MOLECULAR CHARACTERISATION AND DIVERSITY ANALYSIS OF CULTIVABLE GUT MICROBIOTA OF SELECTED MOSQUITO LARVAE FROM THRISSUR DISTRICT, KERALA

Thesis submitted to the University of Calicut for the award of the degree of

DOCTOR OF PHILOSOPHY IN ZOOLOGY

Under the Faculty of Science

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JANUARY 2024

DECLARATION

I hereby declare that the work presented in the thesis entitled **"MOLECULAR CHARACTERISATION AND DIVERSITY ANALYSIS OF CULTIVABLE GUT MICROBIOTA OF SELECTED MOSQUITO LARVAE FROM THRISSUR DISTRICT, KERALA"** is based on the original work done by me under the guidance of Dr. Aneesh E.M. and has not been included in any other thesis submitted previously for the award of any degree. The contents of the thesis are undergone plagiarism check using iThenticate software at C.H.M.K. Library, University of Calicut, and the similarity index found within the permissible limit. I also declare that the thesis is free from AI generated contents.

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Lekshmi Jayakrishnan

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ABBREVIATIONS

^{o}C	-	Degree Celsius
AIDS	-	Acquired Immuno Deficiency Syndrome
ANOVA	-	Analysis of Variance
BHC	-	Benzene hexachloride
BLAST	-	Basic Local Alignment Search Tool
BOD	-	Biological Oxygen Method
CDM	-	Culture Dependent Method
CFU	-	Colony Forming Unit
CHIKV	-	Chikungunya Virus
CO_2	-	Carbon dioxide
DAPI	-	4'6- diamidino-2- phenylindole
DDT	-	Dichloro-Diphenyl-Trichloroethane
DENV-1	-	Dengue Virus 1
DENV-2	-	Dengue Virus 2
DENV-3	-	Dengue Virus 3
DENV-4	-	Dengue Virus 4
DHS	-	Dengue Haemorrhagic Fever
DNA	-	Deoxyribonucleic acid
DO	-	Dissolved Oxygen
DSS	-	Dengue Syndrome Shock
EDGAR	-	Electronic Data Gathering, Analysis, and Retrieval system
Feb	-	February
GC-MS	-	Gas chromatography-mass spectroscopy
HCl	-	Hydrochloric acid
HTS	-	High Throughout Sequencing
ILPs	-	Insulin-Like Peptides
IMM	-	Integrated Mosquito Management
IPM	-	Integrated Pest Management
IRS	-	Indoor Residual Spraying
ISVs	-	Identified Insect -Specific Virus

ITS	-	Internal Transcribed Spacer
IVM	-	Integrated Vector Management
Jan	-	January
KI	-	Potassium Iodide
L1	-	First larval instar
L2	-	Second larval instar
L3	-	Third larval instar
L4	-	Fourth larval instar
LLINs	-	Long Lasting Insecticide-treated Nets
MDV	-	Mosquito Denso Virus
ME	-	Methanolic Extract
MEGA	-	Molecular Evolutionary Genetics Analysis
MFs	-	Methanolic Fractions
ML	-	Maximum Likelihood
MnSO4	-	Magnese Sulphate
MP	-	Maximum Parsimony
MR-VP	-	Methyl Red-Voges Proskauer
MS-DOS	-	Microsoft Disk Operating System
MUSCLE	-	Multiple Sequence Comparison by Log-Expectation
NaCl	-	Sodium Chloride
Na_2HPO_4	-	Sodium Hydrogen Phosphate
NCBI	-	National Center for Biotechnology Information
NEB	-	New England Biolabs
NED	-	N-1 naphthyl Ethylene diamine Dihydrochloride
NFCP	-	National Filarial Control Program
NIV	-	National Institute of Virology
NJ	-	Neighbor Joining
NMCP	-	National Malaria Control Program
NVBDCP	-	National Vector Borne Disease Control Program
OEH	-	Ovary Ecdysteroidogenic Hormone
OMWM	-	Open Marsh Water Management
OTU	-	Operational Taxonomic Unit
PBS	-	Phosphate-Buffered Saline
PCA	-	Plate Count Agar

PCLV	-	Phasi Charoen-Like Virus
PDA	-	Potato Dextrose Agar
PDB	-	Potato Dextrose Broth
RFLP	-	Restriction Fragment Length Polymorphism
RVF	-	Rift Valley Fever
SIM Agar	-	Sulfide Indole Motility Agar
SLE	-	St. Louis Encephalitis
SSA	-	Sub Saharan Africa
TBE	-	Tris Borate-EDTA
TDS	-	Total Dissolved Solids
(Tris-Borate-EDTA)	-	Tris Ethylene diamine tetraacetic acid
TVC	-	Total Viability Count
UN	-	United Nation
UPGMA	-	Unweighted Pair Group Method with Arithmetic Mean
WHO	-	World Health Organisation
YFV	-	Yellow Fever Virus
ZIKV	-	Zika Virus
16S rRNA	-	16S ribosomal RNA

ABSTRACT

Mosquito holds microorganisms in their digestive tract to form gut microbiota. This colonization and acquisition begins from their immature larval stage. Recent research is now peeping into mosquito microbiomes to analyse their role in mosquito biology. This knowledge may provide more information regarding its potential for developing bio-control strategies. Meanwhile, little research is focussed on the fungal entities of mosquito larval gut, as more research has been concentrated mainly on gut bacteria. Therefore the rationale of the present study intended to characterise and analyse the diversity of bacterial and fungal gut content of *Aedes aegypti*, *Culex quinquefasciatus*, and *Anopheles stephensi* of three sites of Thrissur districts across seasons pre-monsoon, monsoon and post-monsoon from 2018-2020, to analyse the phylogeny of bacterial and fungal gut entities as well as physicochemical parameters of larval breeding water during these stipulated time period (2018-2020) from aforementioned sites and seasons. The study proposed to identify bacterial and fungal gut content from these mosquito larvae by 16S rRNA and ITS sequencing, respectively. A total of 1026 bacterial isolates grouped into 63 species and 239 fungal isolates fitting into 64 species were identified from these mosquito larval species by culture-dependent methods. The bacterial isolates belonging to phylum Pseudomonadota and fungal isolates belonging to division Ascomycota were predominant in these mosquito species. This study provides insight into the predominance, abundance, and coexistence of bacteria and fungi within and between the larval gut of mosquito species.

These bacterial and fungal communities identified from the gut of mosquito larval species of *Aedes aegypti*, *Culex quinquefasciatus,* and *Anopheles stephensi* were used for phylogenetic analysis. The organisms were primarily classified based on their

morphological characteristics and biochemical assays. However, the limitations and accuracy of these methods were rectified by molecular characterisation. For the present study, the 16S rRNA gene and ITS were used for molecular characterization and phylogenetic assessment of bacteria and fungi, respectively. Nuclear 16S rRNA gene sequences are beneficial for the differentiation between organisms at the genus level across all major phyla of bacteria, while ITS is a well-recognised marker for systematics and phylogeny. During the study, a total of 63 bacterial isolates and 64 fungal isolates were obtained. The reference sequences from the NCBI database were also used for the study. For phylogenetic analysis, bacteria were clustered into distinct families, including Pseudomonadaceae, Bacillaceae, Enterobacteriacea, Yersiniaceae, Moraxellales, and Paenibacillaceae, using their 16S rRNA sequences. On the other hand, a phylogenetic assessment of fungi was carried out after grouping them into various families, such as Davidiellaceae, Debaryomycetaceae, Pleosporaceae, Trichocomaceae, Aspergillaceae, Cordycipitaceae and Syncephalstracea based on their ITS sequences. The results of the study provide valuable insight into intra and interspecific genetic divergences between members of specific classes. Another finding of the study was the close genetic similarity observed between morphologically dissimilar and geographically distant species.

The abiotic and biotic factors of breeding water influence the survival and proliferation of mosquito larvae. Mosquitoes prefer diverse aquatic bodies for egg-laying and larval survival. During the present study physico-chemical properties of breeding water, across seasons and their influence on the larval abundance of three mosquito larval species, such as *Aedes aegypti*, *Anopheles stephensi*, and *Culex quinquefasciatus* were assessed. Dissolved oxygen, pH, conductivity, total dissolved solids, and temperature were found to have a significant influence on seasonal

variations and played a substantial role in determining the prevalence of these three mosquito larval species. The findings of this study provide valuable insight into important physicochemical parameters that affect the presence of mosquito larval species. The study provides an interconnectedness or idea of the physicochemical parameters of breeding water and the microbial composition of that water, which in turn are linked to larval abundance and their role in shaping the gut microbiota of mosquito species. These results offer valuable information regarding the role of physicochemical parameters and their influence on larval abundance. This information can be used to create new vector control strategies.

Key words: Mosquito larvae, Larval gut, Microbiota, Bacteria and Fungus.

സംഗ്രഹം

ലോകത്തിന്റെ മിക്ക ഭാഗങ്ങളിലും വസിക്കുന്ന പറക്കുന്ന പ്രാണികളിൽ പ്പെടുന്നവയാണ് കൊതുകുകൾ. ലോകമെമ്പാടും ഏകദേശം മൂവായിരത്തി അഞ്ഞൂറിലധികം ഇനം കൊതുകുകൾ ഉണ്ട്. ഇവയിൽ ചില ഇനം കൊതുകുകൾ മനുഷ്യരിലേക്കും മറ്റു മൃഗങ്ങളിലേക്കും രോഗാണുക്കളെ പരത്തുന്ന രോഗവാഹിനികളാണ്. ദശലക്ഷക്കണക്കിനു മനുഷ്യരെ കൊതുകു ജന്യരോഗങ്ങൾ ബാധിക്കുകയും തന്മൂലം മരണം സംഭവിക്കുകയും ചെയ്യുന്നുണ്ട്. വാക്സിനുകളുടെ അഭാവവും, കീടനാശിനികളോടുള്ള പ്രതിരോധവും ഇതിനുകാരണമാകുന്നു. ഡെങ്കിപനി, ചിക്കുൻഗുനിയ തുടങ്ങിയവ ഈഡിസ് ഈജിപ്തി (*Aedes aegypti*) കൊതുകുകളും മന്ത് രോഗം, ജപ്പാൻ ജ്വരം, ക്യൂലക്സ് കവിൻകിഫാസിയാറ്റസ് (*Culex quinquefasciaus*) കൊതുകു കളും മലമ്പനി അനൊഫെലിസ് സടീഫൻസ്സി (*Anopheles stephensi*) കൊതുകു കളും പരത്തുന്നു.

കൊതുകുകൾ പൊതുവെ വൈവിധ്യമാർന്ന ജല ആവാസവ്യവസ്ഥകളിൽ പ്രജനനം നടത്തുന്നു. കൊതുകുകളുടെ ജീവചക്രത്തിൽ നാലുഘട്ടങ്ങളാണ് ഉള്ളത്. അതായത് മുട്ട. ലാർവ, പ്യൂപ്പ, പൂർണവളർച്ചയെത്തിയ കൊതുകുകൾ. ലാർവ ഘട്ടം കൂട്ടത്തിൽ ദൈർഘ്യമേറിയതാണ്. ഈ ഘട്ടത്തിൽ ലാർവ അതിന്റെ ആവാസവ്യവസ്ഥയെ ജലത്തിൽ അടങ്ങിയ ജൈവാവിശിഷ്ടങ്ങൾ ഭക്ഷണമാക്കുകയും തത്ഫലമായി അതേ ജലത്തിൽ ജീവിക്കുന്ന ഒരു കൂട്ടം സൂക്ഷ്മജീവികളെയും ലാർവയുടെ ദഹന വ്യവസ്ഥയിലേക്ക് എത്തിച്ചേരുന്നു. ഈ സൂക്ഷ്മാണുക്കളിൽ ചിലത് കൊതുകുകളുടെ ജീവിതം തുടർന്നും ദഹനവ്യവസ്ഥയുടെ ഭാഗമായി തന്നെ തുടരുന്നു.

സമീപകാല ഗവേഷണങ്ങൾ കൊതുകുലാർവകളുടെ ശ്രദ്ധ കേന്ദ്രീകരിച്ചിരിക്കുന്നു. എന്നാൽ അവയിൽ കൂടുതൽ ഗവേഷണങ്ങളും ബാക്ടീരിയകളെ കുറിച്ചുള്ളതാണ്. ഈ പഠനം തൃശൂർ ജില്ലയിലെ മൂന്നു താലൂക്കുകളിലെ ഈഡിസ് ഈജിപ്തി ക്യൂലക്സ് കവിൻകിഫാസിയാറ്റസ്, അനോഫെലിസ് സ്റ്റീഫൻസ്സി കൊതുകുകളുടെ ലാർവകളിലെ ദഹനവ്യവസ്ഥയിലെ ബാക്ടീരിയകളുടെ ഫംഗസുകളുടെയും വൈവിദ്ധ്യം വിശകലനം ചെയ്യുന്നു. കൊതുക് ലാർവകളുടെ ശേഖരണം മൺസൂണിനു അനുസരിച്ച് മൺസൂണിനു - മഴയ്ക്കു മുമ്പുള്ള മാസങ്ങൾ മൺസൂൺ മഴയുള്ള മാസങ്ങൾ, മൺസൂണിനുശേഷം - മഴയ്ക്ക് ശേഷമുള്ള മാസങ്ങൾ എന്നിങ്ങനെ മൂന്നു ഘട്ടങ്ങളാക്കിയിരിക്കുന്നു. ഈ പഠനത്തിൽ 1026 ബാക്ടീരിയകളെയും, 239 ഫംഗസുകളെയും തരംതിരിച്ചതിൽ നിന്നും 67 വ്യത്യസ്ത ബാക്ടീരിയ സ്പീഷീസുകളെയും, 64 വ്യത്യസ്ത ഫംഗസ് സ്പീഷിസുകളെയും തിരിച്ചറിഞ്ഞു. ഇത്രയും ബാക്ടീരിയകളുടെയും ഫംഗസുകളുടെയും ജനിതക തന്മാത്ര (Molecular Characterisation) 16S rRNA ഭാഗികശ്രേണികൾ, ITS ശ്രേണികൾ കണ്ടെത്തുക വഴി കൈവരിച്ചു. ഇവയ്ക്കൊപ്പം ഇവയുടെ വംശജനിതക വിശകലന പഠനം (Phylogney) കൂടി നടത്തിയിരിക്കുന്നു.

ഇതോടൊപ്പം പ്രജനന ജലത്തിന്റെ ഭൗതികരാസപരിശോധനകളും, ഇവയുടെ കൊതുകുകളിലുള്ള സ്വാധീനവും പഠനവിധേയമായിരിക്കുന്നു.

ഈ അറിവുകൾ ജൈവിക നിയന്ത്രണങ്ങൾ വികസിപ്പിക്കുന്നതിനുള്ള സാധ്യതകളെകുറിച്ചുള്ള കൂടുതൽ വിവരങ്ങൾ നൽകുന്നു.

GENERAL INTRODUCTION

Insects are undoubtedly the most abundant and diverse metazoans in the animal kingdom. The insect classification keeps changing as we explore and deploy new techniques to study their character, habitat, and relationship. We have two accepted patterns of insect classification. One is Imm's classification, an older version that is followed for a long period. The second is by Kristensen's classification, which entomologists prefer. As per Imm's classification, the class Insecta was divided into two subclasses Apterygota and Pterygota. Aptergota includes small agile wingless insects and Pterygota comprises winged insects, which is divided into two divisions: Exopterygota (Hemimetabola) and Endopterygota (Holometabola). Dipterans are among the four largest living organisms pertained to Endopterygota, next to Coleoptera, Lepidoptera, and Hymenoptera, followed by other five orders like Neuroptera, Mecoptera, Trichoptera, Siphonaptera, and Strepsiptera (Richards & Davies, 2013). According to Kristensen's classification, all six-legged arthropods were grouped in the superclass Hexapoda. This superclass is bifurcated into Entognathous hexapoda, which are wingless ametabolous arthropods that can retract mouth parts into the head, and Ectognathous hexapods, winged metabolous arthropods with extended mouth parts outside the head (Kristensen, 1981). The dipterans, true flies with a single pair of wings and a pair halters for equilibrium, comprising more than 1,60,000 species belong to the superclass Ectognathous. Mosquitoes, midges, maggots and bots are the common members of this particular order, which documents the importance of Diptera.

