was removed. Phylogenetic analysis was completed once the alignment has been completed. Also the duplicate sequences were removed.

Sequence Alignment and Genetic Analysis

The genetic variation was determined according to the NJ-K2P method (Kimura 2-parameter model), Needleman-Wunsch algorithm with R and MEGA 10 software (Malesu, Mishra, & Ojha, 2015) . The genetic divergence or genomic differences within and between the bacteria and fungi was determined. The means genetic difference was calculated for each bacterial and fungal strains to get significant information regarding the genetic diversity within and between the microbial consortia.

Visualization and Statistical Analysis

Heat map was constructed to visualize the genetic diversity observed within and between the microbial populations. All the analysis has been completed with MEGA X, SPSS version 27.0 and Software 4.0.

RESULTS

This study investigated the inter and intra genomic variation between fungus and bacteria and the corresponding observations have been given in Figure 5.1 to Figure 5.3. Among the various bacteria investigated, the *Bacillus* species showed moderate intra-specific genetic distances of 0.25–0.30. Moreover, the *Bacillus wiedmannii* and *Bacillus cereus* produced strong genomic relationship compared to the *Bacillus subtilis*. Similar to this observation, *Enterobacter* species (0.20 to 0.25) and *Rahnella* species (0.22 to 0.27) reported with moderate genetic divergence. Among the various fungi investigated, *Aspergillus niger* formed strong genomic similarity with *Penicillium ochrochloron*, while the *W. anomalus* exhibited elevated genetic divergence. The reported findings highlight the significant role of evolutionary forces in altering the genomic diversity in mosquito vector population.

For fungi, *Aspergillus niger* exhibited close genetic similarity to *Penicillium ochrochloron*, reflecting potential phylogenetic overlap, while *W. anomalus* and *Mucor* species showed higher levels of genetic divergence, highlighting unique evolutionary trajectories. These findings illuminate the evolutionary pressures shaping genetic diversity among microbial species in mosquito microbiomes.

Bacillus spp.

The variation observed within the *Bacillus* species may indicate its evolutionary adaptations towards diverse ecological niches allied with the mosquito vector. The mosquito vector usually serves as a base for the bacterial colonization and offers specific forces that may have a principal impact towards the genetic divergence. The differences in genetic variation could represent diverse ecological roles with some of the microbial

population showing extensive adaptations that make fit for the specific environment within the mosquito vector.

Enterobacter spp.

The analysis of genomic variation for the genera *Enterobacter* consists *Enterobacter* cloaca, *Enterobacter hormaechei* and *Enterobacter roggenkampii* with 808 bp, 808 bp and 549 bp respectively and the finding indicated that the genetic distance within studied genera was ranged from 0.20 to 0.25, justifying moderate divergence. In addition to this, the *Enterobacter* cloaca and *Enterobacter hormaechei* produced comparatively more close genetic relationship than the *Enterobacter roggenkampii*. The identical sequence length exhibited by both the *Enterobacter* cloaca and *Enterobacter hormaechei* unveils a strong conservation of genomic characteristic feature between these two organism, possibly indicating the fact that both of them, may shares the pattern of ecolgocal functions within the mosquito vector populations. Relatively the *Enterobacter roggenkampii* would show a shorter sequence length and it indicating the unique way towards the evolutionary path and probably it may assists the same to adapt to altering drastic conditions.



Figure 5. 1 Genetic variation both within and between bacterial strains

Stutzerimonas spp.

The analysis of genomic variation for the genera *Stutzerimonas* consists *Stutzerimonas stutzeri*, and *Stutzerimonas kunmingensis* with 635 bp and 635 bp respectively and the finding indicated that the genetic distance within studied genera was found to be very low from 0.15 to 0.20. As found earlier, the organisms under *Stutzerimonas* shares close similarity. The low genomic variation followed by identical sequence length between these species indicates a possible recent divergence or strong external or internal pressure maintaining the reported genomic similarity.

Rahnella spp.

The analysis of genomic variation for the genera *Rahnella* encompassed *Rahnella aquatilis* and *Rahnella aceris* with 767 bp and 804 bp respectively and the finding indicated that the genetic distance within studied genera was found to be moderate from 0.22 to 0.27. The variation in sequence length indicates that there may be a possible deletion or insertion may happened during the process of divergence. Except the variation in sequence length, both of them may maintained intra specific similarity. The reported evolutionary mechanisms and the genomic variation may slightly allied with their adaptation to specific unique conditions within the studied mosquito vector *Aedes aegypti*.

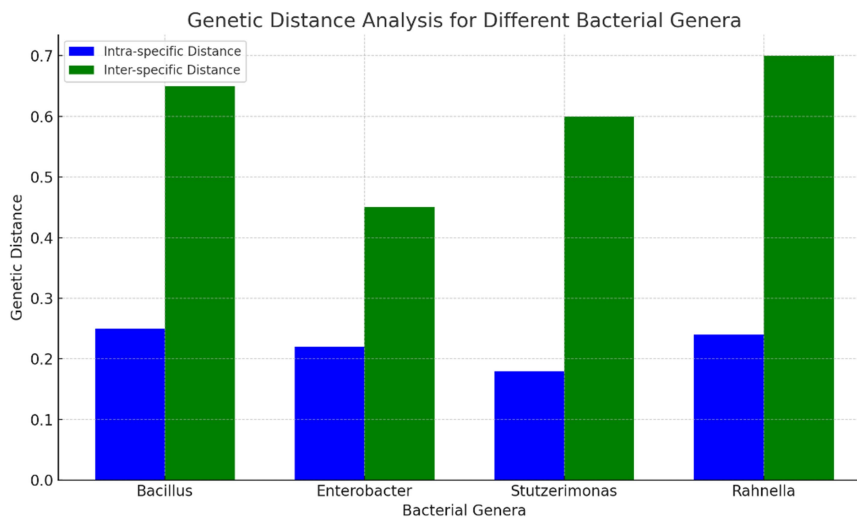


Figure 5.2 Genetic distance for major bacterial genera

Inter-specific Genetic divergence

Inter-specific Genetic divergence in Enterobacteriaceae Family

Enterobacter, *Klebsiella*, and *Rahnella* showed close genetic relationships, with an average genetic distance ranging from 0.35 to 0.45. This suggests a common ancestral

origin for these genera and reflects their evolutionary path within the larger Enterobacteriaceae family.

***Bacillus-Enterobacteriaceae* Relationship**

A higher genetic distance (0.65-0.75) was observed between *Bacillus* species and members of the Enterobacteriaceae family. This deeper genetic divergence is consistent with the distinction between Gram-positive and Gram-negative bacteria and reflects ancient evolutionary events that have shaped their separate biological functions and ecological adaptations.

Mycolicibacterium vaccae

Mycolicibacterium vaccae exhibited the highest genetic divergence (>0.80) and possessed the longest sequence length (1078 bp), indicating a distant evolutionary relationship from the other genera analyzed. This divergence may point to a unique evolutionary trajectory that is not closely tied to the ecological niches occupied by the other bacteria isolated from mosquitoes.

***Stutzerimonas*-Other Genera**

Stutzerimonas showed moderate to high genetic distances (0.50-0.70) when compared with other genera, suggesting that it follows a unique evolutionary trajectory within the mosquito microbiome. These distances highlight *Stutzerimonas*' distinct genetic history, which may be shaped by specific selective pressures encountered within the mosquito host.

The analysis concerning the inter-specific genetic divergence of Enterobacteriaceae Family revealed that *Klebsiella*, *Enterobacter* and *Rahnella* produced extensive genomic relationships ranging from 0.35 to 0.45. The reported findings clearly indicates

that they shares the common ancestral origin earlier and hence this pattern has been observed within the Enterobacteriaceae family.

Bacillus and Enterobacteriaceae

There exists an extensive genetic divergence between the organisms fall under *Bacillus* and Enterobacteriaceae ranging from 0.65 to 0.75. The wide genetic divergence is aligned with their broad group (Gram positive and Gram negative) and justifying the role of evolutionary events happened in ancient times that makes these two groups as separate with diverse ecological and biological functions.

Mycolicibacterium vaccae

It revealed elevated genetic divergence of >0.80 along with highest sequence length (1078 bp). The aforesaid findings distinguish the same from other in terms of the evolutionary characteristic features.

Stutzerimonas and other significant groups

The *Stutzerimonas* exhibited relatively elevated genetic distance raging from 0.50 to 0.70 and this finding unveils the unique phylogenetic track of *Stutzerimonas*. Furthermore, the elevated genetic distance illustrate its genomic history which may be altered by the specific environmental or genetic forces allied with the mosquito vector.

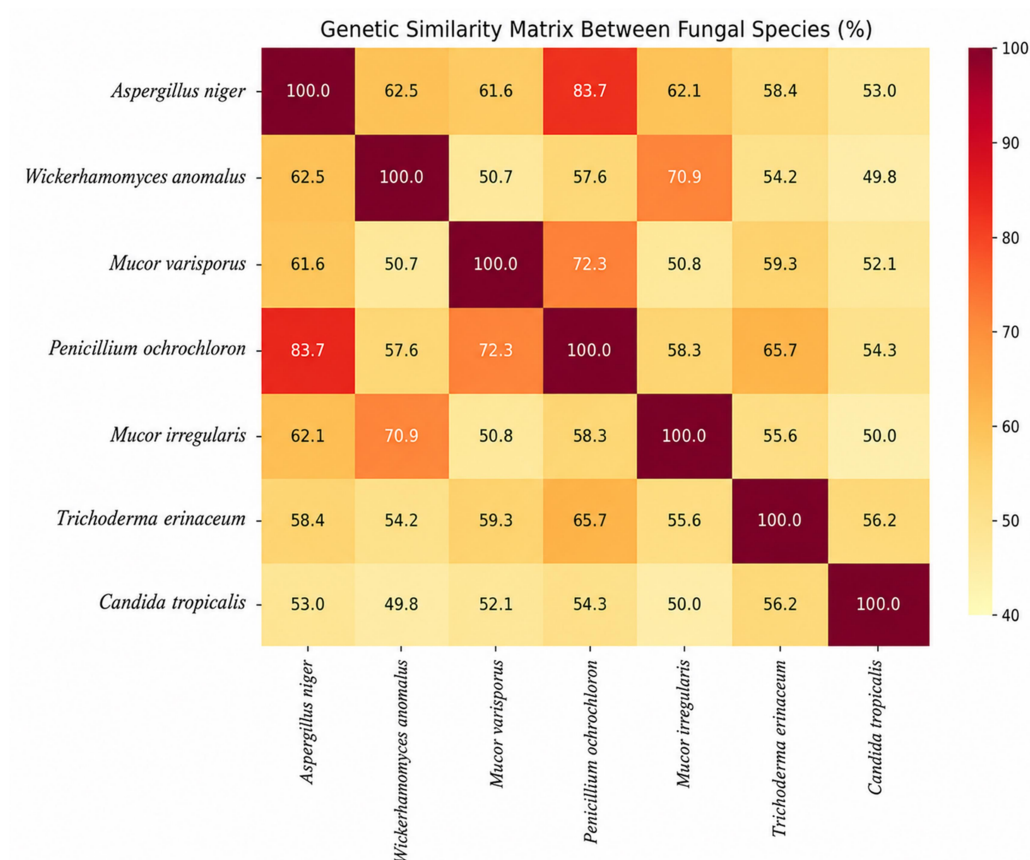


Figure 5.3 Genetic variation both within and between fungal strains

The least intra specific genomic variation was witnessed in *Stutzerimonas* while moderate intra specific genomic variation has been observed in *Enterobacter* species ranging from 0.20 to 0.25. The highest intra specific genomic variation ranging from 0.25 to 0.30 was reported in *Bacillus* species. These observations suggest that various levels of genetic divergence has been observed within each genus, while some of them showing relatively more rapid divergence primarily driven by the ecological parameters allied with the adaptations to the environment inhabited by the mosquito vectors.

Inter-specific Genomic variation

The inter-specific genomic variation clearly distinguish both the Gram-positive and Gram-negative bacteria followed by an average genetic distance of 0.78. The maximum genetic divergence was observed between the Enterobacteriaceae and *Bacillus*.

The variation in sequence length across the various studied organisms indicates that the evolutionary mechanisms including gene rearrangement, deletion and insertion may alter the genetic diversity of the studied microbial population. Also, the specific strong forces or pressures within the mosquito vector may recognized to be the major driving force behind this genetic divergence. The genetic variation observed in the present study unveils a plethora of evolutionary patterns among the microbial consortia and the differences observed in distances and sequence length offers valuable insights concerning the evolutionary history of the studied bacteria, consisting both recent and ancient events.

Genomic variation across selected fungal strains

Aspergillus niger

The *Aspergillus niger* has been recognized as one of the predominant fungi identified from the *Aedes aegypti* mosquito vector and it exhibited strong similarity of 84% towards the *Penicillium ochrochloron*. This circumstance justified the phylogenetic overlap between the studied *Aspergillus niger* and *Penicillium ochrochloron*. The inter specific genetic variation observed in *Aspergillus niger* was ranging from 15.39% to 49.63%. The least inter specific genetic variation was observed between *Aspergillus niger* and *Penicillium ochrochloron* while the highest inter specific genetic variation was observed with *Aspergillus tubingensis*. Furthermore, the evolutionary analysis of *Aspergillus niger* reveals a close association with *Penicillium* than others fall under the

Aspergillus genera and this pattern may suggest a possible divergence within the *Aspergillus*.

Wickerhamomyces anomalus

The *Wickerhamomyces anomalus* showed considerable range of genomic variation (29.08% to 50.80%) with strong association towards the *Mucor irregularis*, while the distant species was reported to be the *Aspergillus tubingensis* (50.80%). The reported unique genetic profile of *Wickerhamomyces anomalus* justifying the capability of the same to manage drastic environmental condition through evolutionary process.

Mucor species

The *Mucor* species produced moderate range of inter-specific genomic variation within a range of 28.18% to 49.33% with strong association towards the *Mucor irregularis*, while the distant species was reported to be the *Wickerhamomyces anomalus* (49.33%). These findings indicated that the the fungi fall under *Mucor* genera are principally shows high degree of genetic diversity, even if they also shares similarity with *Penicillium ochrochloron*.

Penicillium ochrochloron

The *Penicillium ochrochloron* formed noticeable inter-specific genomic variation within a range of 15.39% to 45.49% with strong association towards the *Aspergillus niger*, while the distant species was reported to be the *Aspergillus tubingensis* (45.49%). These findings indicated that the fungi fall under *Penicillium* genera are principally shows genetic diversity and it may shares common ancestor origin in ancient times with *Aspergillus*. The strong genetic relationship of *Penicillium ochrochloron* with *Aspergillus* offers the insights towards the genomic dynamics of the studied fungal isolates along with their environmental settings inhabited by the mosquito vectors.

DISCUSSION

This study contributes the scientific community to explore genetic variation followed by the evolutionary relationships of both fungal and bacterial isolates retrieved from the *Aedes aegypti* mosquito vectors. The reported findings are not only revealing the microbial ecology but also showing the possibilities of exploiting the prominent applications of these isolated microbial consortia for the development of efficient mosquito vector control approaches. The variable genetic diversity observed across the microbial consortia directs the way for the researchers to track the evolutionary inferences and ecological significance influenced by the environmental or genetic forces allied with the surrounding environment of mosquito vectors.

Both the Bacteria as well as the fungi possessed significant roles in altering the ecology and biology of the mosquito vector *Aedes aegypti*, predominantly by impacting the survival, vector competence and reproduction of the target mosquito vector *Aedes aegypti* (Zouache et al., 2022).

The microbial consortia, especially the reside inside the mosquito vector *Aedes aegypti* is always recognized to be predominant element of its physiology by contributing assistance for various following processes required for its life cycle; pathogen resistance, acquisition of nutrients from surrounding and modulation of immune systems (Gabrieli et al., 2021). Similarly, the fungi inhabited within the mosquito vector *Aedes aegypti* can impact the various developmental cycles of its larvae and serving as a biocontrol agents of natural origin (Paula, Ribeiro, Lemos, Silva, & Samuels, 2019). The aforesaid strong associations between the microbial consortia and the mosquito vector illustrates the ecological importance of the both the fungi and bacteria.

The variation in genomic characteristic features reported between and within the bacterial population in this study offers insights towards the adaptive nature managed through the evolutionary processes. The microbes fall under the *Stutzerimonas* genus showed relatively very low genetic divergence with potent genetic conservation. This circumstance indicates either the incidence of recent divergence or prominent environmental genomic-environment forces maintain the genetic stability. This genetic conservation unveiled that *Stutzerimonas* may usually occupy the almost similar niches inside the mosquito vector, with genomic forces supporting the genetic elements retention that are principally essential for the survival.

According to the findings of earlier studies (Frankel-Bricker, Buerki, Feris, & White, 2020; Luis et al., 2019) the fungal species are strongly associated with the *Aedes aegypti* mosquito vectors and are known to produce extensive genetic diversity, that helps the mosquito vectors to persists in the same habitat. As reproved earlier, the *Aspergillus niger* has been recognized as one of the predominant strain isolated from the *Aedes aegypti* mosquito vector. The strong similarity of 84% towards the *Penicillium ochrochloron* justified the phylogenetic overlap between the studied *Aspergillus niger* and *Penicillium ochrochloron*. The reported phylogenetic overlap between *Aspergillus niger* and *Penicillium ochrochloron* justifies the significant similarities according to their functions, especially either in contributing the nutrient cycling or in degrading the organic elements exists in the mosquito environment.

The predominant role of certain fungal strains fall under the genera *Wickerhamomyces* and *Mucor* in mosquito physiology extend beyond to nutrient acquisition to consider Signiant application s in mosquito vector control. For example, the fungal strains can be serve as a prominent pathogen targeting the mosquito larvae or adults thereby disrupting their life cycle and diminishing the mosquito populations diversity.

The evolutionary inferences concerning the genomic variation across both the bacterial and fungal strain evident in this study indicated the significance of this study in the current scenario (Leducq, 2014). The reported genetic variation observed between the different genera and groups of microbial consortia indicates the possible incidence of evolutionary events recently through the gene rearrangements or deletion or mutation that make fit the same to adapt towards specific environmental or genomic forces. The elevated genetic distance observed between the organisms fall under Enterobacteriaceae and Bacillus justifies the evolutionary split happens during ancient times between the Gram- positive and Gram-negative bacteria by the altered ecological adaptation.

The genetic variation reported across the fungal strains offers novel insights regarding the predominant role of evolution in altering the diversity of microbial consortia within the mosquito vector and the surrounding (Naranjo-Ortiz & Gabaldón, 2019). Among the various fungal strains characterized in this study, the *Aspergillus niger* followed by the *Penicillium ochrochloron* exhibited strong genomic association (84.61%). The important phylogenetic overlap may suggests that *Aspergillus niger* and *Penicillium ochrochloron* may have exposed to evolutionary forces that changed their genetic profile and makes fit for the new environment.

The potential of the isolated fungal strains to persists across diverse settings within the mosquito vector may varied according to the mosquito vector species and the surrounding environment. Moreover, certain species like *Penicillium ochrochloron* may share close genomic relationship with *Aspergillus niger* and posses their own unique adaptations that may allow them to fit for the changing environments. The genetic divergence found between the *Aspergillus tubingensis* and *Aspergillus niger* indicated that the evolutionary and environmental pressures can drive specific events thereby the selected species can co-exists within the same organisms while possessing diverse

ecological role. Understating the dynamics allied with aforesaid perspectives with special inference on host biology, environmental elements and microbial diversity is vital for developing efficient strategies for mosquito control. The strong genomic association between the *Penicillium ochrochloron* and *Aspergillus niger* underscore the significance of investigating the microbial consortia allied with the mosquito vectors. The specific genetic and environmental factors shaping diversity of microbial consortia inhabiting the *Aedes aegypti* illustrates the complex link found between the studied microbial strains and the mosquito vectors. This circumstance emphasize the importance of novel and advanced approaches in mosquito vector biology studies.

The relatively low genetic distance observed among *Enterobacter*, *Klebsiella*, and *Rahnella* indicates that these genera share a recent common ancestral lineage within the family Enterobacteriaceae. Since all three belong to the γ -proteobacterial clade, conservation in their 16S rRNA gene sequences is expected, resulting in shorter branch divergence in phylogenetic analysis. Members of Enterobacteriaceae generally retain homologous ribosomal regions because they have evolved from a similar enteric bacterial stock and continue to occupy nutrient-rich host-associated environments. Mosquito microbiome studies repeatedly report that members of Enterobacteriaceae constitute one of the dominant and stable fractions of the mosquito midgut microbiota, particularly in *Aedes*, *Culex*, and *Anopheles* (Yadav et al., 2015). The significantly greater genetic distance observed between *Bacillus* species and Enterobacteriaceae isolates reflects a deep phylogenetic separation at the phylum level. *Bacillus* belongs to the phylum Firmicutes and is Gram-positive, whereas *Enterobacter*, *Klebsiella*, and *Rahnella* belong to Proteobacteria and are Gram-negative. This distinction is ancient in bacterial evolution and is accompanied by major differences in: cell wall architecture, GC content, ribosomal gene organization, sporulation genes, metabolic pathways.