Mosquitoes are nematocerid fly that taxonomically belongs to Culicidae family. This biggest family, Culicidae is grouped into subfamilies Anophelinae, Culicinae, and Toxorhynchitina (Rueda, 2008). The subfamily Anophelinae has worldwide dispersion, having 485 recognized species and several unidentified members of the species complexes that have not been scientifically reported (Foster et al., 2017). In the existing form of Anophelinae classification, it is subdivided into three genera, namely *Anopheles* (472 species in addition to several unspecified members of species complexes, cosmopolitan), *Bironella* (eight species, Australasian) and *Chagasia* (five species, Neotropical). The subfamily Culicinae (true mosquitoes) is the largest troop, comprising 3046 species and Toxorhynchitinae with 90 species.

The studies of World Health Organisation (WHO) marked the presence of more than 3500 species of mosquitoes globally, out of which less than 5% of mosquitoes remain as hematophagous vectors of many debilitating diseases in humans and livestock of their vicious biting (Coon et al., 2016; WHO, 2017). The high toll of morbidity and mortality caused by vector mosquitoes has raised a public health emergency of international concern as there are no treatments for arboviral diseases (Souza et al., 2019). *Aedes*, *Anopheles*, *Culex*, and *Mansonia* genera are more associated with the widespread prevalence of major mosquito-borne diseases across the world, affecting millions of people. More than 950 species of *Aedes*, more than 1000 species of *Culex*, and more than 460 species of *Anopheles* exist worldwide. As per the latest report from WHO, vector-borne diseases account for more than 17% of all infectious diseases, with an annual death of more than 700 000 death cases across the world. Mosquito-borne diseases play a crucial role in this regard. The Outbreaks of chikungunya, Zika, dengue, yellow fever by *Aedes* species, West Nile fever by *Culex* species, and malaria by *Anopheles* species (*Anopheles gambiae* and *Anopheles*

stephensi) the main malaria vectors in Africa and Asia, respectively, has bad effect the tropical and subtropical population of the world (WHO, 2020). WHO guidelines implemented several strategies like chemical and bio-control methods to check the progression of these diseases. A thorough understanding of disease transmission by these mosquitoes requires a better knowledge of the host physiology and pathogenic interaction from the early stages of mosquito development.

These small flies have cosmopolitan distribution with diverse habituation in natural and anthropogenic ecosystems. For example, pools, phytotelmata dikes, swamps, marshes, temporary and permanent pools, holes of rock, trees, lake margins, plant containers, leaf axile, fruits, coconut husk bamboo nodes, artificial containers (tires, tin cans, empty containers, fowl feeders), and other containers inadvertently provided by humans etc. The early life cycle of a holometabolous insect starts with egg laying and hatching within 24-48 hours of duration in an aquatic terrain (Figure 1). Normally eggs are laid in an air-water interface. The female mosquito must consume a blood meal from a vertebrate host after mating for mature egg production. This triggers the release of two neurohormones from the brain: Insulin-Like Peptides (ILPs) and Ovary Ecdysteroidogenic Hormone (OEH), which in turn stimulate the ovaries to produce ecdysone and IPL. These ovarian hormones regulate the production of yolk proteins which gets into the primary oocyte to produce eggs, thereby removing oogenesis arrest (Zhu et al., 2003; Noriega, 2004; Brown et al., 2008; Vogel et al., 2015; Coon et al., 2016).

The elongated eggs are self-assembled into rafts as the preferred orientation within the cluster. The symmetry of their aggregation is always side-side, tip-tip and never side-tip. The succeeding larval stage is the most active and lengthiest in the lifecycle. This stage is subdivided into four instars, namely L1, L2, L3 and L4.

Generally, larvae grow from 1.5mm (first instar L1) up to 10mm during fourth instar L4. Even though larvae lack legs, their body is divided into three tagmata, namely a prominent head with mouth parts, thorax, and abdomen, along with saddle and siphons for breathing in the water (the only exception is for *Anopheles* which lay parallel to the water surface for breathing due to the presence of rudimentary siphon) presence of extensive hairs on the body is yet another, feature of mosquito larvae.

The pupal stage is an immature juncture that links two active phases of the life cycle: larval and adult. Most of the time, it remains on the water surface with the top of the thorax and abdomen hanging down. Anatomical pupae have two body parts: the cephalothorax and the curved abdomen. The cephalothorax and abdomen are formed from the larval thoracic segment and abdomen, respectively. There are ten segments in the abdominal region, bearing the genital pouch on the last segment. There is no general consciousness about sexual dimorphism in this stage of the mosquito life cycle. The pupal stage lasts for 2-3 days. During this stage, they do not feed and rely on the reserved food in the larval stage for pupal existence. They respire oxygen through two respiratory trumpets present on the dorsal surface of the cephalothorax without using the terminal abdominal spiracles (Moorefield, 1951; Langton, 1995; Ha et al., 2017). A small ninth-segment ring (IX) in the pupa lies behind the eighth segment, carrying the tail fins and a small anal lobe.

Adult mosquitoes have slender bodies having three divisions: a head, a thorax, and an abdomen. The head appendages are occupied for gathering sensory information and for feeding. It has compound eyes and a pair of long, many-segmented antennae, maxillary palps, and proboscis. The antennae and maxillary palps are important for detecting olfaction, particularly host odors, as well as odors of breeding sites where females lay eggs. Proboscis acts as a microneedle system for

aspirating blood and nectar by mosquitoes. The main visible difference between male and female mosquitoes is in the antennae of males, which are bushier in comparison to the females and contain auditory receptors to detect the characteristic whine of the female (Krinsky, 2013; Choo et al., 2015).

Adult female mosquitoes feed on plants to get an energy source for flight. They usually mate once and procure blood meal from vertebrate hosts to produce viable eggs, whereas male mosquitoes totally depend on flower nectar as an energy source for their existence and reproduction. Some female mosquito species (anthropophilous) prefer to feed on man, while others (zoophilous) get nurtured by animals (including mammals and birds) other than humans (Harwood & James, 1979). Most of the anautogenous mosquitoes evolved as hematophagous vectors of diseases due to their repeated cycles of blood feeding and egg production from vertebrate hosts. Some autogenous females can also produce viable eggs, even without blood meal (Clements, 1992).

Females feed every 3-5 days by piercing their proboscis into the host. They utilize two pumping organs: the cibarial and pharyngeal dilator pumps situated in the head region for sucking blood (Glenn et al., 2010). In single feeding, a female usually engorges more than its weight of blood. After the digestion of blood meal, gravid females lay eggs at an appropriate aquatic place. The process of egg laying alternating with blood meal continues, and the mosquito dies. The time of blood feeding and the number of eggs produced varies in different species. Some species

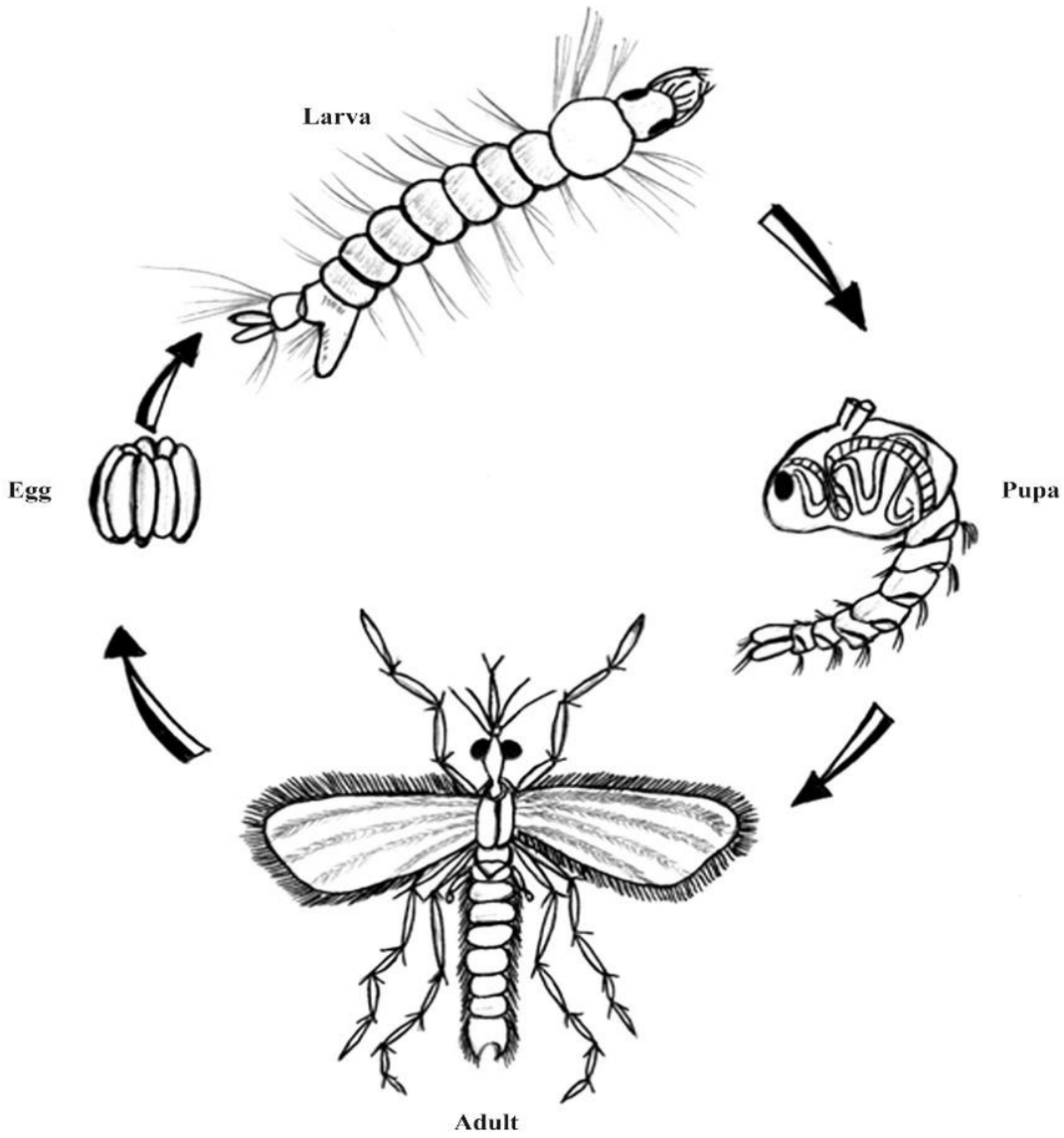

Figure1: Mosquito life cycle; illustrated by the author

of mosquitoes (*Anopheles* and *Culex*) prefer to feed at dusk, twilight, or night time, while others like *Aedes* species bite mostly during the daytime. Female mosquitoes lay between 30-300 eggs each time. Some species of mosquitoes (*Anopheles* and *Aedes*) lay single eggs directly into the water, and some clutch on water (*Culex*). The egg hatches within 2-3 days in the tropics and will more days in temperate regions (WHO,1997). Other species exhibit seasonal switching of hosts that provides a

mechanism for transmitting diseases from animals to humans (zoonotic disease transmission). Diapause (i.e., hibernation, aestivation, overwintering) occurs in various life stages, e.g., as eggs in *Psorophora* and most *Aedes*; as larvae in *Coquillettidia*, some *Culiseta*, *Mansonia*, *Orthopodomyia*, *Toxorhynchites*, *Wyeomyia*, some *Aedes*; as adults, often fertilized females, in *Uranotaenia*, most *Culex*, some *Anopheles* and other *Culiseta*; and as either eggs or larvae in *Culisetamorsitans* (Rueda, 2008).

EXTERNAL LARVAL ANATOMY

Generally, the larval stage is of prime importance in the mosquito life cycle, where continuous feeding takes place before it metamorphoses into pupae and adults. The larval body is broadly divided into the head, thorax, and abdomen (Figure 2).

Head

The larval head protrudes from the thorax bringing the mouth parts anterior to the body. Usually, the larval head is oval but in some species, it is triangular. On the anterior side of the head, a pair of large laterally placed brushes are present. In between these, a small median brush is also present. These three were well supported by labrum. Behind the lateral brushes, is the presence of an unsegmented antenna bearing variously distributed spines and the tuft of long hairs. Posteriorly on each side of the head, there is a large pigmented spot, which will evolve into adult compound eyes growing in the epidermal region of larvae. Below this pigmented eye, there is the presence of functional larval lateral eyes. Posteriorly the head is attached to the occipital foramen using a cylindrical membranous neck but in *Anopheles*, the head is narrowed where it connects the thorax to promote the turning of the head upside down while feeding (Snodgrass, 1959).

The mouthparts of mosquito larvae consist of the labrum, a pair of mandibles, a median hypopharynx, a pair of maxillae, and the labium. The labrum comprises a small transverse median labral plate (which helps in feeding) on the dorsal side of the head and a larger labropalatum on the underside that bears the feeding brushes. The hypopharynx and the labium are joined into a single lobe located between the mouth and dorsomentum on the oral cavity floor. Both the mandibles and the maxillae are diagonally embedded on either side of this lobe (Cook, 1944). The maxilla includes distinctive parts such as the maxillary body, the lateral maxillary palpus, and a basal cardo. In some genera, the cardo is either united with the base of the maxillary palpus, united with the maxillary body, or fused with the palpus and maxillary body to form a single structure. In most larval mosquitoes, the tip of the maxilla bears a collection of independent, flexible spicules called the maxillary brush, which helps to collect food particles from the palatal brushes (Snodgrass, 1959).

The pharynx of mosquito larvae is a small, thin-walled, flattened sac that opens directly from the mouth. It is tilted upward and posteriorly in the head. The posterior ventral surface of the pharynx is continued as a thick-walled oesophagus (Snodgrass, 1959).

Thorax

The larval thorax has a simple oval appearance without any external sign of appendages. In the fourth instar stage, the thorax becomes considerably widened. On its ventral side, beneath the cuticle, the presence of extroverted wings and legs of the future pupa, and on the dorsal side, the pupal respiratory organs were seen. Under the cuticle, long segmented legs flooded in loops are seen against the sides of the thorax.

The forewings are large pads furrowed in their basal parts and smaller slender hind wings are present as tapering free folds of the metanotum (Imms, 1907).

Abdomen

The larval abdomen appears to have eight segments, with the respiratory apparatus on the eighth segment and the terminal segment sometimes considered the ninth (Christophers, 1922). The first seven segments of the larval abdominal region have no distinctive features, except in the case of *Anopheles* the last five or six segments bear on the back pairs of small palmate brushes which aids the larva to suspend from the surface of the water in its usual horizontal feeding position. The respiratory apparatus is present on the posterior part of the eighth segment and bears a pair of large open spiracles, which are water-absorbing organs rather than gills. Other submerged mosquito larva, therefore, breathes through their skin (Snodgrass, 1959).

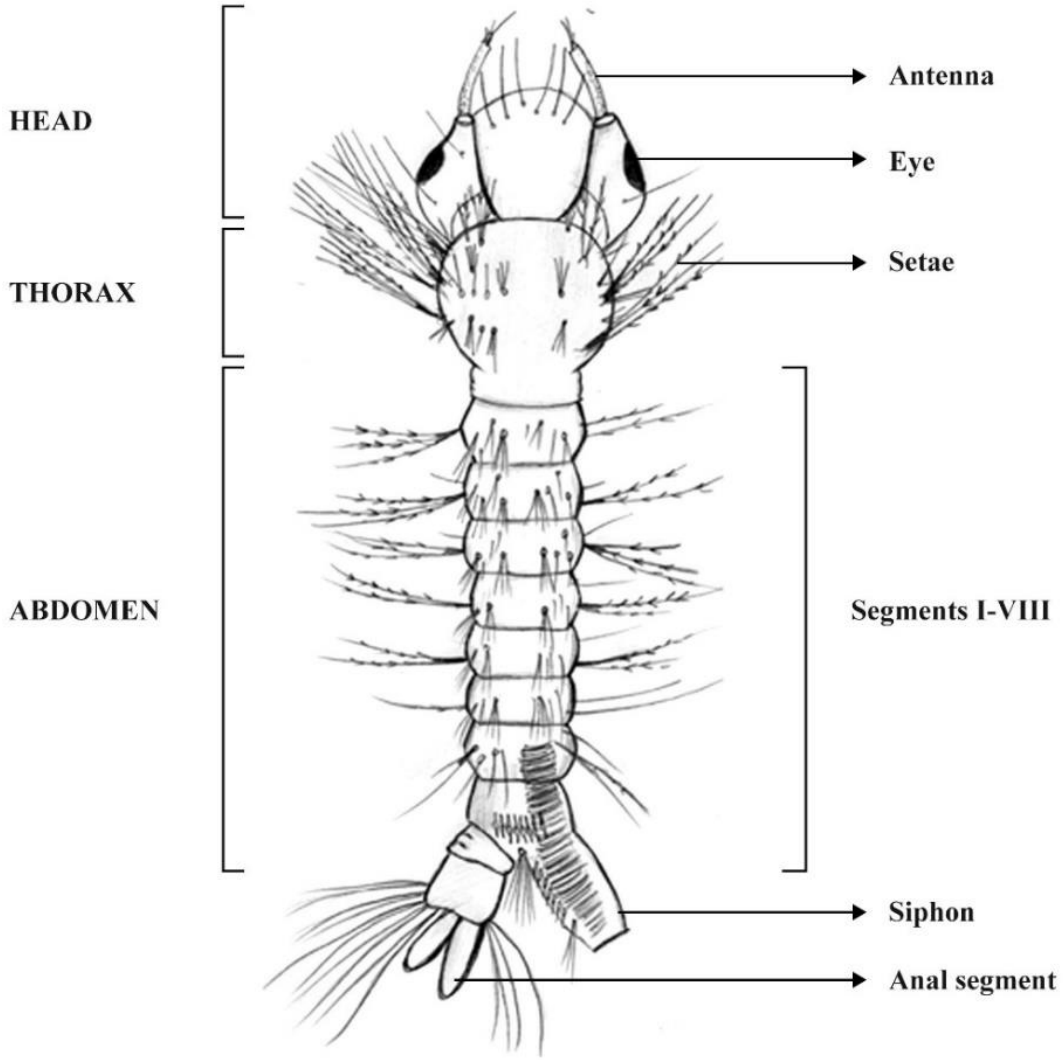

Figure 2: External anatomy of mosquito larva; illustrated by the author

INTERNAL LARVAL ANATOMY

The prime specializations of the mosquito larva are for feeding and breathing; there is little in the internal organization that is essentially different from that of other insects.

Respiratory System

As metaplastic, they utilize dorsal spiracles on the large dorsal trunks for respiration; the lateral trunks associated with the closed lateral spiracles are greatly

reduced. The ninth abdominal segment's dorsal spiracles are secondary respiratory apertures that permit the larva to breathe at the water's surface. Normally, the last pair of lateral spiracles are on the eighth segment (Imms, 1907). The intima of the larval tracheal trunks breaks between the segments, and the pieces adhered to the shed cuticle are drawn out through the new instar's lateral and posterior dorsal spiracles. Later, lateral spiracles are closed again, as they are not functional for larval respiration. The tracheal system of the young larva on hatching is filled with a liquid (Wigglesworth, 1953). Generally, air enters the tracheae only when the end of the respiratory siphon comes above the water surface. One of the dorsal longitudinal trunks fills first, followed by the other. The air gets into the tracheae as the embryonic liquid diffuses through the tracheal walls.

The dorsal blood vessel (Hemolymph circulation)

The dorsal blood vessel of larvae is a simple muscular tube stretching along the midline of the back from the eighth abdominal segment into the head. The dorsal vessel is divided into an abdominal heart and a thoracic aorta without any nervous connection. This hemolymph circulation is driven by the contractile action of the dorsal vessel. The abdominal heart is perforated along the sides by eight pairs of segmental openings, or ostia, whereas the thoracic aorta is imperforate (Jones, 1954). Along the sides of the heart, fan-shaped segmental muscle fibers known as alary muscles that support the heart on the body wall are present. In the head region, the aorta goes underneath the brain, where it is open ventrally, allowing the free discharge of blood into the head cavity, whence it flows backwards to re-enter the heart through the body by ostia. The larval heart beats forward at an average of 85.2 pulsations a minute (League et al., 2015).

Nervous system

The central nervous system of the larva includes a brain and sub-oesophageal ganglion in the head and a ventral chain of segmental ganglia in the abdomen united by paired connectives. The last ganglion is that of the eighth abdominal segment (Snodgrass, 1959).

The reproductive organs

Rudiments of the reproductive organs are present in the young larva in a very elementary state; they slowly develop during the larval life (Snodgrass, 1959).

Gut (Alimentary canal)

The alimentary canal of mosquito larvae is a relatively simple tube. It comprises three parts: an ectodermal stomodaeum, an endodermal mesenteron, and an ectodermal proctodaeum (Figure 3). The stomodaeum starts with the pharynx (in the head region), which is extended by a narrow oesophagus that passes through the neck into the thorax, where it enters the early part of the mesenteron, known as the cardia within the cardia the oesophageal walls are destined to form the entrance funnel of the stomodaeum into the mesenteron. They are lined with exoskeletons made up of chitin and cuticular glycoprotein. This will separate the lumen from the epidermis and is shed during each ecdysis. The cardia is followed by a long straight tube known as the stomach, or ventriculus, that extends back into the seventh abdominal segment. The anterior end of the stomach bears a circle of eight large pouch-like diverticula, the gastric caeca. This midgut region lacks an exoskeleton and is replaced by a peritrophic membrane secreted by the epithelial membrane of the midgut. The tight junctions between the epithelial membrane act as a barrier to the entry of pathogens (Song et

al., 2018). The peritrophic membrane is a non-cellular membrane between the gut lumen and epithelium, composed of glycoproteins and chitin fibrils. The food particles in the stomach are enclosed in a thin tubular peritrophic membrane, which is continuously replaced as it sheds (Wigglesworth, 1930). The peritrophic membrane divides the midgut into the endo and ecto peritrophic space and microorganisms are usually restricted to the former, preventing their direct contact with the midgut epithelium. The peritrophic membrane has two different forms, type I and type II. While type I lines the entire midgut and sometimes is actively produced by female adult mosquitoes during blood feeding and surrounds the blood bolus, type II is only found in a specific region of the anterior larval gut and produced by cardia (Lehane, 1997; Rodgers et al., 2017). The peritrophic membrane has diverse functions, such as a barrier protecting the epithelium from mechanical damage by food particles, safeguarding from toxins present in food, microbial invasion, and concentrating food and digestive enzymes (Shao et al., 2001).

The proctodaeum, or intestine, is bifurcated into a short anterior part and a longer posterior part known as the rectum. The anterior intestine begins as an expansion from the end of the stomach and then tapers to a tube with an S-shaped bend opening to the sac-like anterior enlargement of the rectum, which finally continues as a narrow tube to the anus. The malpighian tubules (excretory organ) are five in number; they originate from the anterior end of the intestine, especially the hindgut. They absorb wastes, such as uric acid, transported to the anterior hindgut. Thus, the hindgut contains a combination of nitrogenous waste and food waste, thereby creating a peculiar nutritive environment for insect gut bacteria than for gut bacteria of animals, in which these two waste products are separated. While water resorption is a function that is well-documented for the hindgut (Chapman, 1998). The

hindgut can also be a site of nutrient absorption, as demonstrated for numerous insect groups, including crickets.

Accessory structure

A pair of small salivary glands of varied shapes is present ventrally in the thorax. The salivary ducts merge in a common outlet duct that enters the head and opens on the labia hypo pharyngeal surface just below the mouth. The glands usually consist of two parts of different shapes separated by a constriction. The spherical anterior part of each gland consists of 12 to 15 large cells; the ovoid-shaped posterior part contains 50 to 60 much smaller cells. The opposite sides of glands are connected by a strand of nephrocytes (Jensen & Jones, 1957).

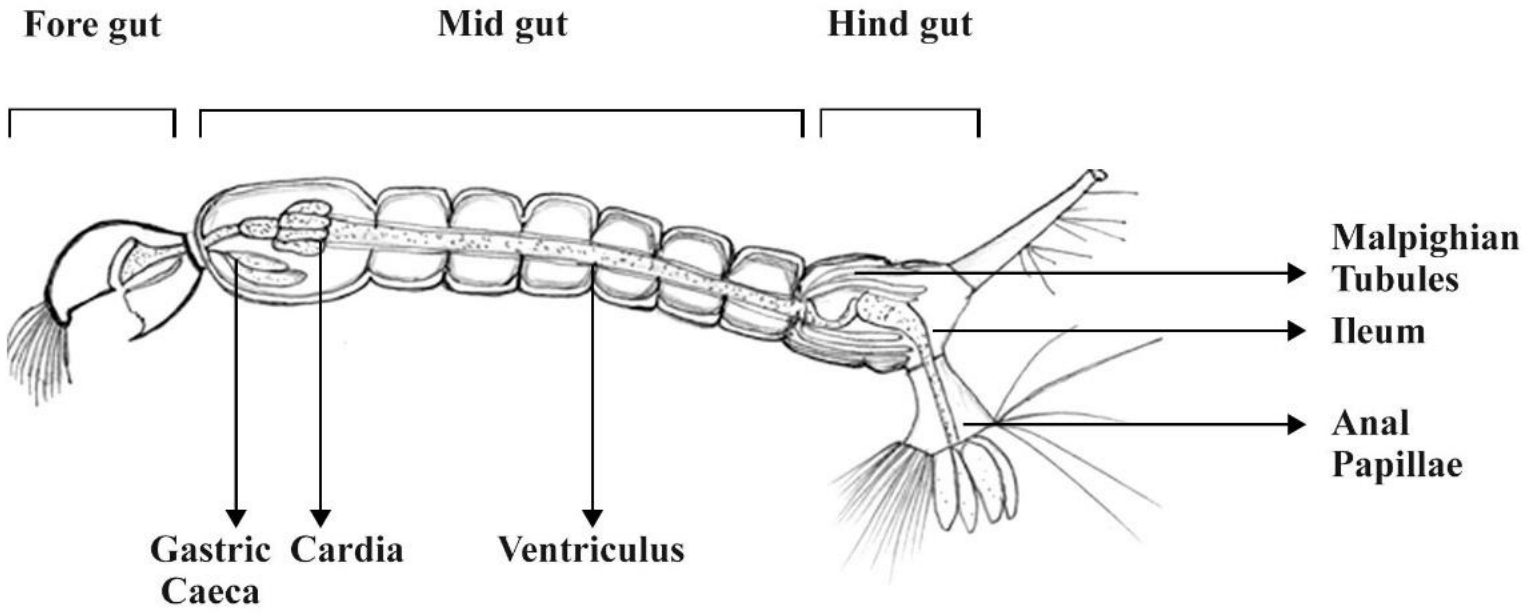

Figure 3: Internal anatomy of mosquito larval gut; illustrated by the author

FOOD RESERVES

The larval stage is the most energetic stage during the mosquito life cycle, where it requires and conserves the nutritive substances for pupation and adult emergence. Under aseptic conditions, the larva requires carbohydrates, fats, minerals (mainly calcium), protein-lipid (cholesterol), vitamin B-complex (especially biotin), and yeast nucleic acid; the promoters may be contained in yeast, liver extract, and micro-organisms (Akov, 1962). The pupal stage is dependent upon the larval stage for everything except the air it breathes. Only when the winged adult finally emerges from the pupal skin can the mosquito again take food and become an independent, self-sustaining insect once more. The elaboration and storage of food reserves in the body of the fourth-instar mosquito larva is the subject of a special study by (Wigglesworth, 1942). The stored materials chiefly include protein, fat, and glycogen for rapid consumption when the larva is subjected to starvation and replenished on subsequent feeding. The nutritional physiology of mosquito larvae has been fairly well investigated. Although most of the algae remain a nutritious food source for larvae, certain blue-green algae remain as larvicide by its toxicity (Marten, 2007).

CLASSIFICATION OF LARVAE BASED ON THE FEEDING PATTERN

The feeding organ comprises the labrum (upper lip), a pair of mandibles, the hypopharynx, (tongue-like) a pair of maxillae, and the labium (lower lip), composed of a united pair of second maxillae. During feeding, the larva does not engulf the water taken into the pharynx with its food but is discharged from the mouth. Thus anal lobes maintain the physiological balance of water in the larval body. The larvae were categorized into filter feeders, browsers, and predators based on their mouth parts, mode of feeding, and habitat. To be more specific, it includes four functional types

(depending upon the feed size and location of food availability) that have been recognized in mosquito larvae: collecting (further subdivided into collector-filtering and collector-gathering), scraping, shredding, and predators (Merritt et al.,1992).

Collector-filterers (e.g., *Culex*, *Anopheles* and *Culiseta*) remove particles that are suspended in the water column or boating on the water surface, whereas collector-gatherers (e.g., *Aedes* and *Wyeomyia*) feed by removing particles deposited on or loosely connected to rocks, vegetation, and other submerged surfaces (Clements, 1992). Filter feeders: They are larvae that strain out small food particles from the water, which are sufficiently small enough to pass directly into the gut without further breakdown e.g., *Anopheles stephensi*. Browsers: They feed on abrade solid material, the particles of which require further manipulation by the mouthparts before entering the digestive tract. Predators: They have strongly chitinized labral brushes. The role of the maxillae has been suppressed and the mandibles are the principal mouth parts. These are very large with strongly chitinized claws and take up most of the oral region of the head capsule. Large, stiff spines that grasp the prey are associated with the strong claws. This is true of the larvae of *Chaoborus* and *Mochlonyx* (Clements, 1992).

Usually, most of the larval species are heterotrophic, which feeds on organic matter (algae) and other microorganisms (bacteria, fungi, and yeast) in the water for about 4-7 days or longer, depending on the water temperature and availability of food. Larvae of mosquito predators (e.g., *Toxorhychites*, *Lutzia*) feed on larvae of other mosquitoes. In some predatory species, the first instar is a filter feeder, and the predaceous feeding structures are not developed until the second instar. The pupae (tumblers), or resting stage, appear after the fourth larval moult. Unlike larvae, pupae do not feed and live for 1-3 days before becoming adults (Clements, 1992). Categories

of larval feeding behaviour have generally been based on two criteria: particle size range and the general location of the food item (Merritt et al., 1992).

GUT MICROBIOME

The insect gut displays specialized paunches that assist microbial persistence. The potential challenge in the insect life cycle is the transmission of microorganisms from one generation to another. Mostly in insects, females leave their eggs after depositing eggs in their outer breeding sites. Hence, the direct transfer of gut symbionts between their conspecifics is more limited in most insects than in mammals, which have extended parent-offspring contact (Martinson et al., 2012). In the case of mosquitoes, the presence of microbes starts from the larval stage onwards. Microbes get colonized in the salivary gland, reproductive tract and gut.

The gut harbours more microbiota due to its extended length starting from the mouth to the anus. However, the vertical transfer of microbes between generations is limited in mosquitoes. While the type and microbial existence largely depends on its habitat. It often remains a hostile environment for microbial survival due to the timely shedding of the exoskeletal lining of both foregut and hindgut, which severely disrupts or eliminates any attached bacterial populations. The midgut produces and frequently sheds the peritrophic membrane and associated microbes, most of which do not cross into the space adjacent to midgut epithelial cells. In holometabolous insects like mosquitoes with distinct life stages, there is a radical remodelling of the gut and other organs at metamorphosis, with the complete expulsion of the larval gut and contents as a meconium that is covered in the peritrophic membrane of the pupal stage. There is a partial or complete void of microbes (especially bacteria) when they transform into adults (Moll et al., 2001).