Because 16S rRNA sequence divergence accumulates over long evolutionary periods, members from these two phyla naturally show wider branch distances in phylogenetic reconstruction. Previous mosquito gut analyses also observed that although both Enterobacteriaceae and Bacillaceae are common in mosquitoes, they occupy distinctly separate phylogenetic clades, indicating independent evolutionary recruitment into the mosquito gut microbiome. *Bacillus* species are spore-forming environmental bacteria with exceptional stress resistance. Their presence in mosquito gut often originates from breeding water, detritus, and nectar sources. Unlike enteric Gram-negative bacteria, *Bacillus* survives via endospore formation and extracellular enzyme production rather than epithelial biofilm-based colonization. Thus, despite sharing the same host environment, *Bacillus* and Enterobacteriaceae represent ecologically coexisting but evolutionarily distant lineages (Pidiyar et al, 2004). *Mycolicibacterium vaccae* showed the highest genetic distance and the longest aligned sequence, suggesting a markedly independent phylogenetic placement. This organism belongs to the phylum Actinobacteriota, which is evolutionarily distant from both Proteobacteria and Firmicutes. Its cell wall contains mycolic acids, giving it a distinct molecular identity, high GC-rich genome organization, and unique ribosomal signatures. Its detection in mosquito gut may indicate acquisition from larval breeding substrates. However, its strong divergence suggests that it has followed an independent evolutionary trajectory unrelated to the core enteric bacteria. Hence, *M. vaccae* likely represents a phylogenetically ancient, ecologically opportunistic, non-core but stress-adapted associate within the mosquito microbiome (Mancini et al.,2018). The lowest genetic distance recorded between *Aspergillus niger* and *Penicillium ochrochloron* indicates a close phylogenetic affinity because both are filamentous ascomycetous fungi belonging to evolutionarily related Eurotiomycete lineages. Their ITS regions commonly retain

conserved ribosomal motifs, resulting in higher sequence similarity. Both species are saprophytic environmental colonizers frequently associated with sugar-rich decaying substrates from which mosquitoes acquire fungal propagules. This low divergence suggests recent lineage separation with partial conservation of ancestral genomic characters. Both fungi may survive in the mosquito gut through similar mechanisms such as: carbohydrate utilization, extracellular enzyme secretion, tolerance to fluctuating pH. Thus their clustering indicates functional as well as evolutionary similarity. Although both belong to the same genus, the unexpectedly high divergence with *Aspergillus tubingensis* suggests marked species-level ITS polymorphism. Black *Aspergilli* are known to possess cryptic genetic diversification despite morphological resemblance, and their ribosomal spacers often evolve rapidly under environmental selection. This represents intra-generic but inter-specific molecular divergence, where same-genus taxa follow distinct adaptive histories. It indicates that all *Aspergillus* members isolated from mosquitoes are not evolutionarily equivalent; some may be transient contaminants while others may be gut-adapted associates. *Mucor* species showed intermediate genetic separation, confirming their placement in a different fungal lineage (Mucoromycota) compared with the dominant ascomycetous fungi. The comparatively lower distance with *Penicillium ochrochloron* may reflect partial ITS conservation, whereas greater divergence from yeast forms indicates distant ancestry. *Mucor* represents an early diverging non-ascomycetous lineage recruited into the mosquito gut from moist breeding habitats. Its occurrence suggests environmental acquisition through larval water sources rather than permanent gut specialization. Moderate divergence indicates ecological coexistence but phylogenetic independence. *Penicillium ochrochloron* showed closest similarity with *A. niger* because both belong to related saprophytic Ascomycota with conserved ribosomal ancestry. However, its

high divergence from *A. tubingensis* indicates that mosquito-associated filamentous fungi are composed of multiple independent ascomycete branches rather than one uniform clade. This demonstrates phylogenetic sub-clustering among filamentous fungi based on ancestral ribosomal conservation. *Penicillium* species are known for strong secondary metabolite production and substrate decomposition; therefore their persistence in mosquito gut may be linked with nutrient transformation and competition with other microbes. *Wickerhamomyces anomalus* displayed substantial divergence because it is a saccharomycetous yeast, taxonomically distant from the filamentous molds analyzed. Unlike *Aspergillus*, *Penicillium*, and *Mucor*, this yeast belongs to a unicellular fermentative lineage with distinct ITS architecture and separate evolutionary history. Importantly, previous studies have specifically reported *W. anomalus* as a stable mutualistic symbiont in mosquitoes with nutritional, protective and antimicrobial roles. Its high divergence reflects functional specialization rather than accidental occurrence. Because *W. anomalus* is known to persist through mosquito developmental stages, tolerate blood meal conditions, produce antimicrobial killer toxins. Its separate phylogenetic position suggests a specialized yeast symbiont lineage within mosquito mycobiota rather than a simple environmental mold contaminant (Cappelli et al., 2021).

CONCLUSION

This study exploits the genomic variation observed across the fungal and bacterial strains within the *Aedes aegypti* mosquito vectors with special reference to ecological adaptations as well as evolutionary inferences. The following bacteria genera were reported to exhibit diverse range of genetic variation; *Bacillus*, *Enterobacter* and *Stutzerimonas*. Among the various bacteria investigated, the *Bacillus cereus* and *Bacillus wiedmannii* showed potent genetic similarity while the *Bacillus subtilis* produced more genetic divergence, justifying the unique functional role of *Bacillus subtilis*. Similar to this observations, *Enterobacter* species unveiled moderate genomic variability and revealing the possible genetic elements conservation according to the ecological role. The fungal strains also produced prominent genomic variation in which *Aspergillus niger* was reported to show the maximum similarity with *Penicillium ochrochloron* indicating the phylogenetic overlap. Moreover, the fungi under the *Mucor* genera also exhibited elevated genetic divergence towards the *W. anomalus* and it validating their unique track on evolutionary path. Altogether, the genomic differences reported in this study illustrates the possibilities of occurring both ancient and recent events allied with their evolutionary track that shaped by the environmental settings of mosquito vector *Aedes aegypti*.

SUMMARY

SUMMARY

This study collectively assessed the diversity of microbial consortia inhabited in the fourth instar larvae of *Aedes aegypti* collected from the Kole wetlands, Thrissur, Kerala, India. The culture dependent approaches followed by the molecular strategies including the 16S rRNA sequencing and metagenomic analysis allow us to differentiate 15 different microbial species including *Enterobacter roggenkampii*, *Bacillus pacificus*, *Rahnella aquatilis*, and *Klebsiella quasivariicola*. The Phylogenetic analysis conducted for the aforesaid selected strains with the aid of Maximum Likelihood method illustrated prominent genetic diversity within each selected genera especially in *Enterobacter*, *Rahnella* and *Bacillus*. The metagenomic analysis constituting the diversity matrices for the selected strains (AAMA, AAMB, and AAMC) of microbes according to various seasons identified predominant bacterial population according to the following phyla: Proteobacteria, Firmicutes, and Actinobacteria. Proteobacteria. This study also implemented the heatmap analysis to illustrate the relative abundance of most dominant genera including the *Clostridium* and *Listeria*. Based on the major findings regarding the fungal community, *Aspergillus niger*, *Candida tropicalis* and *Trichoderma erinaceum* are reported to be the diverse fungal population inhabited in the *Aedes aegypti*. The metagenomic analysis combined with the Shannon and Simpson analysis validated the seasonal dynamics of fungal and bacterial population. Furthermore, both the bacterial population and fungal strains showed significant range of genetic divergence, indicating their unique way in evolutionary paths. The results from the present study offers significant information concerning the role of microbial evolution within the mosquito vectors and the potential implications towards the mosquito vector biology and transmission dynamics.

RECOMMENDATIONS

The current study shed light on different microbes, especially bacteria and fungi present inside the gut of fourth instar larvae of *Aedes aegypti*. But a lot of research gaps are to be addressed in the study which includes,

- ❖ The role of bacteria and fungi in the survival of mosquito larvae is least studied which should be addressed in future studies.
- ❖ More studies on role of bacteria in the development of insecticide resistance in mosquitoes and the gut can be used as a source of insecticide degrading bacteria.
- ❖ Accurate study on different mosquitoes surviving in a habitat like kole wetlands, because the kole wetlands are most avoided habitat of mosquitoes in their control strategies.
- ❖ Use of isolated bacteria from the gut as a weapon against the mosquito itself is more useful to be used as a biological control method.
- ❖ More detailed study on microorganisms inside *Aedes aegypti* larval gut other than bacteria and fungi and their phylogenetic relationships to unveil the evolutionary relationships between them as most studies ignore the fact that it may harbor them too.
- ❖ Novel strategies to control mosquitoes in their larval stage itself in kole wetlands as it will be more difficult to use the current methods in a vast area like kole wetlands.

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PUBLICATIONS



"Symbiotic secrets: exploring the gut microbiome of *Aedes aegypti* larvae and its role in mosquito physiology"

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Abstract

Mosquitoes, particularly *Aedes aegypti*, are of paramount importance in scientific research due to their critical epidemiological significance in transmitting dangerous diseases. Their ability to thrive amidst complex environmental conditions is largely attributed to their symbiotic relationships with microorganisms. In this comprehensive study, we explored the intricate bacterial communities within the gut of fourth instar *Ae. aegypti* larvae sourced from the underexplored Kole wetlands. Our investigation employed a blend of culture-dependent and culture-independent techniques, revealing a diverse array of bacteria, notably Bacillaceae and Enterobacteriaceae. Notably, *Rahnella* was found to be a consistent gut inhabitant across seasons and locales. Metagenomic analysis uncovered the presence of various phyla, with Firmicutes dominating during monsoon and post-monsoon seasons, while Proteobacteria prevailed in the pre-monsoon season. These findings lay a strong foundation for future research into the intricacies of host-microbiota interactions and their implications in mosquito-related diseases, offering insights into public health concerns.

Keywords Gut microbiota · Bacterial diversity · Metagenomic profiling · *Aedes aegypti* · Culture-dependent analysis

Introduction

Mosquitoes obtained more attention than other insect vectors because they could potentially transmit many pathogens, inflicting massive diseases (Ranasinghe et al. 2021). *Aedes* mosquitoes are extremely relevant as they spread deadly diseases such as Chikungunya, Dengue, Lymphatic filariasis, Rift Valley fever, Yellow fever and Zika (Ratmanov et al. 2013). Several methods are followed to obliterate the vector due to the seriousness of the diseases. Habitat manipulation, use of insecticides, biopesticides and release of sterile males (sterile Insect Technique) are some strategies deployed in the past decades.

This kind of traditional mosquito control measures have many limitations, particularly the development of insecticide resistance, which has led to efforts to enhance novel methods for implicit tackling strategies. Even though insecticides have been established to be positive in numerous contexts, the economic fee of their application is logistically challenging in each city and far-flung areas. Their proficiency is unstable due to the resistance evolution in the target insects. Environmental concerns over the toxicity towards non-target organisms are becoming more significant in the current scenario. For instance, a study by Muhammad et al., (2024) unveiled the possible role of microbial consortia in mosquito vector control research. Similar to their perspectives, Costa et al. (2024) analysed the toxic potential towards the *Aedes aegypti* larvae. Microbes inside mosquitoes can invade and colonize unique tissues, perhaps through intracellular routes, especially in the salivary gland, mid-gut and reproductive organs. Microbes residing in the mid-gut or salivary gland interact directly with pathogens, whereas microbes dwelling in distinctive tissues might also affect vector competence. Genetic manipulation of either bacterial symbionts or vectors is a rising technology and *Wolbachia* is a potential weapon observed in 60% of insects as a natural inhabitant. Its discovery has led to a vector control program based

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on *Wolbachia* (infection prevents reproduction between infected male and uninfected female – cytoplasmic incompatibility), which is self-sustaining, affordable and long-term solution barring any harm to humans, animals and surroundings (McGraw and O'Neill 2013). Microbial populations, either acquired through vertical transmission or from their habitat, influence the host's immune activity and mosquito competence by wrecking pathogen attack via competing for resources or by the production of anti-pathogenic molecules (Gajera et al. 2023; Cirimotich et al. 2011; Moreira et al. 2009). The method based on this principle, ie, paratransgenesis, is a highly valued tool for the abolition of mosquito-borne diseases (Favia et al. 2007). Recent studies by Mosquera et al. (2023a, b) unveiled the role of associated symbiotic bacteria in mosquito vectors. According to their findings, the microorganism *Klebsiella* sp. possessed significant role in odorant receptor pathway and various physiological responses in *Aedes aegypti*. They finally concluded that bacteria-linked volatiles can be exploited for mosquito vector control strategies based on mosquito behavior. Similar to their findings, Minwuyet et al. (2023) evaluated the role of *Wolbachia* symbiont with the various mosquitoes such as *Culex*, *Aedes* and *Anopheles* with special reference mosquito-borne diseases eradication.