Virome

Mosquito virome comprises arboviruses that can replicate in mosquitoes and vertebrates and later identified Insect-specific viruses (ISVs) confined to insects and do not replicate in vertebrates (Ohlund et al., 2019). Mosquitoes act as a prominent host for a key group of viruses that are insect-specific (Bolling et al., 2015). Initially, metagenomic approach was used to explore and surveillance specific viruses, such as DENV-1, Phasi Charoen-Like virus (PCLV) in *Aedes aegypti* and CHIKV, DENV-3, and YFV in *Aedes albopictus*. The pilot study using mosquito virus metagenomic sequencing was performed to characterize the heterogeneity of DNA in wild mosquitoes from California (Chandler et al., 2015). This study scrutinized a group of female mosquitoes from different species collected in three geographical sites, comprising *Culex erythrothorax* and other undetermined species. This study affirmed that the virome was highly diverse across each sample and most of its members were uncharacterized. Later, these metagenomic approaches were employed to evaluate viral load (Shi et al., 2018) in two mosquitoes *Aedes* and *Culex* genera.

The comparison presented a remarkable difference in the virome of mosquitoes, in which the genus *Aedes* has a low viral diversity and less abundance than *Culex*. This metagenomic approach leads to the sighting of different viral families Bunyaviridae, Reoviridae, Orthomyxoviridae, Rhabdoviridae, unclassified Chuvirus Flaviviridae, Mesoviridae, and Negevirus groups in mosquitoes. Most resident virome act as commensal microbes due to their inability to infect vertebrate cell lines, prolonged host infection, and vertical transmission. For example, densovirus, a single-stranded DNA virus can disseminate and persist naturally in mosquito populations. Mosquito denso virus (MDVs) can cause systemic infection in mosquitoes and replicate in many different tissues, including the midgut, malpighian tubules, fat body,

musculature, neurons and salivary glands. So, this commensal virome can be used as an excellent candidate for paratransgenesis, a bio control method for vector mosquitoes (Mukha et al., 2006).

Bacteria

Bacteria account for the major percentage of mosquito microbiota, occupying various organs mainly in the gut and to a lesser extent in salivary glands, ovaries, testes, male accessory glands, and hemolymph. The larval gut harbors a diverse and enormous number of microbes, especially bacteria of both positive and negative strains. These microbial food resources can act as limiting factors for larval development reasons. For example, axenic (free of microbes) larvae are found dead during the first instar itself compared to larvae colonized with bacteria. A substantial fraction of microbes get transferred horizontally into the gut by larval feeding during their aquatic life stage. The presence of a continuum of bacteria is documented during the juvenile, pupal, and adult stages. The bacterial communities vary, overlap, or are reduced when they transform from one stage to another (adult). Normally larvae meet their nutritional requirements by feeding on heterotrophic bacteria, fungi, and protozoa (ciliates and flagellates) (Carpenter, 1983; Walker et al., 1991). This paves the initial way for microbial colonization in the gut. The bacterial survivorship in the larval gut is highly dependent upon the microbial content of its habitat driven by leaf material input and other allochthonous organic debris, along with the prevailing physicochemical parameters of the breeding water.

Core microbiota (microbiota shared by the same species from different geographical areas) and pan microbiota (different microbiota shared by different mosquito species regardless of their geographical location) are the two concepts that are solemnly influenced by the biogeochemical properties of breeding water. From

the earlier studies, it was found that midgut is dominated by Gram-negative bacteria for example, in the case of *Anopheles stephensi* a causative agent of malaria, Gram-negative *Myroides, Chryseobacterium, Aeromonas, Pseudomonas, Klebsiella, Enterobacter* and *Shewanella* and the rest were Gram-positive *Exiguobacterium, Enterococcus, Kocuria*, *Microbacterium*, and *Rhodococcus.* Significant variation can be reflected in microbiota due to the effect of environmental habituation (Chavshin et al., 2012; Jones et al., 2013; Jones et al., 2021).

In the case of *Aedes aegypti*, Proteobacteria is the predominant phylum present in larvae, pupae, and adults. Phylum Firmicutes, Actinobacteria and Bacteroidetes, as well as members of Proteobacteria, were found to be consistently present in fourth th instar larvae (Coon et al., 2014; Wang et al., 2018). Other genera frequently found in the larvae gut include *Chryseobacterium, Elizabethkingia, Pseudomonas, Neisseria* and *Enterobacter* (Osei-Poku et al., 2012). The *Actinobacteria, Leucobacter* and *Microbacterium*, both belonging to the *Microbacteriaceae* family, are abundant in *Aedes aegypti* larvae but nearly absent in adults (Coon et al., 2014). In contrast, Chryseobacterium (Flavobacteriaceae) was a common component of mosquito microbiota at all life stages. A proteobacterium of the genus *Asaia* is stably associated with larvae and adults of *Anopheles stephensi* and earlier studies documented that the microbiota changes according to the existing ecological niche of larvae (Favia et al., 2007; Rani et al., 2009) whereas in the case of *Culex quinquefasciatus Bacillus* sp., *Staphylococcus* sp. and *Pseudomonas* sp., are more frequently encountered in the larval gut (Vasanthi & Hoti, 1992).

Fungus

As far as mycobiota is considered, it is the least analyzed gut entity compared to larval gut bacterial microbiota (Guegan et al., 2018). Mosquitoes can encounter

fungi during different phases of their life cycle. During the larval stage, they are exposed to fungi on the plant detritus, on the water column, and on the water in their aquatic breeding sites, whereas adults are exposed to fungi during their resting period (both indoor and outdoor), blood-feeding, oviposition, etc as ubiquitous eukaryote fungi are found in all mosquito habitats. They enter into the larval body either by ingestion through different feeding behaviours (collecting-filtering, as well as grazing on and shredding of decaying and living organic matter) or by active and passive breaches in the cuticle (Merritt et al., 1992). Recent studies have pointed to the presence of a core fungal community dominated by yeast in all mosquitoes (Luis et al., 2019). The yeast intake benefits larvae as it holds the nutritional value required for development.

On the contrary, the ingestion of entomopathogenic fungi is lethal to mosquitoes. The fungal spore's ingestion is a common route to reach the larval gut. Ingestion of *Culicinomyces*, *Beauveria bassiana*, *Metarhizium anisopliae* and *Aspergillus clavatus* spore was reported for *Aedes*, *Culex,* and *Anopheles* mosquito larvae. These spores remain in the midgut and release toxins, which can eventually cause larval death. In general, many of these fungi germinate and penetrate the different parts of gut epithelium by various paths. For example in the case of *Aedes aegypti*, *Beauveria bassiana* blastopore colonizes the foregut, midgut, and hindgut region within 24 hours of post-infection, While in the case of *Culex quinquefasciatus* the ingestion of *Aspergillus clavatus* spore leads to its accumulation in the gut thereby disrupting the gut epithelial tissue for facilitating fungal dissemination into mosquito body (Scholte et al., 2004; Bawin et al.,2016).

It can also enter the larval body through cuticular penetration. Fungi in the genus *Coelomomyces* are known to infect mosquito larvae by cuticular penetration. In

addition, soil entomopathogenic fungi like *Metarhizium anisopliae* and *Beauveria bassiana* are noted for infecting multiple species of mosquito larvae through respiratory apparatus by direct cuticle penetration. Active cuticular penetration involves various steps like attachment of spore to host epithelium, germination to form a fungal tube, differentiation of fungal tube into attachment organ (appressoria), followed by the formation of penetration tube to cuticular layer, degradation of cuticular layer by degrading enzymes, succeeded by the proliferation of fungus in the hemolymph as blastopore or hyphal bodies. As the infection progresses, the insect host succumbs to infection through fungal toxin or starvation (Scholte et al., 2004; Seye et al., 2009; Lovett & St. Leger, 2017). These qualities of entamophagenic fungi can be convincingly used as a bio-control measure against malaria *Anopheles stephensi* (Bukhari et al., 2011). Generally, the feeding behaviour also leads to the formation of commensals in the hindgut. Not less than four fungal species in the genus *Smittium* sp., belonging to the order of Harpellales, can attach and replicate in the hindgut of various mosquito species with no effect on larval development or survival (Sweeney, 1981; Piyaratne et al., 2005). The presence of the genus *Penicillium* was reported many years back from mosquito larvae of *Aedes* sp., *Anopheles* sp., *Culex* sp. and *Mansonia* sp., based on the morphological analysis of fungal colonies (Lara da Costa et al., 1998).

By using the culture-dependent method, the presence of fungi like *Candida parapsilosis* and *Meyerozyma guilliermondii* were spotted in larvae, pupae and adults (in both gut and gonads) of *Aedes aegypti* and *Aedes albopictus* in both laboratory strains and wild-collected strains by culture-dependent method from countries like Brazil, Bangladesh and Italy (Bozic et al., 2017). Identifying these fungi in *Aedes* sp., which complete their life cycle in anthropized environments, suggests these

mosquitoes could contribute to the dissemination of pathogenic yeasts, thus increasing their public health relevance (Bozic et al., 2017). Metabolic interactions between members of the mycobiota and the mosquito host are being discovered. As an example, a fungus from the *Talaromyces* genus was identified to be naturally present in the midgut of field-caught *Aedes aegypti* females from Puerto Rico (Anglero-Rodríguez et al., 2017) using a combination of microscopy and sequencing of the rRNA internal transcribed spacer (ITS) (Schoch et al., 2012). *Talaromyces* enhanced DENV2 infection by transcriptional and enzymatic inhibition of trypsin in the midgut, thus increasing mosquito vector competence (Anglero-Rodríguez et al., 2017).

PHYSICOCHEMICAL PARAMETERS

The selection of breeding sites by gravid female mosquitoes is an innate behavioural trait that profoundly influences mosquito survival and population dynamics. Along with these physical and chemical characteristics, lentic aquatic breeding sites influence the probability of hatching, immature larval development, pupation, and adult emergence by specifying the niche of a mosquito species. Typically, larval mosquitoes are found in different aquatic habitats, including freshwater and saline environments. Based on the type of aquatic habitats larval mosquitoes were categorized into three types: fresh water osmoregulators, euryhaline osmoregulators, and euryhaline osmoconformers (Bradley, 1987). For example the mosquito *Ochlerotatus taeniorhynchus* has the ability to tolerate salinities with the help of salt-secreting rectal segments, while the obligate freshwater forms like *Aedes aegypti*, *Culex quinquefasciatus* lack salt glands and limited their distribution in freshwater habitats. At the same time, earlier studies have revealed the existence of *Anopheles* mosquitoes in brackish and freshwater found in rural, urban and coastal areas (Piyaratne et al., 2005).

The instigation for ovipositional flight is related to the breeding site's environmental parameters like temperature, relative humidity, wind speed and rainfall. The selection of a breeding site involves visual, olfactory, and tactile responses. Physico-chemical properties of the mosquito breeding habitat, such as optimum temperature, total suspended solids, total dissolved solids, electrical conductivity pH, ions and dissolved oxygen, have an effect on larval development and survival. The data regarding geochemical properties is a potent key factor for larval surveillance for vector management. Apart from this, these parameters can also influence the microbial composition of breeding water. Fluctuations in pH, temperature, ions and dissolved oxygen content can cause variations in the microbial content of breeding water (Kaufman et al., 1999). This positively or negatively influences the larval survival as they consume these tiny organisms as food. This implies an indirect relation between physico-chemical parameters in shaping the gut microbiota of mosquito larvae. *Anopheles stephensi* an urban vector for malaria thrives in clean water collected in overhead tanks, fountains, cisterns, etc. The profiling of *Anopheles stephensi* breeding water quality provides more insight into the positive correlation of water parameters such as total dissolved solids, electrical conductivity, total hardness, phosphate, fluorides and chlorides on larval survival (Thomas et al., 2016). *Aedes aegypti* displays a great deal of distinctiveness in choosing their breeding site, limiting their distribution to preferred sites.

The chemical cues such as microbial metabolites like specific bacteria-associated carboxylic acids, methyl esters and kairomones serve as potent oviposition stimulants for gravid *Aedes aegypti*. More over, female mosquitoes are inexorably linked to laying eggs in artificial containers, especially discarded tires, which resist desiccation, the eggs then hatch after being transported in used tires from different

countries, which results in cosmotropical distribution, for example, from Asian countries to Western countries (Ponnusamy et al., 2008; Takumi et al., 2009). The chemical preference varies among mosquito species for example *Culex* and *Anopheles* mosquitoes are more inclined to turbid water with high alkalinity and less oxygen content. Earlier studies have marked the prominent chemical factor in each mosquito species respective breeding site, especially *Aedes*, *Culex*, and *Anopheles*. Sulphate content was lower in the *Culex* and *Anopheles* breeding sites than in the *Aedes* breeding sites, whereas the chloride content was higher in the *Culex* and *Anopheles* breeding sites and lower in the *Aedes* breeding sites. The phosphate content was less in *Aedes aegypti* breeding sites. A thorough understanding of breeding ecology is inevitable for larval control management if the mosquito is of epidemiological importance.

PHYLOGENETIC ANALYSIS

To fully assess the role of vector mosquitoes in the epidemiology of human diseases, it is essential to understand the diversity of microbiota harboured by natural flies during each phase of their life cycle. DNA sequencing and molecular phylogenetics are increasingly being used in microbiology to study the transmission of microbes. By reconstructing the evolutionary history of microbial genomes, the behaviour of microbial populations can be modelled, and the future of epidemics may be forecasted (McCormack & Clewley, 2002). Phylogenetic analysis explores the evolutionary relationships between organisms and is a vital foundation for microbial studies. The development of reliable phylogenetic trees is an important step in characterising new pathogens and developing new treatments in biomedicine. Trees based on gene sequences are maps with which to articulate the elusive concept of

biodiversity. Thus, comparative analyses of small-subunit rRNA (16S or 18S rRNA) and other gene sequences show that life falls into three primary domains, Bacteria, Eucarya and Archaea (Woese, 1987; Woese et al., 1990). Recent studies have highlighted host-microbiota phylosymbiosis, i.e. a congruency between the host phylogeny and the divergence in its associated microbial community composition.

Even though mosquitoes lead to several deadly diseases in mankind, it cannot be completely ruled out from the ecosystem. From an ecological perspective, the role of mosquitoes is inevitable as they are members of aquatic and terrestrial food chains, serving as food for higher trophic levels like birds and fishes (Vijayan, 2010). In populous countries like India, there is a surge in mosquito-borne diseases like malaria (*Anopheles* mosquito), dengue (*Aedes* mosquito) and filariasis (*Culex* mosquito), which are capable of affecting public health, the social and economic status of the country. National Vector Borne Disease Control Programme (NVBDCP) of India reported malaria as the most life-threatening disease in the country, with 95% of the population in the country residing in malaria endemic areas. Filariasis and dengue follow this. For control of these epidemics, vector control is considered to be one of the important master plan to reduce transmission. The use of chemical insecticides can restrain adult mosquitoes. However, larval control is more economical and provides sustainable control by eradicating the emergence of adult mosquitoes. A thorough understanding of larval gut content with special emphasis on dominant members like bacteria and fungi is essential in formulating an effective biocontrol to check these outbreaks and epidemics. The present study aims

- To investigate gut microbiota (bacterial and fungal communities) in *Aedes aegypti*, *Culex quinquefasciatus* & *Anopheles stephensi* using culture dependent method.

- To delve into the molecular phylogeny of the microorganisms residing in the mosquito guts by employing advanced molecular techniques, to unravel the evolutionary relationships among these microbial communities and gain insights into their genetic diversity.
- To analyse (comparative) physicochemical parameters in natural habitat of these selected mosquitoes across seasons will shed a comprehensive view of the seasonal conditions that influence the mosquitoes' breeding sites.

REVIEW OF LITERATURE

Generally, mosquitoes are considered annoying creatures that act as bridge vectors for diseases. Out of more than 3500 species, only a few female mosquitoes act as carriers of deadly viruses and parasites that result in human mortality and disease. Their positive role in the biosphere is purely neglected. Being a member of the food chain, the role played by mosquitoes is worth appreciating. Mosquito larvae, which occupy the base trophic level in the aquatic food chain, are being eaten by *Gambusia* (mosquito fish), which in turn are taken by medium-sized fish. These medium-sized fish are food for large fish. Alligators, birds and humans are consuming the large fish (Rueda, 2008).

The interdependence between prokaryotes and eukaryotes, as well as between eukaryotes and eukaryotes, is inevitable for the existence of life on Earth. For example, chemical signalling occurs between microbes and insects in ample instances. Like that, mutualistic interaction occurs between plants and mosquitoes in which mosquitoes act as excellent pollinators and plants as providers of nectar. Floral nectar serves as a habitat and server of nutrients to microorganisms. These microbes can alter the inflorescence odour by acting as semi chemicals (message-bearing chemicals). It is thereby strengthening plant-pollinator signalling system in pollinators like mosquitoes. Normally mosquitoes are attracted both by visual inflorescence displays odour as well as by carbon dioxide released by microbes. These floral nectar are the chief source of dietary nutrients besides extra floral nectar, aphid honey dew and fruit juices needed for adult mosquitoes for fight, mating, blood-feeding, and fecundity. The *Aedes communis* and *Aedes canadensis* behave as pollinators of a particular

variety of orchids, *Habenaria obtusata*, in North American swamps and in Silene otitis, a perennial and dioecious plant mostly seen in Europe and Central Asia (Manguin & Boete, 2011). *Tanacetum vulgare*, commonly called Tansy, is a perennial herbaceous flowering plant that attracts *Culex pipiens* by semiochemicals for pollination (Peach et al., 2021). The mosquito plant interactions were justified by the studies of (Beier, 1996; Nyasembe & Torto, 2014) in mosquitoes collected from Western Kenya, in which 6.3% of the indoor-resting mosquitoes and 14.4% of host-seeking *Anopheles gambiae* and *Anopheles funestus* showed positive result for fructose(a form of carbohydrate present in plants).

Since mosquitoes hold a place in the food chain, the elimination of mosquitoes will affect the ecological balance. Over time globally, a lot of death due to mosquito-borne diseases has been reported next to tuberculosis and AIDS. On a world-wide basis, mosquito-borne diseases are a key group of concern. More than 400, 000 people die every year from malaria and other serious illness epidemics like dengue, yellow fever, chikungunya, and Zika in numerous metropolitan cities.

By 2050, half of the world's population is expected to be exposed to the risk of arbovirus transmission (Viglietta et al., 2021). The principal methods available for reducing the public health burden of most mosquito-borne diseases are vector-based interventions. Among the vector mosquitoes, the genera *Aedes*, *Anopheles* and *Culex* play an important lead in disease transmission. *Aedes* (Stegomyia) *aegypti* (Linneaus), a day-bitting mosquito, more intimately associated with humans than other vertebrate hosts for blood feeding during the gonotrophic cycle. This preference makes it more adaptable to survive in human habituations to feed and rest. Out of 950 species of *Aedes* mosquitoes, *Aedes aegypti* has been the vector of dengue virus, yellow fever virus, Zika virus, and chikunguniya virus. This single species of mosquito remains the

main threat, causing inestimable suffering to mankind (Powell, 2018). *Aedes aegypti*, was an indigenous species to Africa, whose ancestral population was still found in the forest zone, whose larval forms were found in tree holes, and whose adults depend on non-humans for blood meals. The mosquito shows cosmo tropical distribution due to its greater adaptability to avoid the effects of transient climatic conditions. By the start of civilisation, humans began to use containers and utensils for water storage all year around, making it an interesting breeding place for *Aedes aegypti* to obviate adverse conditions like flood for oviposition (Jansen & Beebe, 2010).

The sylvatic yellow fever virus (YFV) originated in Central African rain forest terrain and extended along riverine forests. There was no documentation of yellow fever outside Africa before 1500. The primary vector mosquito was *Aedes africanus* for sylvatic YFV. Other mosquito species with identical larval habitats and biting options are also involved in YFV transmission, including *Aedes furcifer-taylori*, *Aedes vittatus, Aedes luteocephalus*, *Aedes opok*, *Aedes metallicus*, and *Aedes simpsoni* sensu lato and *Aedes bromeliae* (Hanley et al., 2013). The 15th-19th centuries witnessed large-scale epidemic inflation of yellow fever by trade in port cities of North and South America, Africa, and Europe, where a lot of mortalities and morbidities were recorded. During 1900-1901, studies by Walter Reed found that yellow fever was transmissible among humans by *Aedes aegypti* mosquitoes (Bryant et al., 2007).

Dengue fever was first mentioned as “water poison” connected with flying insects in a Chinese medical encyclopaedia in 992 from the Jin Dynasty (265-420 AD). By 1823, the term ‘denga’ or ‘dyenga’ was used to name a particular disease on the east coast of Africa. 1870 witnessed an outbreak in Zanzibar (Swahili Unguja), an island in the Indian Ocean, lying 35 km off the coast of east-central Africa. The

affected people manifested sudden cramp-like seizures. The term ‘dengue’ was assumed to have originated from Swahili word ‘ki denga pepo,’ which means “a sudden, cramp-like seizure caused by an evil spirit”. It is believed that dengue spread by slave trade from East Africa and Cuba to Caribbean islands during the outbreaks in 1828. During the time of II World war, the pandemic spread too many parts of the world. The virus was isolated by Japanese scientists Hotta and Kimura in 1943 by inoculating the brain of suckling mice using the serum from dengue patient as culture media. In the succeeding year (1944) virus was isolated by Albert B. Sabin from U.S. soldiers in India, New Guinea, and Hawaii. These isolations laid the foundation for the classification of the virus into serotype 1 (DENV1) and serotype 2 (DENV2), respectively. At the time of epidemic of Manila, Philippines in 1956, Hammond isolated other two serotype, i.e serotype 3 (DENV3) and serotype 4 (DENV4). The year 1980 was marked by resurgence of dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS) in many parts of the world especially it radiates from Southeast Asia and the Western Pacific to the American region. Globally by the end of 90s and in the early beginning of 2000 an unprecedented rise in the number of dengue disease with a high rate of mortality and morbidity was recorded (Gubler et al., 1997).

The epidemiological data on dengue in India is quite intriguing and interesting. In India, the first confirmed sporadic outbreak of DHF occurred in 1956 in Vellore district, Tamil Nadu. Later, in 1963-1964, a massive blow of DHF occurred in Calcutta, West Bengal; from there, it took about twenty to thirty years to spread to other states of the country. The first major nation-wide epidemic occurred in 1996, caused by DENV-2, a genotype IV strain of the virus. Almost 10,252 cases and 423 deaths were reported at that time. Following this, a gradual expansion in viral

transmission has been noticed in the country. At present, four serotypes of DENV are prevalent in India (Chakravarti et al., 2012).

During 1914-1970, the Rockefeller Foundation conducted research programs on yellow fever; the discovery of ZIKV and numerous other arboviruses was its outcome. Zika was discovered during a course of study on vectors responsible for sylvan YFV in Uganda. In 1947, the ZIKA virus was isolated in Ziika forest near Entebbe, a city in Uganda, from non-human primate (Rhesus monkey) and mosquitoes (*Aedes africanus*) in 1948. This virus was named after the geographical location from which it was isolated. By 1954, the first infection of ZIKA was reported in humans residing in Nigeria (Africa). This particular ZIKV was isolated from an *Aedes aegypti* mosquito during the 1960s in Asia. By 2007, hundreds of ZIKA infections in humans were reported from Yap State (one of the four states of the Federated States of Micronesia). It became a worldwide concern when it hit Brazil in 2015. However, globally, geographic distribution of ZIKV, its incidence and prevalence of disease, are likely to be underestimated (Waggoner & Pinsky, 2016).

The geographic expansion of ZIKA led to reports of cases from areas of zero or minimal transmission. Serological confirmations of the ZIKA virus in India were reported shortly after 1954 in the states of Gujarat and Tamil Nadu. After years of dormancy in 2018, the first compiled report of the largest outbreak of ZIKA occurred in Jaipur, Rajasthan, and Madhya Pradesh in the months of September and October 2018. A total of 153 cases were reported from Jaipur, and 130 confirmed cases of ZIKA were reported from Madhya Pradesh and 70 confirmed cases were reported from Kerala (Saxena et al., 2019; Sah et al., 2023).

Likewise, the chikungunya virus (CHIKV) was first isolated in 1952 in Tanzania, a country in East Africa. The disease was named from a Makonde word

portraying the bent posture of people with severe arthralgia, which is a trait of chikungunya fever. In earlier times, viruses disseminated in forest regions of Sub-Saharan Africa, and enzoonotic transmission occurred between non-human primates and vector mosquitoes. Later, the pandemic was reported on the Asian continent during the 1950s and 1960s, after the opening of the Suez Canal for trade. This disease was reported in many parts of South Africa, like Zimbabwe, Cameroon, Uganda, and Senegal. The year 1999 was endorsed by an outbreak in Port Klang, Malaysia. In 2004, a severe outbreak occurred in coastal Kenya and spilled out on the islands of the Indian Ocean, infecting millions of people. Consequently, the infection spread and flourished in other countries like Italy, France, America, Europe, and Asia. The reason for this unprecedented surge and spread of the pandemic is mainly due to air travel. *Aedes albopictus*, a native of Asia, was considered the second chikungunya virus vector besides *Aedes aegypti*. From its native land, it spread to islands in the Indian Ocean, then to southern European countries and to Africa. In 1963, the first outbreak occurred in Kolkata, India, which claimed to be the first Asian country to have chikungunya. Later on, it gets reported from other states of the country, like Maharastra, Tamil Nadu, Kerala, etc. India is estimated to contain the highest number of chikungunya virus infection cases from Asia. Even though *Aedes albopictus* acts as a vector, its implication on disease transmission is less in Asia when compared to that of *Aedes aegypti* (Chandak et al., 2009; Weaver & Lecuit, 2015).

Culex quinquefasciatus Say, 1823 (the southern house mosquito) is a member of the *Culex pipens* species complex. The *Culex pipens* species complex comprises *Culex pipiens Linnaeus* 1758, *Culex quinquefasciatus* Say 1823, *Culex pipiens pallens Coquillett* 1898, and *Culex australicus* (Dobrotworsky & Drummond, 1953). *Culex quinquefasciatus* (nocturnal biting mosquito) was the prime vector of the nematode

Wulchereria bancrofti (agent of bancroftian flariasis) and the West Nile virus (WNV). As anthropophilic species, adults feed on both bird and human blood and have the vigour to transmit sylvatic arboviruses from migratory birds to humans in urban areas. *Culex quinquefasciatus* was also potent enough to transmit the protozoan *Plasmodium relictum*, the causative agent of bird malaria (Lopes et al., 2019). As an opportunistic mosquito, it can survive in a bit of breeding water, which makes its geographical expansion easier. It acts as a prominent vector nematode, causing lymphatic filariasis in semi-urban areas of Asia. It vectored the Rift Valley virus in the African region and West Nile, St. Louis encephalitis virus, and Western equine encephalitis virus in the United States (Vadivalagan et al., 2017).

To date, in 1866, Otto Wucherer described the first *Wuchercheria bancrofti* microfilaria as the agent of lymphatic filariasis in Brazil. Lymphatic filariarsis is a neglected tropical disease and has an epidemic spread in countries like Asia, Africa, and America. In terms of its geographical distribution (Fontes et al., 2012). Lymphatic filariasis is one of the most important health concerns in India. The disease was documented in India as early as the 6th century B.C. by the eminent Indian physician Susruta in his book 'Susruta Samhita'. In India, *Wuchereria bancrofti*, vectored by *Culex quinquefasciatus*, was responsible for 99.4% of infectious problems in the country. To overcome this, India launched the National Filaria Control Programme (NFCP) in 1955 with the rationale of controlling the epidemic (Agrawal & Sashindran, 2006).

Rift Valley Fever (RVF) is considered a global menace, affecting a wide array of animal species, including humans. The epidemic mainly causes abortions in livestock. Up until the late 20th century, the RVF was restricted to Sub-Saharan Africa (SSA). The first report of RVF outside SSA came from Egypt in 1977; in the

succeeding year (2000) it was first reported outside the African continent and then from the Arabian Peninsula(Clements et al., 2007).

In 1937, WNV was first isolated from the West Nile district of Uganda from the blood of a native Ugandan woman. The zoonotic transmission of WNV occurs through the bird-mosquito-bird transmission cycle. The period 1950-1980 was marked by the geographical endemic spread of WNV throughout Africa, the Middle East, Southern Europe, Western Russia, South Western Asia, and Australia due to its viability in infecting numerous mosquito and bird species. It became a global public health concern after its discovery in North America in New York City in 1999. It causes neurodegenerative outbreaks in the United States. WNV infections have been linked to a high prevalence of long-term effects like memory loss, confusion, and fatigue. Nearly 1% of WNV infections result in neuroinvasive diseases such as meningitis, encephalitis, or acute poliomyelitis. It was evident that neurological impairments can persist for years following infection (Petersen et al., 2013). In India, WNV has been isolated from human beings, frugivorous bats (Rousettus leschenaulti), domestic pigs (NIV, unpublished data), and mosquitoes. In India, among *Culex* mosquitoes, *Culex vishnui*, *Culex quinquefasciatus*, *Culex tritaeniorhynchus*, *Culex bitaeniorhynchus*, and *Culex univittatus* act as potent vectors of WNV(Paramasivan et al., 2003).

St. Louis encephalitis (SLE) is an arboreal disease present in the New World (North America) and transmitted between bird-mosquito cycles. By 1600, Europeans had settled in North America, and large-scale agricultural development caused a shift in North America's landscape topology. This creates a perfect habitat for *Culex* mosquitoes and avian finches like doves, house finches, etc. By 1933, thousands of SLE cases were reported in St. Louis, Missouri. In the succeeding years, it spread to

many parts of North America (Reisen, 2003). Malaria is a common vector-borne disease present in tropical and subtropical regions of the world, including Africa, Asia, and America. In 1880, Charles Laveran discovered the presence of malaria parasites in human blood. The role of mosquitoes in transmitting malaria was discovered by Sir Ronald Ross in 1898. It is caused by protozoan parasites belonging to four *Plasmodium* species, namely *Plasmodium vivax, Plasmodium malariae*, *Plasmodium falciparum* and *Plasmodium ovale* and is vectored by *Anopheles* mosquitoes. Not all anophelines can transmit malaria. Only mosquitoes belonging to the Anopheles sub-genus *Cellia* can transmit the disease. There are around 460 recognised species so far, of which over 100 can transmit human malaria. Some species of vector mosquitos comprise *Anopheles culicifacies, Anopheles sacharovi*, *Anopheles superpictus*, *Anopheles stephensi*, *Anopheles arabiensis*, *Anopheles pulcherrimus*, and *Anopheles hyrcanus* (Raghavendra et al., 2011).

India, the most populous country in the world, faces serious public health challenges when it comes to the matter of accurate estimation and proper control of malaria. The year 1950 was marked in the history of India as having the highest incidence of malaria, with an approximate 75 million cases and 0.8 million deaths per year. Many reasons for the disease's prevalence include the country's geographical topography and favourable climatic conditions that sustain the parasite and the vector mosquitoes. Two major human malaria species are reported in India: *Plasmodium falciparum* and *Plasmodium vivax. Plasmodium malariae* has been reported in Odisha, the eastern state of India, whereas *Plasmodium ovale* is absent. To overcome the epidemic, the government of India introduced the National Malaria Control Programme (NMCP) in 1953 with the vision of declining and controlling the disease's progression (Das et al., 2012).

According to the World Health Organisation (WHO), around 1/6 of illnesses and diseases are caused by vector-borne diseases. Every year, over one million mortality cases and almost one billion cases were reported from various parts of the nation. Mosquito-borne diseases include dengue, yellow fever, chikungunya, Zika virus (*Aedes aegypti*), chikungunya, dengue, and WNV (*Aedes albopictus*). Lymphatic filariasis is vectored by *Culex quinquefasciatus* and *Anopheles* (more than 60 known species can transmit diseases), malaria and lymphatic filariasis in Africa. Dengue remains the fastest-spreading mosquito-borne disease in the world. *Aedes aegypti* was the principal vector, and *Aedes albopictus* was the secondary vector of the disease (WHO, 2014).