Mosquito larvae acquire a large fraction of the bacterial population from their aquatic environment and it varies significantly in accordance with site of collection (Coon et al. 2016b). Analyzing the intrinsic gut microbiota will be beneficial for figuring out doable microbial candidates and exploring the feasibility of implementing these bacteria in novel vector control tactics. In this article, we have focused on the identification and analysis of bacterial species residing inside the mid-gut of fourth-instar *Aedes aegypti* larvae collected from Kole wetlands of Thrissur district, Kerala, India by using both culture-dependent and culture-independent approaches. Wetlands are areas containing marsh, ponds, and swamps, whether they are organic or man-made, long-lasting or transient, with water that is still or moving, fresh, brackish, or salty, including marine water areas with a maximum depth of six metres at low tide according to Convention on Wetlands of International Importance especially as Waterfowl Habitat held at Ramsar, Iran, in 1971 (Mo 1994). The protection of animals, recreation, sediment control, flood prevention, education, and science all depend heavily on wetland ecosystems. These Low-lying tracts, called the Kole fields are situated 0.5–1 m below mean sea level. In a significant portion of the region, the land is still flooded for a few months out of the year. This region stretches parallel to the Arabian Sea from the lowlands on the Chalakudy River's bank in the south to Thavannur in the north. These lands were previously shallow lagoons that eventually grew in size (Sarath et al. 2017).

Many creatures, especially endemic species, find it to be an excellent environment, and the number of ecosystem interactions that take place between the resident flora and fauna reflects how important it is to the stability of the ecosystem. Small plants and grasses that live along the banks of these wet places provide an ideal substrate for mosquito egg laying. Our research is unique because, despite being an ideal environment for mosquito development, no studies have been done on the gut bacteria of *Aedes aegypti* obtained from kole wetlands and it is well established that lifestyle and environmental factors (diet, length of the day, infections, exposure to xenobiotics) influence the composition of the gut microbiota. All of these variables change with the seasons, particularly in places where the climate is highly varied from season to season. Numerous earlier investigations have documented seasonal variations in the microbiome (Koliada et al. 2020).

Seasonal differences affect the physiology of mosquitoes, which in turn may affect their vector competency. A study was just carried out in 2023 to investigate this idea. In order to investigate the seasonal effects on mosquito physiology, they focused on *Culex pipiens*, a key vector of the West Nile virus (WNV) in the temperate United States. They did this by looking at known bacterial and immunological components linked to mosquito arbovirus infection. Semi-field studies were conducted in the spring, summer, and late summer, which correspond to the low-, medium-, and high-intensity WNV transmission periods in the past, respectively. Through these tests, they were able to observe variations in the distribution and abundance of *Wolbachia* in the mosquitoes between seasonal cohorts, as well as variations in the expression of immune genes and components of the RNA interference (RNAi) pathway. All of these data, point to the conclusion that seasonal variations have a major impact on mosquito physiology and microbiome components. They also imply that seasonality may affect mosquito susceptibility to pathogen infection, which may explain the temporal patterns in the spread of mosquito-borne diseases (Field and Smith 2023). In this study we have analyzed the bacterial composition during three different seasons.

Methods

Study area

The study area includes the Kole wetland areas of Thrissur district. The district has an area of 3032 sq. km and is situated in south-western India 10.52°N 76.21°E and is in the central part of Kerala. Mosquito larvae were collected from the Kole wetlands from June 2019 to 2020.

Cultured microbiome profiling

Isolation of bacteria from mosquito larva

The specimens (midgut dissected from a single larvae) were surface sterilized using 70% ethanol, followed by two rinses in 250 µl of phosphate buffer saline (PBS) (Apte-Deshpande et al. 2012). Midgut from *Aedes aegypti* larva (instar IV) was dissected under a dissecting binocular microscope in a sterile condition. The dissected midgut of each mosquito larva was individually transferred into a 2 ml microcentrifuge tube containing 500 µl of PBS and homogenized with a micropestle. Homogenized lysate was transferred into 10 ml of nutrient broth and incubated at 28–37 °C for 24–48 h. A control was also set up without a dissected gut and maintained the experimental condition for the previous step.

Bacteria isolation from plate cultures

The Culture dependent method was followed for the isolation of bacteria. The obtained culture was serially diluted in sterile distilled water (900 µl) and 100 µl volume from each dilution was plated on a sterile plate and incubated at 28–37 °C for 24–48 h. Growth of microbes were evaluated based on the total number of colony-forming units (CFU). Morphologically dissimilar colonies were repeatedly identified and subcultured in nutrient agar plates to obtain pure bacteria cultures (Pereira et al. 2011).

Molecular identification of bacterial cultures using 16 s rRNA sequencing

Genomic DNA was isolated using NucleoSpin® Tissue Kit (Macherey–Nagel) following the manufacturer's instructions. A 1.5 ml of culture was taken in a microcentrifuge tube and added 180 µl of T1 buffer and 25 µl of proteinase K and incubated at 56 °C in a water bath until it was completely lysed. 5 µl of RNase A (100 mg/ml) was added and incubated at room temperature for 5 min after lysis. Incubated at 70 °C for 10 min after adding 200 µl of B3 buffer. 210 µl of 100% ethanol was added and mixed thoroughly by vortexing. The mixture was pipetted into NucleoSpin® Tissue column, placed in a 2 ml collection tube and centrifuged at 11,000 × g for 1 min. The NucleoSpin® Tissue column was transferred to a new 2 ml tube and washed with 500 µl of BW buffer. Washing was repeated using 600 µl of B5 buffer. The NucleoSpin® Tissue column was placed in a clean 1.5 ml tube after washing and DNA was eluted out using 50 µl of BE buffer.

Agarose gel electrophoresis for DNA quality and quantity check

The quality of the DNA isolated was checked using agarose gel electrophoresis. 1 µl of 6X gel-loading buffer (pH-8.0, 30% sucrose in TE buffer, 0.25% bromophenol blue) was added to 5 µl of DNA (Akilabindu (2019)). The samples were loaded to 0.8% agarose gel prepared in 0.5X TBE (Tris–Borate-EDTA) buffer containing 0.5 µg/ml ethidium bromide. Electrophoresis was performed with 0.5X TBE as electrophoresis buffer at 75 V until bromophenol dye front migrated to the bottom of the gel. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using a Gel documentation system.

PCR for amplification of isolated DNA

PCR analysis were performed in a reaction mixture containing 2X phire master mix (5 µl), distilled water (4 µl), forward primer (0.25 µl – CAGGCTAACAC ATGCAAGTC), reverse primer (0.25 µl – GGGCGG WGTGTACAAGGC)(5' 3') (Jiang et al. 2020). The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems). PCR cycling programme consisted of an initial denaturation at 95 °C for 5 min; then denaturation at 95 °C/30 s; annealing at 60 °C /30 s; extension at 72 °C/60 s (35 cycles) and a final extension at 72 °C for 7 min and stored at 4 °C. Amplified product was visualized in a 1% agarose gel containing ethidium bromide using UV transilluminator.

16s rRNA sequencing

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following manufactures protocol. The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1 (Terminator 2002).

Phylogenetic analysis

Basic Local Alignment Search Tool (BLAST) in the GenBank were used for searching.

Metagenomic analysis

Library preparation

20 ng of DNA was used to amplify V3&V4 hyper variable regions of 16S gene using KAPA HiFi HotStart ReadyMix PCR Kit (Catalog: 0370, KAPA BIOSYSTEMS). The gene-specific sequences used in this protocol target only the 16S V3 and V4 region. They were selected from the Klindworth et al. publication (Witt, Neumann, Zierdt, Gébel, & Röscheisen). The reaction includes KAPA HiFi Hot Start Ready Mix (2X), 100 nm final concentration of modified 341F and 785R primers and 20 ng of DNA. The PCR involved an initial denaturation of 95 °C for 5 min followed by 25 cycles of 95 °C for 30 s, 55 °C for 45 s and 72 °C for 30 s and a final extension at 72 °C for 7 min and holding at 4 °C. The amplification was confirmed by loading 3 µL of the PCR product on 2% agarose gel and the presence of a band at ~456 bp. Upon the confirmation on the amplification, the PCR products were cleaned up, using 0.9X AMPure XP beads (Catalog: A63881, Beckman Coulter) to remove unused primers and eluted in 10 µL of 0.1X TE buffer. Further unique P7 and P5 barcoding was performed for the individual samples with an additional 8 cycles of PCR by taking 4 µL of the purified PCR product. The final PCR products were again purified using 0.9X AMPure XP beads and the final library was eluted in 15 µL of 0.1X TE buffer.

Library quantification

The library concentration was determined in a Qubit.3 Fluorometer (Catalog: Q33216, Life technologies using The Qubit™ dsDNA BR (Broad Range) Assay Kit (Catalog: Q32853, ThermoFisher Scientific). The Qubit™ dsDNA BR Assay Kit contains DNA reagent, buffer, and two DNA standards. DNA BR assay reagent is one of the most sensitive detection dyes for the accurate quantitation of DNA/library in solution, with linear fluorescence detection in the range of 100 pg/µl to 1000 ng/µl of DNA. Dye and the buffer was diluted at a 1:200 ratio and 1 µl of the library was mixed with the dye mix and incubated at RT for 2 min, and the readings were taken in the Qubit.3 Fluorometer (Catalog: Q33216, Life technologies). Prior to the sample's measurement, the instrument was calibrated using the two standards provided in the kit.

Library validation

The library quality assessment was done using Agilent D1000 ScreenTape System (Catalog: 5067- 5582, Agilent) in a 4150 TapeStation System (Catalog: G2992AA, Agilent),

which is designed for analysing DNA molecules from 100 to 1000 bp. 1 µL of the above normalized and pooled library was mixed with 3 µL of D1000 sample buffer (Catalog: 5067- 5583) and vortexed using IKA vortexed at 2000 rpm for 1 min and spin down to collect the sample to the bottom of the strip. The strip was then loaded on the Agilent 4150 TapeStation system.