Viruses get circulated to humans by the bite of infected female mosquitoes. Once the virus is incubated for a period of approximately 1 week, the mosquito can transmit the virus until its end. Later, the infected mosquito acts as a vector and disseminates the virus by biting. In keeping with WHO statistics, about 40% of the total population is at risk of dengue. Out of the affected cases, about 2.5% of deaths were reported. The major cause of this massive blow was the lack of effective antiviral medication and vaccines. As stated by the WHO, every year, about 200000 yellow fever cases were reported, resulting in more than 30000 deaths worldwide. At present, there are no specific treatment norms for diseases. The treatments were provided for existing symptoms shown by the patients. Chikungunya was less severe when compared to dengue and yellow fever.

WHO has listed malaria (vector-*Anopheles*) as a dreaded disease affecting more than 97 countries across the world, with more than 3.4 billion people at risk. The disease severely hit Sub-Saharan Africa, with more than 90% of deaths. Lymphatic filariasis, also known as elephantiasis, is transmitted mainly by *Culex* mosquitoes.

About 120 million people are presently infected with lymphatic filariasis, of which 40 million are disFigureured and debilitated by the disease. It affects more than 25 million men with genital disease and more than 15 million people with lymphoedema. About 65% of those infected live in the Southeast Asia Region, 30% in the African Region, and the remainder in other tropical areas (WHO, 2014).

Unplanned urbanisation, environmental changes, increased global travel and trade, and other societal challenges have escalated the emergence or re-emergence of vector-borne diseases. The United Nations (UN) specifies urbanisation as the process by which a large number of people permanently congregate in comparatively small regions, producing a city (Desa, 2018). In line with data from 2018, 55% of the world's population lived in cities, and it was anticipated that by 2050, that number is expected to rise to 68% (Desa, 2018). The reports from the WHO stated that urbanisation presumably brings about the emergence of new vector-borne diseases, particularly viral diseases spread by mosquitoes (WHO, 2017).

Rochlin and co-workers in 2011 stated the role of bio-indicators, like bacteria, plankton, plants, and animals, to assess the state of an ecosystem's normal habitat. They have been used to evaluate the environmental status and existing biogeographic changes. Significant mosquito habitats are frequently found close to populated regions, particularly residential areas. The environmental changes brought on by human activities have a major impact on the diversity and abundance of many mosquito species. Urban environments have become the principal breeding grounds for invasive species like *Aedes albopictus* and the common yellow fever mosquito, *Aedes aegypti*. The presence of a large area of breeding habitats, lots of human hosts, and little mosquito control creates an ideal mosquito hotspot (Rochlin et al., 2011).

One of the most significant abiotic elements affecting the physiology, behaviour, ecology, and, consequently, the survival of insects is the ambient temperature. As poikilotherms, insects have variable internal temperatures that are influenced by the temperature of their environment. Due to fluctuations in thermal variation, insects have to face challenges like desiccation, changes in metabolism, and even losing the ability to move. *Aedes aegypti* mosquitoes can withstand temperatures between 15°C and 32°C (Reinhold et al., 2018).

Similar findings have been reported in Asia, where climate change could worsen mosquito-borne diseases. Studies have reported that an increase in temperature (1-2°C) results in rapid and higher viral replication, making the situation more favourable for the acute spread of dengue and chikungunya, especially in areas with a large number of *Aedes* mosquitoes (Lounibos & Kramer, 2016).

The vector-borne ailment episystem includes biological and environmental units and aspects of the overall epidemiological vector-borne disease system within particular spatial or temporal scales. An important facet of the Anthropocene is the shift in land use from naturally occurring to human-dominated landscapes, which modified disease risk by affecting the interrelationship between people, pathogens, vectors, and vertebrate hosts. The major root cause of the worldwide dissemination of vector-borne diseases is the immediacy and intensity of unscientific land use and its impacts on local ecology. Arthropod vectors are able to quickly adjust to local climatic and ecological changes. For instance, drought can have a significant impact on vegetation, aquatic predators, and water table levels, all of which affect vector populations. Lack of precipitation can influence mosquito populations both at the larval and adult stages. Flood water mosquitoes have shown a variety of adaptations for surviving and growing in transient habitats, particularly transient pools of water

produced by drought conditions. Notably, several species of mosquitoes can produce drought-resistant eggs that are capable of withstanding many years without water. Hatching occurs when prompted by environmental factors such as changes in water table levels. Whereas urban mosquitoes are naturally adaptable and can take advantage of habitats made in response to drought (such as water storage facilities), this adaptable property of invasive aedine mosquitoes to container settings helps in the local transmission of viruses in temperate regions.

Vector control and surveillance are the most productive methods to check for mosquito-borne diseases. While choosing the most appropriate vector control method, or combination of methods, consideration should be given to the local ecology, behaviour of the target species, the availability of implementation resources, the cultural context in which control interventions are carried out, the feasibility of applying them in a timely manner, and the adequacy of coverage. Methods of vector control include the elimination or management of larval habitats, larviciding with insecticides, the use of biological agents, and the application of adulticides. Frank and colleagues in 1980 pointed out that integrated pest management (IPM) has been practiced by mosquito control organisations for many years. These elements of mosquito control are present in practically all programmes. Integrated Mosquito Management (IMM) is a reframing of IPM; its goal is to manage mosquito populations to lower the risk of mosquito-borne disease transmission and to achieve a low level of mosquito annoyance by using the most efficient and cost-effective techniques with the least detrimental environmental impact (Frank et al., 1980; Floore, 2006).

During the last century, malarial vector control strategies were solely based on chemical agents like dichloro-diphenyl-Trichloroethane (DDT). For example, the use of long-lasting insecticide-treated nets (LLINs) and indoor residual spraying (IRS) of

DDT has prevented 75% of malarial deaths globally by controlling vector mosquitoes (*Anopheles*). Duly, WHO recommended that mosquito control be the sole intervention that can curtail malaria transmission from peak levels to close to zero (Shaw & Catteruccia, 2019). Malaria incidence in India was reduced by 99.8% from 75 million cases annually in the 1930s to 110,000 cases annually in the 1960s. In addition, extensive spraying programmes have generated behavioural resistance in some countries' vectors. This may be a direct reaction to the irritating pesticides (DDT or pyrethroids), which can have an effect on control attempts, or it may be a genetic feature that has evolved as a result of selection due to the presence of insecticides in homes.

A few Indian malarial vector species exhibit physiological resistance to DDT, posing major issues in vector control programs. In India, indoor insecticide spraying is used to combat malaria. After the eradication programme slackened in the 1970s due to the development of physiological resistance issues, there was a massive surge of malaria. This was made worse by the construction of irrigation systems, the relocation of susceptible human populations into risk zones, and the presence of outdoor resting populations of *Anopheles culicifacies*, the main malaria vector. The 1980s witnessed the reintroduction of DDT in India, a country where *Anopheles culicifacies* is DDT-resistant. Due to DDT's irritating impact, which lasts considerably longer than its poisonous effect, malaria transmission has been reduced (Pates & Curtis, 2005).

Smith and Webley (1969) demonstrated the irritating impact of DDT in verandah trap houses, where mosquitoes become irritated and leave the huts more quickly than those who weren't sprayed. (Smith & Webley, 1969). Sadasivaiah and co workers in 2007 suggested the combined use of IRS with DDT or other insecticides

as a pivotal part of the national malaria control programme. Synthetic pyrethroids are a safe substitute for DDT, especially in new housing areas and in places with low and seasonal transmission. The use of pesticide-impregnated traps and aerial insecticide sprays, improved vector control, and surveillance have achieved great progress against vectors such as tsetse flies, mosquitoes, sandflies, etc. However, the elaborate use of chemical control strategies has caused insecticide resistance to develop and spread in naturally occurring populations of *Anopheles*, *Aedes*, *Culex*, and *Phlebotomus*. For example, malaria vectors developed resistance to DDT or pyrethroids and demanded the use of shorter-duration insecticides like carbamates and organophosphates as control measures. Although the public health effects of this phenomenon are still unknown, it raises strong concerns for the future of these vector control methods. In the case of malaria, LLIN and IRS techniques exclusively target *Anopheles* species that feed and rest indoors. Aside from a restricted application of larvicidal substances, no tools are currently available to stop transmission by outdoor biting and feeding mosquito populations. The development of alternative strategies to effectively reduce the transmission of pathogens by insect vectors is therefore a high priority (Hoffman et al., 2002).

To combat *Culex* or *Aedes* mosquitoes, residual home spraying is no longer frequently utilised. This is partially due to the fact that many vector species have outdoor resting habits or rest indoors on materials that haven't been sprayed, including clothing, curtains, etc. The ability of *Aedes* mosquitoes to adapt to new breeding grounds creates a serious threat, especially in urban areas where high population densities of humans and mosquitoes expose a lot more individuals to the risk of disease. The strategic techniques of vector control notified by WHO are known as Integrated Vector Management (IVM), which mainly target the management of

dengue vectors. The aim of the process was to make the best use of available resources for vector control. As *Aedes aegypti* occupies a variety of natural and man-made ecosystems, vector control should be designed by considering these aspects. It may not be practical or cost-effective to control mosquito breeding in all such ecosystems. Here, vector propagation is managed by removing or recycling non-essential containers that serve as larval breeding grounds. These measures ought to be the mainstay of dengue vector control. Another method employed in this aspect is vector control by chemicals. Chemicals are frequently used to treat *Aedes aegypti* larval habitats; larviciding should be limited to containers that cannot be otherwise removed or controlled and should be seen as a complementary practice to environmental management, unless a crisis arises. It's difficult to point out and reach natural habitats like leaf axils and tree holes, which are *Aedes* mosquito's typical habitats, or in deep wells, larvicides may be impractical to use. Another key obstacle is tracing the indoor *Aedes aegypti* larval habitat in many urban areas. Moreover, chemical measures can be used only if environmentally friendly management techniques or other non-chemical procedures are impractical or too expensive to implement (McCall et al., 2009).

Lopes and co-workers in 2019 illustrated that the use of hexachlorobenzene (BHC), DDT, and dieldrin as residual action insecticides as part of a Brazilian governmental campaign to control Bancroftian flariasis during 1951-1955 was incipiently effective (Lopes et al., 2019). In the following years, it was found to be ineffective due to the development of resistance in mosquitoes, and subsequently, their use was suspended. *Culex* mosquitoes showed resistance to a wide array of chemical insecticides like chlorpyrifos, malathion, carbamate, propoxur, themephos, etc. (Lopes et al., 2019).

Since many years, interventions that target mosquito larval stages have been successfully utilised, but their efficacy varies greatly from species to species. In general, interventions are successful when habitats are large and sensitive to environmental change, but they are less effective when habitats are small, widely distributed, and transitory. Rogers (1967) recognised that the most beneficial mosquito control was to impede adults from emerging, particularly through water management and larviciding. Yet supplemental adulticide would often be required to protect population centres. Source reduction or habitat modification of larvae acts as an effective method for the eradication of mosquitoes. It is a practical and long-term method for controlling mosquitoes in production areas. Additionally, it lessens or completely replaces the requirement for other mosquito control strategies. For example, any low, wet, muddy region, salt marsh, or other breeding grounds for mosquitoes may be ditched or drained. However, the main drawback of this approach is that habitat modification may not always be an ideal option in economically important wetlands (Rogers, 1967). Carlson and co-workers discussed habitat management by impoundment, an additional strategy for source reduction. Impoundments are mosquito breeding grounds that have artificial or natural dikes built around them to confine water poured into the marsh. This inundation prevents floodwater species from ovipositing, which significantly lowers their populations (Carlson et al., 1991).

Ditching has been used for a long time as a source-reduction mosquito control method. Open marsh water management (OMWM) is a contemporary source reduction approach used in several salt-marsh coastal regions of the United States. This technique uses short ditches in the marsh to connect mosquito breeding grounds to habitat (tidal, creek, ditches) in deep water. Thus, mosquito broods can be managed

without the use of pesticides by allowing larvivorous fish to access these depressions. On the other hand, these areas might be drained before adult mosquitoes emerge (Carlson et al., 1991).

If standing water cannot be drained or removed, biological control agents, larvicide applications, or a combination of these methods may be employed to control the problem. Such potential sites include any area with standing water, such as marshes, swamps, streams, flowerpot saucers, roof gutters, wells, old tyres, ponds, etc. The following factors should be taken into account when using larvicides: To reduce effects on organisms other than the target species, the substance should be tailored specifically for mosquitoes, and the larvicide must be able to penetrate thick vegetation canopies. The formulation of the larvicide (liquid, granular, solid, etc.) must be suitable for the habitat being treated, applied precisely, and based on monitoring data. An efficient larviciding programme is a vital component of integrated mosquito control operations. Application precision is crucial because even missing a relatively small region might cause a big mosquito brood to emerge, necessitating repeated broad-scale adulticide (Floore, 2006). New approaches have to be implemented to replace the use of pesticides. IPM is one of them. IPM guidelines are based on environmental planning, public awareness campaigns, and biological control. It intends to effectively control the mosquito population by protecting the environment from contamination (Wilke & Marrelli, 2015).

Biological control has the ability to be host-specific with almost no non target consequences. Service in 1983 claimed that biological control agents may successfully lower mosquito populations; however, at the time, there was debate over their efficacy. Legner and Sjogren in 1984, provided examples of successful

management by various fish species. Large populations of the mosquito-eating fish species *Gambusia* can be raised before being released into areas where mosquitoes thrive, where they eat a variety of insects in addition to mosquito larvae. In areas where the water freezes or where there isn't year-round water, mosquito fish must be released every year. Other biological control species comprise *Toxorhynchites* sp. (mosquito larvae) that consume other container-breeding mosquito larvae. *Toxorhynchites* sp. inoculative releases, however, have been made without establishing control with the intention of colonisation and efficient predation (Schreiber & Jones, 1994). Mosquito larvae are also consumed by microcrustaceans (predaceous copepods, such as *Macrocyclops longisetus*). For example *Mesocyclops*' effectiveness was demonstrated to be increased when combined with biorational larvicides. The fungus *Lagenidium giganteum* has been developed as a biological mosquito control agent (Roberts & Panter, 1985).

Benelli mentioned in 2015 the usage of organophosphates, insect growth regulators, and microbial control agents against target mosquito larvae. Indoor residual spraying and insecticide-treated bed nets are commonly used to lessen the spread of diseases carried by mosquitoes in tropical regions. However, these compounds cause resistance in a variety of vector species and have detrimental consequences for human health and the environment. Based on this, environmentally friendly techniques have lately been used to improve the control of mosquito vectors. In addition to traditional biological control strategies.

Benelli and colleagues in 2016 highlighted the discovery of the plant-based drug artemisinin for the treatment of malaria and the subsequent granting of the Nobel Prize in 2015. The study also upholds the importance of screening plants and fungi as sources of metabolites for parasitological and mosquitocidal qualities. Strikingly,

plant-borne molecules are much more effective at a few parts per million (ppm) against *Aedes*, *Anopheles*, and *Culex* young larval instars. More than 80 plant species have currently been used to successfully synthesise nano-mosquitocide, with a focus on larvicidal applications. However, research on ovicidal and ovideterrent nanoformula is limited.

In aquatic habitats, natural enemies that feed on mosquito larvae and pupae can significantly contribute to the decline of Culicidae populations. Indeed, many aquatic creatures, such as fish, amphibians, copepods, young odonate water bugs, and even the larvae of other mosquito species, prey on mosquito young instars. The relevance of larvivorous fish in the biological control of mosquitoes has received the greatest attention. Fish predation was monitored in a variety of habitats, ranging from tiny plastic containers to intricate natural ecosystems like coastal wetlands. In a wide range of habitats and environments, larvivorous fish have been shown to be particularly successful at reducing mosquito larval populations. More than 60 countries have implemented mosquito control measures by introducing larvivorous fish, specifically those from the genera *Gambusia* and *Poecilia* (Poeciliidae). Even though introduced, larvivorous species often remain a risk to native aquatic fauna, comprising amphibians. This instigates the need to think precisely about ecological menaces through the introduction of predatory species as a part of vector control (Louca et al., 2014).

By mid-1970, after the discovery of Bacillus thuringiensis *subsp. israelensis* (Bti), many researchers have investigated the properties of mosquitocidal endotoxin proteins and their role as an alternative to chemical pesticides. *Bacillus thuringiensis* is a complex of multiple subspecies, all distinguished by the production of a crystalline parasporal body during sporulation. The parasporal body of the majority

of subspecies comprises cry (for crystal) proteins, which are referred to as Cry4Aa, Cry4Ba, and CryllAa, and non-specific CytlAa protoxins, which are extremely toxic to some insect species but safe for the majority of non-target organisms. Upon ingestion, the crystals are dissolved by insect midgut digestive enzymes. Proteases then cleave the protoxin to release the active toxin. In order to cause an osmotic imbalance and consequent lysis of the midgut cells, the toxin attaches to proteins on the midgut microvillar membrane, inserts into the membrane, and creates pores. This results in paralysis and eventual host death. The distinctive feature of these bacteria is that their potential is high when they act synergistically. These synergistic effects are thought to also lessen the possibility of resistance developing. Due to Bti's effectiveness and safety, isolates of several subspecies presently serve as the active ingredient in commercial insecticides used for insect control in the U.S. and many other countries. The primary bacteria in these products are *Bacillus thuringiensis subsp.Kurstaki* (Btk) is used to control lepidopterous pest larvae, and *Bacillus thuringiensis* (Bti) is used to treat mosquito and blackfly larvae. *Bacillus thuringiensis* subsp. morrisoni, strain tenebrionis, a different species that is toxic to pest beetles like the Colorado potato beetle, was created but is no longer commercialised. *Bacillus sphaericus* (Bs) has recently entered the market under the brand name VectoLexH, while *Bacillus thuringiensis* is offered under a variety of trade names, including VectoBacH and TeknarH. *Bacillus sphaericus* is exclusively effective against a narrower range of mosquitoes than Bti. Even against these, it performs poorly against a number of significant species, including *Aedes aegypti*. However, Bs has better residual action in polluted environments and is marginally more harmful to some *Culex* species than Bti. Specifically against *Culex quinquefasciatus,* the primary vector of lymphatic filariasis, which typically breeds in highly polluted water bodies

in urban areas. As a result, it is used in China, India, Brazil, Central America, and the USA to combat *Culex* species in contaminated environments (Federici et al., 2007).

Norbert Becker commented that the fundamental requisite for the successful use of bacterial control agents was the production of effective formulations befitting the ecosystem of target organisms. The unique characteristics of *Bacilli* include their adaptability, relative ease of mass manufacturing, formulation, and application, as well as their safety for the environment. The rapid development and use of these *Bacilli* in many mosquito and blackfly control programmes was due to the comparatively low costs for development and ease of application (Becker, 2000).

Over the past 400 million years, insects and fungi have coexisted and formed several types of interactions. Out of it, pathogenicity is a characteristic interaction evolved between fungal families and arthropods. In contrast to other insect pathogens like bacteria or viruses, entomopathogenic fungi can infect their hosts by piercing through the cuticle without the requirement to be ingested. As a result, they have a great chance of controlling sucking insects, which can be pests in agriculture (like aphids, leafhoppers, stink bugs, and thrips) or disease carriers (like mosquitoes, kissing bugs, and tse tse flies). Generally, entomopathogenic fungi exist in filamentous form or as yeast. A widely accepted mode of action of filamentous fungi is that their asexual spores adhere to the exoskeleton of mosquitoes, deteriorate chitin-containing cuticles with the help of secreted enzymes, and invade insect hemocoel. This has been validated by the studies of (Mannino et al., 2019). Another mode of action is the oral ingestion of fungal conidia. For example, *Culicinomyces* sp. mostly infect mosquito larvae through the midgut after ingestion, in contrast to most entomopathogenic organisms. Larval mosquitoes consumed massive conidia of fungi and oomycete floating in the water, and then their digestive tracts filled with conidia.

It was even observed that the larvae of *Culex pipiens pallens* quickly consumed *Pythium guiyangense* even in an environment with sufficient food. Ingestion of conidia leads to disruption of the internal structure of larvae, which ultimately leads to larval death (Shen et al., 2020).

The available literature survey shows that mosquito microbial pathogens can be categorised into soil-dwelling fungi and aquatic entomopathogenic oomycete. The genus *Lagenidium* comprises several saprotrophic species that are pathogenic to algae, phytoplankton, pollen, barnacles, blue crabs, mosquito larvae, shrimp, and mammals. Out of these species, *Leptolegnia chapmanii*, *Pythium guiyangense*, and *Lagenidium giganteum* belonging to the kingdom Chromista are considered effective mosquito larvicides because these water moulds can grow and produce zoospores repeatedly in the aquatic environment, lengthening the infection periods. All three species are extremely tolerant of environmental conditions and exhibit significant virulence towards mosquito larvae belonging to the genera *Anopheles, Culex,* and *Aedes*. *Lagenidium giganteum* mosquito larvicidal activity was confirmed by Couch and Romney in 1973 by infecting mosquito larvae. Later, *Lagenidium giganteum* was registered under the trade name Laginex by the US Environmental Protection Agency in 1995 for commercialization as a biological control agent. Soon, Laginex was deregistered and is no longer available for purchase or usage today because of the infections caused by *Lagenidium giganteum* in healthy canines and its near phylogenetic affinity to isolates from mammals that can infect humans and animals and cause life-threatening infections. *Leptolegnia chapmanii* and *Pythium guiyangense* are two promising control agents that still need to be further studied (Shen et al., 2020; Vilela et al., 2015).

The soil-borne entomopathogenic fungi focused mainly on the members of the genera *Coelomomyces*, *Trichoderma*, *Tolypocladium*, *Fusarium*, *Culicinomyces*, *Beauveria*, *Metarhizium*, *Aspergillus*, and *Conidiobolus*. Throughout history, the members of the genus *Coelomomyces* are known for their life history's diploid phase as pathogens of aquatic culicine and anopheline mosquito larvae, yet their haploid life history affects aquatic microcrustacean-copepod or ostracod. Due to the intrinsic life cycle of fungi, alternating diploid and haploid generations between mosquito and microcrustacean hosts, respectively, make it difficult to maintain and manipulate them in the laboratory (Rueda-Páramo et al., 2017). Previous studies by Sharma and Marques (2018) commented on the role of *Fusarium oxysporum* as an entmopathogen in *Galleria mellonella* larvae. Larval death occurs by active infection of the fungus through its penetration, proliferation, and interaction through the host hemocoel rather than by mere physical adherence caused by the fungal conidia (Sharma & Marques, 2018). *Tolypocladium cylindrosporum* was isolated for the first time from larvae of *Aedes aegypti* in a stagnant breeding site situated in a secondary tropical forest in South America. In vivo studies were conducted to test the activity using fungal conidia against the third instar and on adults through the feeding technique. Findings emphasised antagonistic potential against larvae and adults of *Aedes aegypti* (Montalva et al., 2019).

From the available literature survey on the species *Trichoderma asperellum*, Podder and Ghosh justified the application of fungus as an eco-friendly larvicide by their in vitro experimentation on anopheline larvae (malarial vector). Their experimentation was designed exclusively on crude methanolic extract (ME) and on different methanolic fractions (MFs) of the fungus against anopheline larvae. It was scientifically proven that after the interaction with fungal extracts in ME, there is a

reduction in the phenol oxidase content inside the cuticle and hemolymph of the anopheline larvae. This reduction causes the degeneration of hemocytes, thereby reducing insect immunity. Out of this, Methanolic Fraction 8 (MF8) showed the strongest larvicidal activity. The GC-MS analysis of the particular fraction indicates the presence of 49 compounds like 2, 3-di hydro thiopene, p-cymene, alpha-pinene, hexadecanoic acid, 8-methyl quinoline, (Z, Z)-9, 12-octa decadienoic acid, etc., which were previously proven to be efficient insecticides by a literature survey (Podder & Ghosh, 2019).

A previous investigation on the activity of *Aspergillus clavatus* on laboratory-reared *Culex quinquefasciatus* (S-lab strain) larvae was investigated. In the experimental setup, multiple concentration assays on spore suspension were prepared by using heat-deactivated and non-heated spores. The larval mortality was measured after 48 hours of exposure. In comparison to non-heated spores, heat-deactivated spores generated a reduced mortality rate, indicating insecticidal properties. Larvae with and without spore treatment were prepared for scanning and transmission electron microscopy to analyse histological damage to *Culex quinquefasciatus* larvae upon exposure to *Aspergillus clavatus* spores. It was revealed that the spores enter the digestive system through the mouth, and upon fungal exposure within 8 to 24 hours, internal tissues of the larvae, such as the midgut wall, the skeletal muscles, and the cuticle-secreting epidermis, were gradually destroyed. These findings imply that toxins released by *Aspergillus clavatus* active germinating spores in the digestive system changed the larval tissues, resulting in necrosis and larval death (Bawin et al., 2016).

When it comes to adult mosquito control, the situation is quite different. In fact, many efficient biological larvicides are available, but there haven't been any

validated biological control measures for adult mosquitoes. Addressing this gap Ernst-Jan Scholte and coworkers reported positive findings showing that an entomopathogenic fungus from the *Hyphomycetes* (imperfect fungus) may infect and kill adult *Anopheles gambiae* sensu stricto mosquitoes in laboratory containers through tarsal contact. These fungi can infect and kill insects without being consumed, when compared to other mosquitocidal bio control agents like bacteria, microsporidia, and viruses. Additionally, commercially generated *hyphomycetous* insect-pathogenic fungi are employed all over the world to combat a variety of agricultural insect pests, such as *Metarhizium anisopliae* and *Beauveria bassiana* (Scholte et al., 2005).

The management of mosquitoes is still challenging due to their high genetic flexibility and great reproductive potential. This necessitated the development of better control measures. The concept of paratransgenesis is a promising alternative method in this regard. The concept was based on the use of symbiotic bacteria to express effector molecules inside the target vector. Understanding mosquito microbiota is crucial for paratransgenic systems to work, and it's crucial to recognise the spectrum of well-established bacteria in mosquitoes that can be passed on to the next generation. The effectiveness of a paratransgenic system relies mainly on the scrutiny of commensal bacteria that are not pathogenic to humans or animals among the varied organisms that insects harbour, notably in their digestive systems. These bacteria are mostly found in the midgut of mosquitoes and play various biological roles in digestion. A close relationship exists between blood-dependent insects and symbiotic microorganisms that support the anabolic processes of vitellogenesis and ovogenesis. When these bacteria are eliminated, the fecundity rate and growth rate decrease. In addition to bacteria, paratransgenic approaches also operate with fungal species because they have the benefit of living in the environment for months as spores

and can infect mosquitoes directly through the cuticle, as opposed to bacteria, which need to be ingested to infect mosquitoes. As per the literature survey, mosquito larval management is an ideal strategy for vector management. By complying with the microbes, the strategy becomes more productive. A better understanding of the gut microbiota is inevitable (Wilke & Marrelli, 2015).

It has been known for a long time that bacteria inhabit mosquito guts. The presence of microbiota is evident from the mosquito larval stage onward. Both larval and adult stages harbour a group of living microbes in their digestive tract that form a gut microbiota. It includes unicellular organisms like bacteria and viruses and unicellular eukaryotes like fungi, algae, and protozoa. The microbiome in the mosquito gut is intricately linked and affects a wide range of host properties, including immunity, fecundity, longevity, and fitness. A thorough knowledge of microbial community dynamics is necessary for a better understanding of the symbiotic relationship between the host and its gut microbial residents. Three lines of evidence reflect the acquisition of a gut microbiota by mosquito larvae. As per the first line of experimental research, mosquito larvae hatch in axenic form. The second line suggests that most of the microbes identified within the larvae overlap with the microbes present in their aquatic breeding sites. Another justification is that adult mosquitoes lodge several types of bacteria in their reproductive tract; some are on the surface of eggs where females lay. Larvae obtain these bacteria by ingesting egg shell fragments while hatching. The third line indicates that a large number of microbes reside in the larval gut, which would not be expected if these microbial communities were obtained directly from parents or congeners. The majority of gut microbes were identified by culture-dependent and independent methods. The overall findings of the culture-independent approach suggest that adult mosquitoes have a low diversity of bacterial

communities that vary between individuals and species. Functional research suggests that the gut microbiome can decrease vulnerability to infection by human pathogens by altering the immune response of mosquitoes or producing antiparasite molecules. Considerable variation in bacterial taxa found in field-collected adults strongly suggests the influence of environment in shaping the gut microbiome. However, it is still unknown whether adult mosquitoes primarily acquire bacteria through transmission from larvae or through their own feeding behaviour (Coon et al., 2014; Strand, 2018).

Initial research on mosquito gut content analysis relies on 4'6-diamidino-2-phenylindole (DAPI) staining. DNA-bound DAPI fluoresces blue at a wavelength of 365 nm and can be observed with high magnification. Even the quantitative estimation of very small bacteria, below the limit of resolution of a light microscope <1.0µm, can be analysed. Walker and colleagues revealed qualitative and quantitative differences in bacteria in the larval gut among the fourth instars of *Aedes triseriatus* (Say), *Anopheles quadrimaculatus* (Say), and *Coquillettidia perturbans* (Walker). The technique can be used to study the gut composition of various filter-feeding arthropods (Walker et al., 1988).

From the literature survey, it was found that metagenomics sequencing studies are apt to derive information about mosquito gut microbiota. The majority of bacteria found in mosquito larvae and adults are Gram-negative aerobes or facultative anaerobes, and they primarily belong to four phyla: Bacteroidetes, Firmicutes (Clostridia, Actinomycetes, Spirochetes, and other species) Proteobacteria(Gammaproteobacteria, Alphaproteobacteria, and Betaproteobacteria), and Actinobacteria. Generally, the presence of similar gut microbiota in insects

surviving in different environmental conditions suggests that their prevalence may be partly due to their abundance in habitats where insects lived (Dickson et al., 2017).

Wang and co-workers in 2011, using high-throughput pyrosequencing of the bacterial 16S rRNA gene, systematically profiled the gut bacterial assemblage of different life stages in *Anopheles gambiae*, Kenya. Their data revealed that the phyla Cyanobacteria, Proteobacteria, Bacteroidetes, Actinobacteria, and Firmicutes together constituted 90.7-99.9% of the bacterial communities across all life stages. Meanwhile, in the larval and pupal stages, photosynthetic Cyanobacteria were notably more numerous, representing approximately 40% of the communities. During the transition from pupa to adulthood, there is a radical remodelling in the gut community structure (Wang et al., 2011).

For instance, Aeromonadaceae, Comamonadaceae, Erythrobacteraceae, and Rhodobacteraceae represented 74.4% of the community in pupae and were reduced to undetectable levels in newly emerged adults (24 hours post-eclosion, no sugar feeding). In adult mosquitoes, Enterobacteriaceae and Propionibacteriaceae were prominent. Remarkably, Enterobacteriaceae accounted for 69.4% of the community, dominated by *Thorsellia anophelis*, a species originally isolated from the gut of *Anopheles arabiensis* (Wang et al., 2011).