Results

Isolation and molecular identification of culturable bacteria

Plating mosquito gut contents from *Aedes aegypti* larvae collected during different seasons was used to isolate culturable microflora. Serial dilution was done to reduce the initial number of colonies. An ideal separation of bacterial colonies was observed at 10^{-3} dilution. Bacterial colonies were selected based on morphological variations and conventional microbiological techniques (colony size, color, shape, opacity, margin and elevation). Basic biochemical methods were used for the characterization of the isolated bacteria. 16 s rRNA sequencing and BLAST analysis have revealed the presence of bacteria belonging to the family Enterobacteriaceae, Bacillaceae, Aeromonadaceae and Pseudomonaceae (Fig. 1). Among the isolated organisms, bacterial strains belonging to the family Bacillaceae were the most dominant ones, followed by Enterobacteriaceae. Different bacterial species identified during three different seasons are depicted in Fig. 2. Seven species were members of the gram-positive family, Bacillaceae, and 4 were in the family Enterobacteriaceae (Fig. 3 and Table 1).

Metagenomics analysis

The Metagenomics analysis performed using the *Aedes aegypti* mosquito gut content revealed the presence of many bacterial species belonging to the following phylum; Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Firmicutes, Gemmatimonadetes, Planctomycetes, Proteobacteria, and Verrucomicrobia. The number of reads of the analysed strains such as AAMA, AAMB and AAMC was detected to be 292,254, 202,272 and 238,910 respectively; while the Q20 values of AAMA, AAMB and AAMC were reported as 99.73, 99.72, and 99.78. The higher values of Q20, specifically more than 90%, represent that the gene assembly had maximum integrity along with good quality. It also ensures the researcher's maximum credibility and can proceed with subsequent species-wise diversity analysis according to the various seasons. As given in Table 2, the frequency-wise distribution according to the

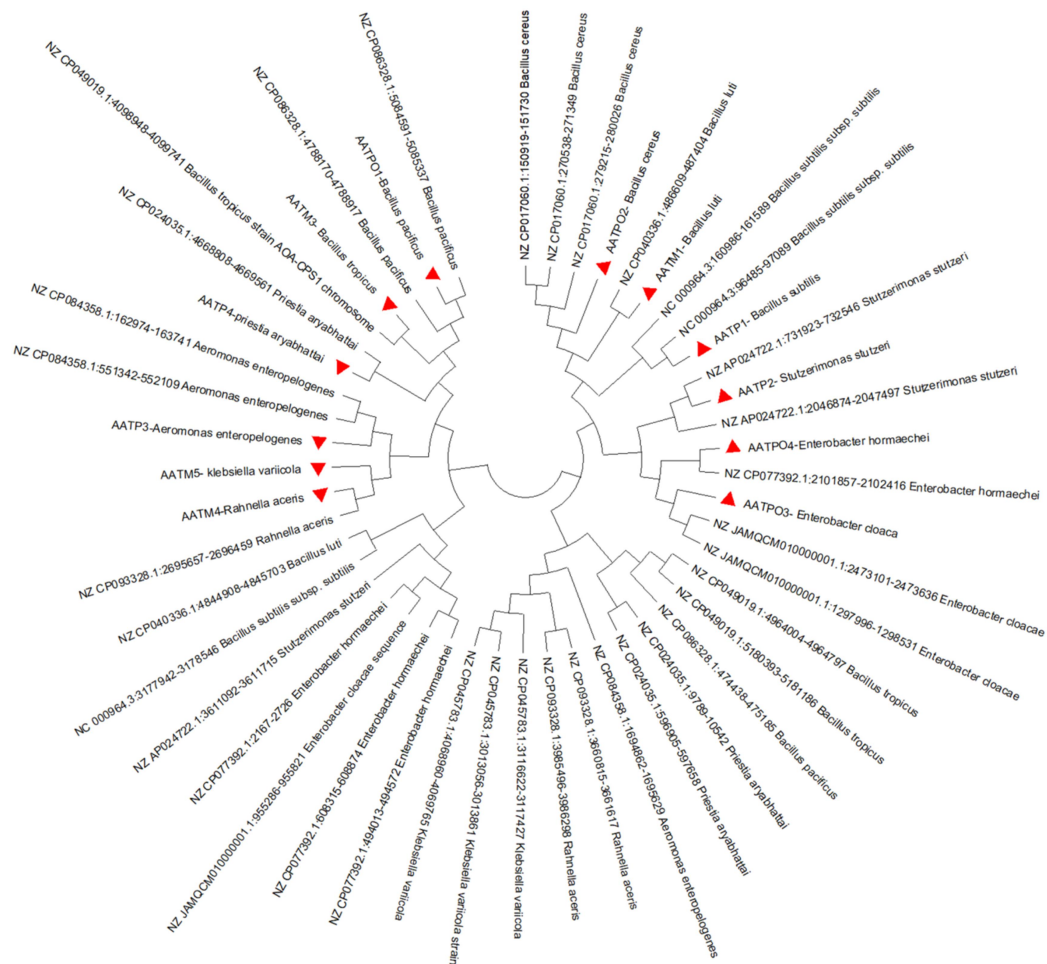


Fig. 1 The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei 1993). The tree with the highest log likelihood (-8999.14) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor and BioNJ algorithms to a matrix of pairwise distances

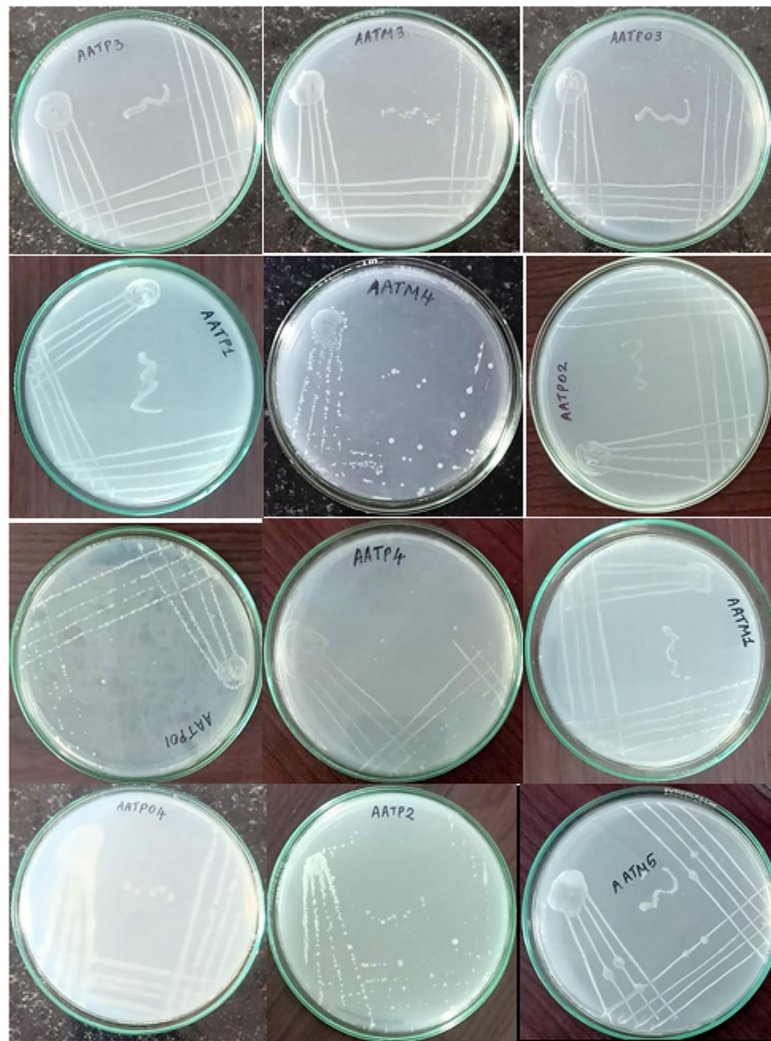
estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. This analysis involved 35 nucleotide sequences. There were a total of 1548 positions in the final dataset. Evolutionary analyses were conducted in MEGA11

Phylum revealed that Actinobacteria, Firmicutes and Proteobacteria are the predominant group with a total of 351 species Tables 3 and 4.

The season-wise distribution of the reported phylum such as Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Firmicutes, Gemmatimonadetes, Planctomycetes, Proteobacteria, and Verrucomicrobia was given in Fig. 4. The AAMA, AAMB and AAMC strains designated in Fig. 4 correspond to the various seasons,

such as monsoon, pre-monsoon, and post-monsoon, respectively. According to the monsoon season and post-monsoon, the Firmicutes represent the majority, while the pre-monsoon season detected the presence of Proteobacteria in greater intensity. The prevalence of microbes belonging to the Chloroflexi at a greater level in the pre-monsoon indicates how the Kole wetlands represent the maximum diversity in *Aedes aegypti* mosquito gut microbiota. In addition to the formerly discussed phyla, the heat map analysis represents (Fig. 5A) the presence of

Fig. 2 Culture plates of various bacteria isolated from *Aedes aegypti* larvae



20 different phyla in the analysed strains. At the genus level, the top 10 genus accounted for the mosquito larvae includes *Clostridium*, c39, *Hydrogenophaga*, *Leucobacter*, *Listeria*, *Mycobacterium*, *Propionispora*, *Rhodoplanes* and *xanthobacter* (Fig. 6). The heat map representing the distribution of 20 predominant genera for the respective strains has been given in Fig. 5B. As given in Fig. 5, several species were present according to the distribution of species abundance; among these, the following species are identified as predominating; *Clostridium bifermantans*, *Anaerospira hongkongensis*, *Clostridium butyricum*, *Clostridium intestinale*, *Clostridium ljungdahlii*, *Clostridium*

pasteurianum, *Clostridium tyrobutyricum*, *Listeria weihenstephanensis*, *Rhodoplanes elegans* and *Xanthobacter flavus*. In addition to the reported species, Fig. 5C has accounted for the 20 most dominant species found in the respective strains.

Diversity analysis

The composition profile of all the selected strains, such as AAMA, AAMB and AAMC was analysed using the Alpha diversity matrices. The gene profile from the analysis was considered the input for the Simpson 1-D index and

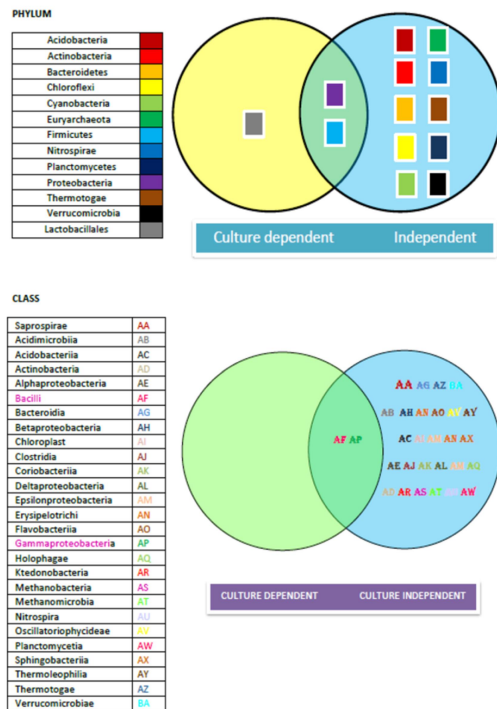


Fig. 3 The microbial inhabitants shares the culture dependant and independent pools

Shannon (H), commonly designated as the Alpha diversity matrices. Figure 7 embodies different Alpha diversity indices of which Chao1 and ACE represent the richness of the analysed strains, while the Shannon, Simpson, InvSimpson and Fisher denote both the richness and relative abundance. Across the three strains, as provided in Table 5, The Shannon diversity analysis indicates that the AAMB strain ($H' = 3.11813661$) was the most abundant than the AAMA ($H' = 2.58097801$) and AAMC ($H' = 2.29624848$). It may assign that the climate change and seasonal factors are may be responsible for this variation in population diversity. The high diversity score obtained from the Simpson diversity analysis of AAMB also agreed with the former statements.

The diversity analysis sensitive to singletons and doubletons, i.e., rare OTUs was provided in Table 6. The beta diversity analysis of the AAMA, AAMB and AAMC strain using other approaches, including the Fisher's exact test has also been given in the Table 6. The Microbiome beta-diversity profiling showed significant differences between the different combinations of various strains such as AAMA_vs_AAMB,

AAMA_vs_AAMC, and AAMB_vs_AAMC, according to the different seasons. The heat map representing the distribution of top 20 significant OTUs in AAMA_vs_AAMB was given in Fig. 5.

Discussion

Classical, culture-dependent methods and metagenomic analysis were used to determine the microfauna inside the larval gut, and both methods have many benefits (PCR based methods). The present study by culture-dependent methods have revealed the presence of 11 bacteria belonging to the genus, *Enterobacteria*, *Bacillus*, *Aeromonas*, *Stutzerimonas* and *Rahnella*. Nowadays, studies are applying next-generation sequencing data from 16 s rRNA gene hypervariable regions for qualitative studies on gut bacteria, but we were using the conventional methods based on plate culture also, as we will be able to use these isolated species for further trials or investigation for developing a vector control strategy and to analyze its role in various life activities (Duguma et al. 2013). Maintenance of isolated bacteria is necessary for such investigation, which will be more applicable and easy with these procedures. Additionally, culture-dependent techniques can be used to isolate bacteria that are only present in trace amounts.

Qualitative analysis for the identification of bacteria from *Aedes aegypti* for the first time was done by (Luxanani et al. 2001) and identified two *Bacillus cereus* strain and they hypothesized that their impervious retention in mosquito larvae guts and the feasibility of genetic manipulation shows these traces possess great capability to be employed as a mosquito control agent. Six bacterial strains belonging to the genus Bacillaceae were identified in our study. Many bacterial strains have been identified from previous studies in the mosquito gut. Some of them are widely used as biological control agents as most of the chemical pesticides are environmental contaminants and insects quickly develop resistance against them. Lack of vaccines also makes it an immediate need to develop an efficient vector control method and using microbes for this purpose will be obliging as it is species-specific and environment-friendly (Balakrishnan et al. 2015). Many bacillus species like *Bacillus thuringiensis*, *B. cereus* and *B. subtilis* are reported to have mosquitocidal activity. *B. subtilis* is a non-pathogenic species, that produces an endospore that can overcome extreme conditions of heat and dessication and it is easy to culture, rendering it to be used as a vector controlling agent and they can be preserved for long periods (Kimelman and Shemesh 2019). *B. thuringiensis* produce protoxins (Cry4A, Cry4B, Cry11A, and Cyt1A) which get activated by larval

Table 1 Distribution of microbial inhabitants according to order

		Valid Percent	Cumulative Percent
Valid	[Saprospirales]	.9	.9
	Acidimicrobiales	.3	1.1
	Acidobacteriales	.3	1.4
	Actinomycetales	21.4	22.9
	Aeromonadales	.6	23.4
	Alteromonadales	.9	24.3
	Bacillales	10.6	34.9
	Bacteroidales	2.6	37.4
	Bdellovibrionales	.6	38.0
	Bifidobacteriales	.9	38.9
	Burkholderiales	3.7	42.6
	Campylobacteriales	.3	42.9
	Caulobacteriales	.9	43.7
	Chlorophyta	.3	44.0
	Chroococcales	.3	44.3
	Clostridiales	17.4	61.7
	Coriobacteriales	1.1	62.9
	Desulfobacteriales	.3	63.1
	Enterobacteriales	1.4	64.6
	Erysipelotrichales	2.0	66.6
	Flavobacteriales	.9	67.4
	Gaiellales	.3	67.7
	Gemmatales	.9	68.6
	Holophagales	.3	68.9
	Ktedonobacteriales	.3	69.1
	Lactobacillales	2.0	71.1
	Legionellales	.3	71.4
	Methanobacteriales	.3	71.7
	Methanomicrobiales	.3	72.0
	Methylophilales	.3	72.3
	Myxococcales	1.4	73.7
	Neisseriales	.3	74.0
	Nitrosomonadales	.6	74.6
	Nitrospirales	.3	74.9
	Pasteurellales	.9	75.7
	Pseudomonadales	1.4	77.1
	Rhizobiales	8.6	85.7
	Rhodobacteriales	2.3	88.0
	Rhodocyclales	.3	88.3
	Rhodospirillales	4.0	92.3
	Sphingobacteriales	.9	93.1
	Sphingomonadales	3.1	96.3
	Syntrophobacteriales	.3	96.6
	Thermotogales	.3	96.9
	Verrucomicrobiales	.9	97.7
	Xanthomonadales	2.3	100.0
	Total	100.0	

gut enzymes, making epithelium pores. However, insects may develop resistance to the toxin due to its persistence for longer period in lower quantities in certain conditions, these four protoxins are not easy candidates to overcome (Bruehl et al. 2020). Bacterial symbionts often trigger the moulting process by bacteria-mediated hypoxia inside the larval gut and, do not render their development even when fed with a nutritionally complete diet without the microbiota (Coon et al. 2017). Identification of *Wolbachia* was a breakthrough in mosquito research, and a lot of studies were concentrated in manipulating the bacteria as a vector control agent and scientists are trying to infect *Aedes aegypti* with the bacteria, which normally do not carry them. *Elizabethkingia* is a mutualistic and transstadially present bacteria that outnumber other members of bacterial communities, which is introduced by the female mosquito during egg laying and it can be considered as a part of ovitraps. A single method-based approach for mosquito control is not adequate in the present scenario. An integrated approach is better to counteract resistance (Chen et al. 2015). Organisms belonging to the family Bacillaceae were abundant during these three seasons, their expansive physiology and spore forming ability may be the factors allowing them to colonize in the gut and they are abundant in soil, air and water (Elshaghabee et al. 2017). In our study, we found that *Rahnella* was also present in the gut of larvae collected during these three seasons and it existed in larvae collected in the same season but from different sites, reflecting the fact that it may be a natural inhabitant of the gut. The same bacteria was reported in previous studies in the malaria vector *Anopheles stephensi* (Cirimotich et al. 2011). Among the identified bacterial strains, species belonging to family Bacillaceae showed higher diversity predominance (Duguma et al. 2013) and Overlapping in certain phyla and classes of bacteria isolated by culture dependent methods and metagenomic profiling are depicted in the Fig. 5.

Many factors are shown to influence the microbial consortia of different mosquito species and the environment play an imperative part in specifying the microbial population. Zhao et al. (2023) recommended a cryopreservation approach to recover microbial consortia from the larval habitats of mosquitoes. In addition to this, Malik et al. (2023) implemented the possibilities of using omics-based assessment of microbial consortia in the environment as a symbiotic link. Similar to their inferences, Mosquera et al. (2023a, b) analysed the influence of microbial consortia with Eggs in *Aedes aegypti* shapes mosquitoes. Larvae have been shown to feed on microbes in their habitat and it implies that they acquire them orally and get transmitted from larvae to adults (Coon et al. 2014, 2017). So that, geographical location may also have profound influence in modeling the microbial population inside gut. In this study, we are focusing on *Ae. aegypti* larvae collected from kole wetlands. Wetlands are the most

Table 2 Showing various bacteria isolated from fourth instar larvae collected during three seasons

	ENTEROBACTERIACEAE	PSEUDOMONACEAE	AEROMONADACEAE
<i>Bacillus tropicus</i>	<i>Enterobacter cloaca</i>	<i>Stutzerimonas stutzeri</i>	<i>Aeromonas enteropelogenes</i>
<i>Bacillus luti</i>	<i>Enterobacter hormaechei</i>		
<i>Bacillus subtilis</i>	<i>Klebsiella variicola</i>		
<i>Priestia aryabhatai</i>	<i>Rahnella aceris</i>		
<i>Bacillus pacificus</i>			
<i>Bacillus cereus</i>			

Table 3 Species Diversity of microbial inhabitants according to its seasons

	Observed	Chao1	se.chao1	ACE	se.ACE	Shannon	Simpson	InvSimpson	Fisher
AAMA	671	873.843373	39.8991837	838.233284	14.4863412	2.58097801	0.76289486	4.21753821	98.5758471
AAMB	632	831.105263	37.8071371	844.500761	15.4236605	3.11813661	0.88626298	8.7922121	95.2304462
AAMC	603	772.166667	33.7856416	766.229948	13.8074396	2.29624848	0.71246356	3.47782007	90.3457864

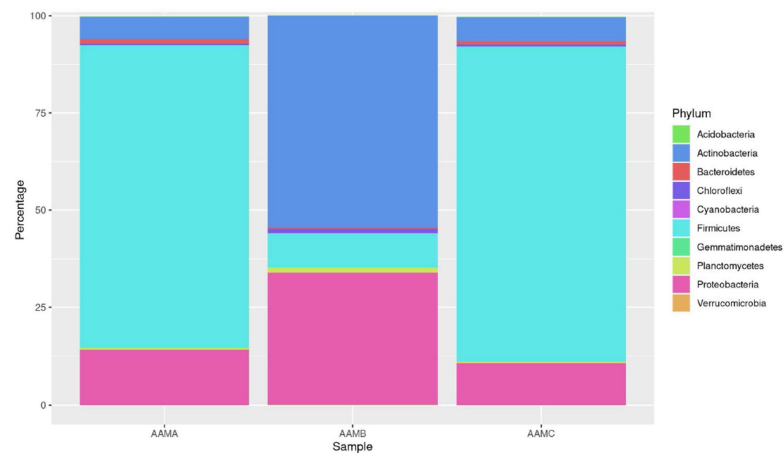
Table 4 OTU of samples

S.No	Comparison	Significant OTUs (p-value <= 0.05)	Significant OTUs (p-value(corrected)<= 0.05)
1.	AAMA_vs_AAMB	286	220
2.	AAMA_vs_AAMC	145	63
3.	AAMB_vs_AAMC	257	201

productive habitats covered by water and are constantly or periodically flooded, with more aquatic vegetation, forming a breeding ground for mosquitoes (Junk et al. 2012).

The role of microorganisms in ecosystems and the physiology of many organisms belonging to humans, plants and animals have been recognized in metagenomics focussed studies. The studies focused on profiling mosquito midgut microbiota using metagenomics can deliver appropriate information to fight against the mosquito vectors and various diseases impacts spread by the Aedes mosquitoes. It was obvious that the outcomes of this study could be considered as noteworthy information because of the presence of microbial species belonging to the following phyla in the Aedes mosquitoes gut; Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Firmicutes, Gemmatimonadetes, Planctomycetes, Proteobacteria, and Verrucomicrobia. The presence of microorganisms belonging to these phyla in high-altitude freshwater was reported by (Liu et al. 2014). In addition to this, (Duguma et al. 2013) characterized the microbiome associated with the Culex mosquito vectors with special reference to the wetland habitats. Validation of the predominant phyla in Ae. aegypti mosquitoes from Guadeloupe and French Guiana was accomplished through a study by (Hery et al. 2021), indicating the existence of Proteobacteria,

Actinobacteria, Firmicutes, and Bacteroidetes as the primary phyla. The current study marks the pioneering application of metagenomics in vector control research to investigate the microbiome present in the gut of Aedes mosquito larvae and their associated habitats, such as the "Kole wetland". As per the findings from (Duguma et al. 2013), mosquitoes consume microbial communities during their life cycle and this study inevitably underestimated the diversity of microbiome present within both mosquito guts and their surrounding environments, according to the various seasons such as premonsoon, monsoon and post-monsoon. As obtained in this study, (Ramos-Nino et al. 2020) reported that the major microbiome constituents by metagenomic analysis of Culex quinquefasciatus and Ae. aegypti was the bacteria. Based on their research, it was found that the dominant bacteria in Cx. quinquefasciatus and Ae. aegypti mosquitoes were Proteobacteria. The significance of Proteobacteria in both studies underscores its crucial involvement in the mosquito life cycle. This observation holds true despite the fact that the prior study originated in Grenada, a location geographically distant from the site of the current study. In terms of prevalence, the most abundant phyla observed here were the Firmicutes and AAMC and AAMA strains, while the AAMB was observed with the Chloroflexi. In accordance with these findings, a prior investigation conducted by (Adegoke et al. 2020) exposed that Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria stood out as the most dominant phyla. The fact is that none have piloted as study on these aspects according to the various seasons in Ae. aegypti mosquito vectors. To date, no studies have been conducted to explore these aspects specifically across different seasons such as Pre-monsoon, monsoon and post-monsoon in Aedes aegypti mosquito vectors. Nonetheless, previous studies, including an investigation by (Parab et al.

Fig. 4 Phyla abundance distribution

2022) from the Indian region, have reported a prevalent abundance of Firmicutes during the monsoon and the post-monsoon seasons. In a study (Hery et al. 2021) encompassing diverse urban areas in Guadeloupe and French Guiana, Firmicutes were identified as the dominant phylum. Likewise, (Zouache et al. 2011) successfully identified the organisms from two phyla, namely Firmicutes and Proteobacteria, which are agreed as the major findings of the current research. At a greater level, the dominance of microbes belonging to the Chloroflexi in the pre-monsoon season specifies how the kole wetlands represent the maximum diversity in *Ae. aegypti* mosquito gut microbiota. A study conducted by (Samson et al. 2023) reported that *Bacteroidetes* and *Chloroflexi* demonstrated an increase in abundance during the pre-monsoon season compared to other investigated seasons such as monsoon and post-monsoon seasons. These findings support the results of (Maestre et al. 2015) that specific factors have an impact on the composition of *Chloroflexi* and *Acidobacteria*. Upon conducting a phyla-level analysis, the subsequent phase of the study identified the top 10 genera present in *Aedes aegypti* mosquito larvae. These genera encompass *Clostridium c39*, *Hydrogenophaga*, *Leucobacter*, *Listeria*, *Mycobacterium*, *Propionispora*, *Rhodoplanes*, and *Xanthobacter*.

Especially, in a study steered by (Arévalo-Cortés et al. 2020), numerous species were found to belong to the following genera: *Parabacteroides*, *Bifidobacterium*, *Ruminococcus*, *Alistipes*, *Macellibacteroides*, *Campylobacter*, *Mucispirillum*, *Pseudomonas*, *Bacteroides*, and *Clostridium*. Among the various reported genera, the organisms belonging to the *Clostridium* are widely recognised as the predominant genus in the present investigation and this highlights the notable contribution of the *Clostridium* genus to the *Ae.*

aegypti mosquito vectors, highlighting its significant role. Nevertheless, as evident in this research, a study conducted by (Arévalo-Cortés et al. 2020) discovered prominent variations in the abundance levels across distinct seasons. Furthermore, they also reported that particular microbiomes found in mosquito vectors possess the capability to safeguard against pathogen infections in various mosquito species.

The *Clostridium* genus mentioned above is primarily associated with the degradation of fenpropathrin, a newly developed synthetic pyrethroid used predominantly against different insects. Moreover, a study by (Wang et al. 2021) revealed that *Hydrogenophaga* exhibited the highest richness. Similar to the results of this study, research led by (Coon et al. 2016a) reported that *Leucobacter* was identified as major microbial genera found in the gut of *Ae. aegypti* mosquito vectors. No studies have documented the presence of *Rhodoplanes* genus microbes within the gut of *Ae. aegypti* mosquito larvae worldwide. This observation highlights the distinctiveness of the current study. The previous study (Hery et al. 2021) has already established a significant association between *xanthobacter* and *Aedes aegypti* mosquito larvae. This underscores the importance of documenting the presence of *xanthobacter* in *Ae. aegypti* mosquito larvae.

Following the analysis of the reported microbiomes at the genus level, a more detailed examination at the species level identified *Clostridium bifermentans*, *Clostridium intestinale*, *Clostridium ljungdahlii*, *Clostridium pasteurianum*, *Clostridium tyrobutyricum* and *Clostridium butyricum* as the prevailing microbial species within the gut of *Aedes aegypti* mosquito larvae. As previously stated, this particular strain of *Clostridium* is known for its engrossment in the degradation of newly developed synthetic pyrethroids, which are commonly used to control various insects like fenpropathrin. Consequently, it is probable that *Clostridium bifermentans*

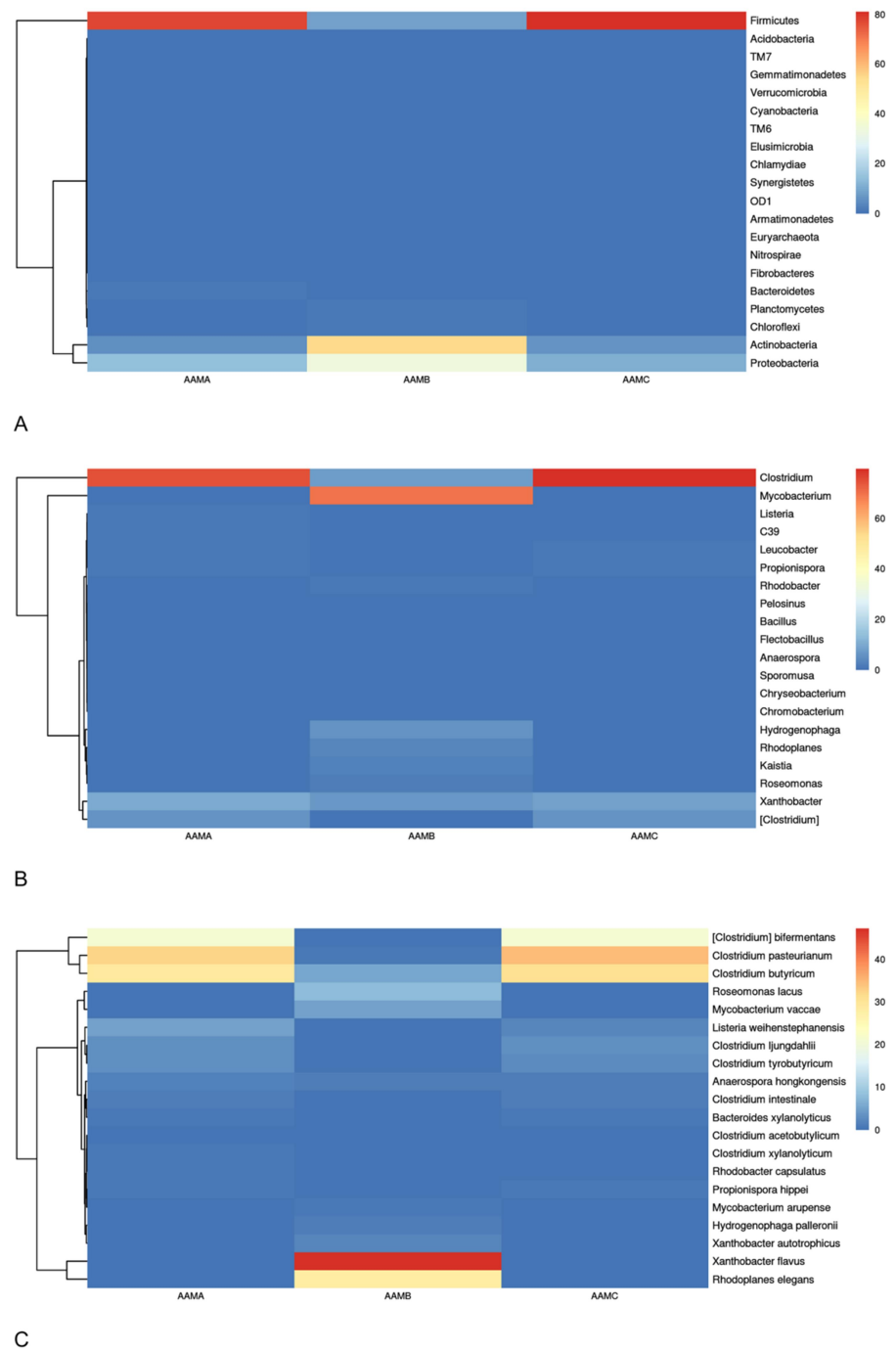


Fig. 5 Heat Map Depicting the OUT directed analysis according to the various seasons. A. Genus Distribution B. Phylum Distribution C. Species Distribution

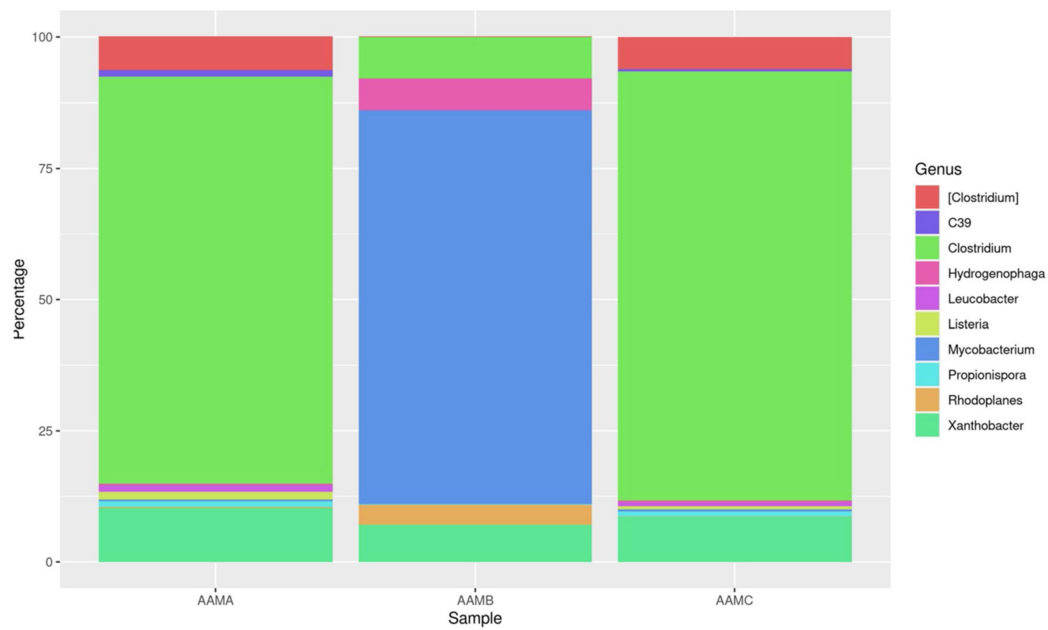


Fig. 6 Genus wise distribution of microbial inhabitants according to the various seasons

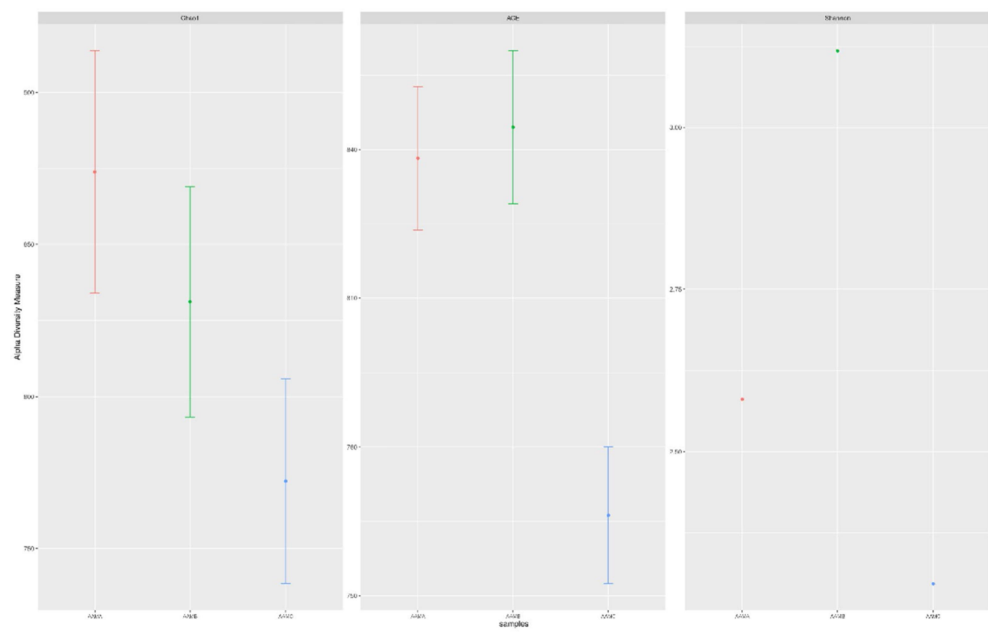


Fig. 7 Alpha diversity analysis

Table 5 Distribution of microbial inhabitants according to class

		Valid Percent	Cumulative Percent
Valid	[Saprospirae]	.9	.9
	Acidimicrobiia	.3	1.1
	Acidobacteriia	.3	1.4
	Actinobacteria	22.3	23.7
	Alphaproteobacteria	18.9	42.6
	Bacilli	12.6	55.1
	Bacteroidia	2.6	57.7
	Betaproteobacteria	5.1	62.9
	Chloroplast	.3	63.1
	Clostridia	17.4	80.6
	Coriobacteriia	1.1	81.7
	Deltaproteobacteria	2.6	84.3
	Epsilonproteobacteria	.3	84.6
	Erysipelotrichi	2.0	86.6
	Flavobacteriia	.9	87.4
	Gammaaproteobacteria	7.7	95.1
	Holophagae	.3	95.4
	Ktedonobacteria	.3	95.7
	Methanobacteria	.3	96.0
	Methanomicrobia	.3	96.3
	Nitrospira	.3	96.6
	Oscillatoriothricaceae	.3	96.9
	Planctomycetia	.9	97.7
	Sphingobacteriia	.9	98.6
	Thermoleophilia	.3	98.9
	Thermotogae	.3	99.1
	Verrucomicrobiae	.9	100.0
	Total	100.0	

play a role in supporting the mosquito vectors in their ability to withstand and adapt to diverse toxic circumstances and their associated effects. Findings by (Qureshi et al. 2014) highlight a captivating observation that the *Clostridium bifermentans* were renowned for their capacity to elicit harmful effects on various mosquito vectors. This diverse outline suggests that identical strains of bacteria can generate varied impacts. The unique findings of this study are that the *Anaerospira hongkongensis* and was discovered in the gut of *Ae. aegypti* mosquito larvae for the first time from the world. Especially, no former reports have recognized the presence of *Anaerospira hongkongensis* and *Listeria weihenstephanensis* in any part of the world. This absence further underscores the unique nature of the current research. In this study, another major microorganism identified was *Rhodoplanes elegans*. Corresponding to the previously said strains, there is a scarcity of research on *Rhodoplanes elegans* specifically within the gut microbiota of *Ae. aegypti*

Table 6 Phylum based distribution of microbial inhabitants

Phylum	Valid Percent	Cumulative Percent
Valid		
Acidobacteria	.6	.6
Actinobacteria	24.0	24.6
Bacteroidetes	5.1	29.7
Chloroflexi	.3	30.0
Cyanobacteria	.6	30.6
Euryarchaeota	.6	31.1
Firmicutes	32.0	63.1
Nitrospirae	.3	63.4
Planctomycetes	.9	64.3
Proteobacteria	34.6	98.9
Thermotogae	.3	99.1
Verrucomicrobia	.9	100.0
Total	100.0	

in the current context. However, earlier studies exploring metagenomic analysis of soil have confirmed the presence of microbial species from the *Rhodoplanes* genus (Singh et al. 2022), underscoring the novelty of this current investigation. In addition, the study underlined the striking involvement of *Xanthobacter flavus* in certain insects or blowflies (Tomberlin et al. 2017), surpassing its role in vector control research. Significantly, this study is the first to report *Xanthobacter flavus* as a gut inhabitant in *Ae. aegypti* mosquito larvae, providing novel insights into vector control research.

The unique aspect of the current research is further enhanced by identifying *Xanthobacter flavus* in *Ae. aegypti* mosquito larvae using metagenomics. This detection was conducted across different seasons, including pre-monsoon, monsoon, and post-monsoon, emphasizing the dynamic nature of *Xanthobacter flavus* presence in the mosquito larvae. The season-specific diversity analysis also illustrated the prominent microbiome diversity in the *Ae. aegypti* mosquito larvae. The gene profile obtained from the analysis served as the input for calculating the Simpson 1-D index and Shannon (H), which are widely recognized as the Alpha diversity metrics. (Hery et al. 2021) emphasized the reliability and integrity of employing these approaches for diversity analysis in metagenomics. Among the various strains analysed, the mosquito vectors representing the Pre-monsoon (AAMB strain ($H' = 3.11813661$)) was noted as the most abundant than the AAMA ($H' = 2.58097801$) and AAMC ($H' = 2.29624848$) representing the seasons such as monsoon and post-monsoon season respectively.

Previous studies have primarily concentrated on the diversity of vector mosquitoes (Chaves et al. 2011; Forrester et al. 2014) or the distribution of microbes (Guégan et al. 2018).

However, none have explored the concept of how seasonal variations influence the development of mosquito vectors in relation to the specific microbiome inhabiting their bodies. The high diversity of microbiome inhabited in the *Ae. aegypti* mosquito vectors revealed through the alpha and beta diversity matrices assign that the climate change and seasonal factors are may be responsible for this variation in population diversity. The Chao1, ACE, Shannon, Simpson, and Fisher analyses yielded remarkably high diversity scores, providing robust evidence in strong support of the statements under discussion. As reported by (Villegas et al. 2018), the diversity analysis based on operational taxonomic units (OUTs) was notably associated with the presence of singletons and doubletons. In this regard, the operational taxonomic units (OUTs) based diversity analysis in context with the various seasons such as pre-monsoon, monsoon and post-monsoon season was analysed as depicted in Fig. 8. The distribution of the reported microbial consortia (Ferreira

Altogether, the results of the present study highlight the microbiome's significant influence on mosquito vectors' life cycle. One significant impact of microbes on the mosquito life cycle is their involvement in larval development by enhancing the growth and survival rate. Similar findings were reported by (Villegas and Pimenta 2014). Some of the microbes also can potentially affect mosquito reproduction mosquito's reproductive capabilities. Furthermore, microbes can influence mosquito host-seeking behavior,

feeding preferences, and even the ability of mosquitoes to transmit pathogens. For instance, certain bacteria present in the mosquito gut can modulate the mosquito's attraction to human hosts by producing volatile compounds that influence host-seeking behavior. Understanding the intricate relationship between mosquitoes and their microbial partners is crucial for developing effective strategies to control mosquito populations and mitigate the spread of mosquito-borne diseases. The findings of this study hence can be considered as a base that can be used to unravel the specific mechanisms underlying these interactions and explore their potential for novel vector control approaches, including manipulating the mosquito microbiota to disrupt their life cycle and reduce disease transmission.

Conclusion

The midgut microbiota of disease vectors is a critical factor in the efficient transmission of human diseases. Yet, the complex interplay between mosquito species, their environment, and climate in shaping the composition of this microbiota remains poorly understood. To bridge this knowledge gap, our research has been dedicated to profiling the microbiome of *Aedes aegypti* larvae. These larvae represent a pivotal stage in the mosquito's life cycle and offer a unique opportunity for in-depth microbiome investigations. This findings have shed light on the presence of a rich diversity of microbial species coexisting in symbiosis with mosquito populations. These microorganisms exert significant control over various aspects of the mosquito's biology, including its growth, reproduction, and even its immune defense mechanisms. However, despite these insights, a comprehensive understanding of the identity and diversity of gut bacteria in mosquitoes remains elusive. Additionally, delving into the intricate interactions within microbial networks, examining the resilience of these communities, and assessing their impact on mosquito viability requires further, in-depth investigation. This research has provided valuable insights into the microbial communities dwelling within *Aedes aegypti* larvae from the overlooked Kule wetlands. The studies concerning on these direction found scanty in the current scenario since many of the studies have just isolating the strain while the current study have implemented advanced approaches including the metagenomics. Gathering additional data regarding the impact of the gut microbiota on mosquito physiology and meticulously dissecting the roles of individual community members will be instrumental in effectively addressing this crucial research question.

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Statements and Declarations

Author contribution All authors contributed to the study conception and design. Embalil Mathachan Aneesh designed, reviewed, edited, supervised and validated the research work. Honey Sebastian and Ambalaparambil Vasu Sudhikumar reviewed and supervised the research work. Material preparation, data collection and analysis were performed by Sini Francis C F involved in completing the aforementioned work and writing the original draft. All authors read and approved the final manuscript.

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Data availability The datasets used in this article are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

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