Coon and co-workers studied the role of gut bacteria in egg production of *Aedes aegypti* (anautogenous) and *Aedes atropalpus* (facultatively autogenous) larvae by 16S rRNA sequencing. In their study, they produced gnotobiotic forms from axenic larvae of *Aedes aegypti* and *Aedes atropalpus* that were singly colonised by several members of the larval gut microbiota. They correlate female fitness parameters such as egg production in the first ovarian cycle by comparing them with the respective conventionally reared mosquito larvae. The assay focused on significant bacterial

phyla previously identified from the guts of laboratory-reared mosquitoes. *Microbacterium* (Actinobacteria : Microbacteriaceae), *Paenibacillus* (Bacteroidetes : Paenibacillaceae), *Comamonas* (Proteobacteria: Comamonadaceae) and *Aquitalea* (*Proteobacteria*: Neisseriaceae). Two of these genera, *Aquitalea* and *Comamonas*, belong to the Betaproteobacteria and were formerly identified as dominant members of the *Aedes atropalpus* larval gut community by pyrosequencing. All other genera were previously isolated from *Aedes aegypti*. Members of the Bacteroidetes (Flavobacteriaceae, Sphingobacteriaceae) and Actinobacteria (Microbacteriaceae) are generally found in autogenous species both in laboratory-reared and field-caught forms. As specified by the results, 87% of *Aedes aegypti* and 82% of *Aedes atropalus* larvae reared conventionally developed into adults within 8 days. As previously stated in the literature, the development of axenic *Aedes aegypti* or *Aedes atropalpus* larvae was halted after the first instar (negative control). Gnotobiotic forms from axenic *Aedes aegypti* and *Aedes atropalpus* inoculated with *Microbacterium* or *Leucobacter* died within 4-5 days post-inoculation as first instars. No significant change was observed in other isolates tested (survival to adulthood and development times) for conventionally reared *Aedes aegypti* larvae. Only *Aquitalea* and *Comamonas*, in contrast, supported *Aedes atropalpus* larvae that had similar survival and development rates to those of *Aedes atropalpus* that had been raised conventionally. *Paenibacillus*, *Chryseobacterium*, or *Sphingobacterium* administered *Aedes atropalpus* larvae survived between the negative and positive controls; however, development durations for survivors were greater than for *Aedes atropalpus* that were raised normally. Perhaps this was due to the higher proportion of individuals dying as larvae (Coon et al., 2016).

To estimate the body size of female adult *Aedes aegypti* and *Aedes atropalpus*, the measurement of forewing length was considered. There was no size difference between the conventionally reared adult mosquito and adult female *Aedes aegypti* that emerged from larvae colonised by *Paenibacillus*, *Chryseobacterium*, *Sphingobacterium*, *Aquitalea*, or *Comamonas*. Only gnotobiotic *Aedes atropalpus* larvae inoculated with *Aquitalea* or *Comamonas*, on the other hand, evolved into adults that did not significantly differ in size from *Aedes atropalpus* adults raised conventionally. Adult *Aedes atropalpus* larvae produced from *Paenibacillus*, *Chryseobacterium*, or *Sphingobacterium*-infected larvae were substantially smaller (Coon et al., 2016).

In a previous study, *Chromobacterium* sp. (Gram-negative) was isolated from the midgut of field-collected *Aedes aegypti* mosquitoes from Panama. To assess the effect of *Chromobacterium* sp. on susceptibility to malaria parasites and dengue viruses. *Chromobacterium* sp. was introduced along with artificial nectar meal; it inhibits the growth of other resident midgut microbiota. Colonisation in the midgut triggers mosquito immune responses, and *Chromobacterium* sp. exposure drastically decreases the survival of both larval and adult mosquitoes. To investigate the susceptibility of *Chromobacterium* sp., *Anopheles gambiae* was infected with *Plasmodium falciparum* and *Aedes aegypti* with dengue virus DENV2 two days after ingestion of *Chromobacterium* sp. through feed. Seven days after post-infestation, parasitic infection in *Anopheles gambiae* was assayed by counting oocyst-stage parasites on the basal side of the mosquito midgut. DENV2 infection of the midgut of *Aedes aegypti* was assayed through standard plaque assays. In these experiments, the morality rate was higher in adult female mosquitoes exposed to *Chromobacterium* sp. It was found that *Chromobacterium* sp. has anti-plasmodium and anti-dengue activity

independent of the mosquito. It was also found that surviving mosquitoes displayed significantly increased resistance to *Plasmodium falciparum* infection and dengue infection (Ramirez et al., 2014).

The prevalence of gut bacterial communities was assessed by recent studies on the comparison between the diversity of midgut bacteria in larvae and in female adults of *Aedes aegypti* and *Aedes albopictus* by 16S rRNA sequencing. The study projected the presence of bacteria belonging to six families like Erwiniaceae, Flavobacteriaceae Microbacteriaceae, Neisseriaceae, Staphylococcaceae and Micrococcaceae from field-caught *Aedes albopictus* mosquitoes. Whereas bacteria belonging to five families (Enterobacteriaceae, Staphylococcaceae, Bacillaceae, Erwiniaceae, and Moraxellaceae) were detected in field-collected *Aedes aegypti* adults. The most common bacterial species isolated from adult *Aedes aegypti* and *Aedes albopictus* were *Bacillus endophyticus* and *Pantoea dispersa*, respectively. Species belonging to the Moraxellaceae family and Microbacteriaceae were identified in the larvae of *Aedes aegypti* and *Aedes albopictus*, respectively. While considering bacterial load in mosquito larvae, *Bacillus fexus* predominated in *Aedes aegypti*, while *Bacillus megaterium* dominated in *Aedes albopictus* (Ranasinghe et al., 2021).

To explore bacterial diversity in the midgut of *Aedes aegypti* mosquitoes, screening and evaluation of the gut microbes were done culture-dependently in field-collected and laboratory-reared mosquitoes. The results showed that the microbial flora in the midgut of *Aedes aegypti* larvae and adults is diverse and dominated by Gram negative Proteobacteria. About 13 isolates were obtained from fields collected and laboratory-reared mosquito larvae, belonging to three major groups: Gamma Proteobacteria, Beta-Proteobacteria, and Firmicutes. About 64% of identified bacteria belong to the group Gamma Proteobacteria, with the dominant genera being

Aeromonas, *Enterobacter*, and *Pseudomonas*. *Serratia odorifera* was present both in adult females (laboratory-reared and field-collected) and laboratory-reared larvae. The potential activity of *Serratia odorifera* on DENV-2 susceptibility was checked by co-feeding *Serratia odorifera* with DENV-2 to axenic adult females. The observations showed that the viral susceptibility of these female mosquitoes enhanced significantly when compared to solely dengue-2-fed and another gut-inhabitant, *Microbacterium oxydans*-co-fed females. Based on the results, they proposed that the enhancement in the DENV-2 susceptibility of *Aedes aegypti* females was due to the blocking of the prohibitin molecule present on the midgut of female mosquitoes by the polypeptide of *Serratia odorifera* (Apte-Deshpande et al., 2012).

Dagne Duguma and colleagues analysed microflora associated with the developmental stages (early and late larval instars, pupae, and adults) of *Culex tarsalis* mosquitoes by sequencing the bacterial V3 region from 16S rRNA genes to check the hypothesis that bacteria spotted in the larval stage of *Culex tarsalis* are transstadially disseminated to the adult stage and to compare the microbiomes of laboratory-reared mosquitoes in contrast to field-collected mosquitoes by beta diversity analysis. Proteobacteria, Cyanobacteria, Bacteroidetes, and Firmicutes were the most prevalent phyla present in all phases of the life cycle. While considering abundant genera shared across developmental stages, *Thorsellia* accounts for 40%, followed by *Cyanobacteria* (18%), and *Dysgonomonas* (13%). *Thorsellia anophelis* was recovered as the dominant bacterial species in all developmental stages of the field-collected *Culex tarsalis*. However, late-instar larvae from laboratory-reared *Culex tarsalis* were dominated by Proteobacteria (87%), followed by Actinobacteria (8%) and Bacteroidetes (4%). The most predominant family in late-instar laboratory-reared mosquitoes was Enterobacteriaceae (77.8%). The dominant bacterial communities

include *Rahnella* (Gammaproteobacteria: Enterobacteriaceae) (64%), unclassified Enterobacteriaceae (12%), and unclassified Microbacteriaceae (Actinobacteteria) (8%). *Thorsellia* was rare in laboratory-reared mosquitoes. A significant difference in bacterial communities was observed between laboratory-reared and field-collected mosquitoes, which may be due to differences in their habitat and feeding behaviour (Duguma et al., 2015).

Culex quinquefasciatus mainly inhabits polluted breeding sites like cess pits and drains. The sullage water is likely to possess abundant microorganisms. Due to the filter-feeding nature of the larvae, these microbes will get into the larval gut. In this study, quantification of larval gut microflora was taken to study gut microbial influence in growth and development. Larval sampling was done from cess pits located in 10 different regions of Pondicherry, viz., Kadirgamam, Kottakuppam, Mudaliarpet, Muthialpet, Vanarpet, and Jeevananthapuram. A total of 54 distinct types of bacteria, 47 different forms of fungus, and 10 different types of actinomycetes are found in the gut flora. Out of these common bacterial genera, *Escherichia coli* and *Proteus* were found to occur in higher numbers, followed by *Bacillus*, *Staphylococcus*, *Shigella serratia*, and *Enterobacter*. A higher abundance of *Aspergilli* fungi were found in the larval guts compared to other genera like *Fusarium*, *Heterosporium*, *Pullularia*, and *Alternaria*. Among the actinomycetes, *Streptomyces* were more abundant, followed by *Nocardia*, *Micromonospora*, and *Nocardiopsis.* To evaluate the impact of gut microflora on the growth and development of *Culex quinquefasciatus*. The larvae were raised from the first instar to the adult stage in the presence of specific gut microorganisms and feed. The larvae were monitored daily for calculating the number of larval survivors (immature stage, their respective stages of development, and the number of adults that emerged) and larval mortality.

Bacillus, *Pseudomonas*, *Shigella*, and *Staphylococcus* exhibit 100% mortality during the early stages of larval development. The actinomycete *Nocardiopsis* species also displays 100% mortality. In contrast, the fungal isolates cause 100% mortality (Vasanthi & Hoti, 1992).

Next-generation 454 pyrosequencing is used to figure out the whole-body microbiota of *Culex quinquefasciatus* in different larval stages and following exposure to common environmental contaminants (pharmaceuticals and personal care products) found in wastewater. PPCP treatments included environmentally relevant concentrations: 1) a fusion of common antibiotics (Oxytetracycline, Lincomycin, and Ciprofloxacin); 2) a mixture of mammalian hormones (17α-Ethynylestradiol, 17β-Estradiol, 19-Norethindrone, and Estrone); 3) a blend of the antibiotic and hormone treatments plus acetaminophen and caffeine; and 4) an untreated control. Within the control group, despite similar rearing conditions and feeding patterns, the dominant bacterial family changes when it moults to the next instar. There is a significant difference in bacterial families between the second and third instars, i.e., Cytophagaceae is mainly dominant in the second instar of larvae, where the presence of bacterial families like Microbacteriaceae, Enterobacteriaceae, Sinobacteraceae, and Acetobacteraceae Comamonadaceae showed a greater predominance in the third instar than the second instar. There is a resurgence of bacterial families from the third and fourth instars; for example, Microbacteriaceae and Enterobacteriaceae are also present in the fourth instar larvae. Interestingly, the Rickettsiaceae family is predominantly present in all instars. Operational taxonomic units (OTUs) assigned to the family Rickettsiaceae were present in most treatments and instars, despite the fact that they fluctuated in hormone-treated and antibiotic-treated samples when compared to control. Notably, Rickettsiaceae dominated antibiotic and mixture treatment groups

across all instars. Sphingobacteriaceae was the second most prevalent bacterial family found in all instars in antibiotic and mixture treatment groups. Between instars, hormone treatments altered the bacterial populations, but not as significantly as the control group. Oxalobacteraceae predominated in second instars exposed to hormones; in the third instar, Microbacteraceae and Rickettsiaceae dominated with a small proportion of Rickettsiaceae; Microbacteraceae was the most common family of fourth instars exposed to hormones (Pennington et al., 2016).

Chandel and co-workers investigated the microbial diversity of aerobic bacteria from adult female *Culex quinquefasciatus* during the post monsoon season by using a 16S rRNA gene sequence-based approach. Indoor resting mosquitoes were collected from the study site at dawn and dusk and opted for analysis. The study site includes 10 locations with different climatic zones. Coastal regions with Bhuj and Jamnagar, arid zones comprising Jodhpur and Barmer of the Thar Desert, Amritsar and Bathinda as semiarid climatic zones, Nagrota and Leh (mountainous climatic zones), Hathigarh and Masimpur representing the tropical climate of Assam, were studied. The study projected the presence of 83 bacterial species belonging to 31 bacterial genera, which in turn belong to three phyla, namely Proteobacteria, Firmicutes, and Actinobacteria. Phylum Proteobacteria was the predominant phylum with 37 species, followed by Firmicutes (33 species) and Actinobacteria (13 species). In the phylum Proteobacteria, the c-proteobacteria class remains the dominant member. The largest genus, represented by 11 species, was *Staphylococcus*, whereas *Enterobacter* was the most common genus, found in all field sites except Leh. The highest bacterial ubiquity was observed in Bhuj (22 species), followed by Nagrota (18 species), Masimpur (18 species), and Hathigarh (16 species). Whereas, the fewest species were reported from Leh (8 species). It has been ascertained that each mosquito

harbours intensely diverse gut bacteria with a very small overlap of bacterial taxa within their gut. The presence of midgut microbial alteration may be one of the aspects responsible for vector competence within the mosquito population (Chandel et al., 2013).

Chavshin and co-workers., screened the midgut microbiota of larvae and adult *Anopheles stephensi* collected from two districts, namely Bandar-Abbas (urban) and Bashagard (rural), major malaria foci in southern Iran by applying cell-free culture technique and 16S rRNA gene sequencing. A total of forty species in 12 genera, comprising five Gram-positive *Exiguobacterium*, *Enterococcus, Kocuria*, *Microbacterium*, and *Rhodococcus* bacteria, and seven Gram-negative *Myroides*, *Chryseobacterium*, *Aeromonas*, *Pseudomonas*, *Klebsiella*, *Enterobacter*, and *Shewanella*, were disclosed from the larval midgut. While the inspection of the adult midgut microbiota exposed the presence of 25 Gram-negative species from five genera, including *Pseudomonas*, *Alcaligenes*, *Bordetella*, *Myroides*, and *Aeromonas*, The most prevalent midgut symbionts were *Pseudomonas* and *Exiguobacterium* (51% and 14%, respectively) at the larval stage, and *Pseudomonas* and *Aeromonas* (54% and 20%, respectively) at the adult stage. *Pseudomonas*, *Aeromonas*, and *Myroides* have been isolated from both larval and adult stages, implying probable trans-stadial transfer from the larval to the adult stage (Chavshin et al., 2012).

Stable association of alpha *Proteobacterium* (*Asaia* sp.) is seen in larvae and adults of *Anopheles stephensi*, prime vector of *Plasmodium vivax*, a main malaria agent in Asia. This was evaluated by the relative abundance of *Asaia* sp. in different organs of *Anopheles stephensi* by 16S rRNA gene abundance, quantitative real-time PCR, transmission electron microscopy, and in situ hybridization of 16S rRNA genes. Following the results, it was found that in adult mosquitoes, *Asaia* sp. showed

abundance in the female gut and in the male reproductive tract, which have been cultured in cell-free media and later transformed with foreign DNA. The presence of green fluorescent protein-tagged *Asaia* sp. in the female gut and salivary glands was pivotal for *Plasmodium* sp. development and transmission. While colonisation of the larval gut and the male reproductive system by the transformed *Asaia* sp. suggests bacterial species as candidates for malaria prevention since it is an effective inducible coloniser of mosquitoes that carry *Plasmodium* sp. (Favia et al., 2007).

Mosquito larvae ingest microorganisms from their aquatic breeding site. In order to assess transstadial transmissibility, the present study examines the midgut microbiota of *Anopheles stephensi* during its developmental stages and adults from wild and laboratory-reared populations and compares it with the bacterial richness of its breeding site. Wild larval populations were sampled from the puddle (FS-1) and curing waters on cement slabs (FS-2) in a construction area in Ponda city, Goa, India. In total, 298 isolates of bacteria from 21 genera in the four major phyla, Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria, were identified. Proteobacteria and Firmicutes made up 49% of the bacterial isolates in the FS-1, while Actinobacteria made up 51%. Bacteroidetes and Firmicutes made up 99% of the isolates in FS-2. Firmicutes made up 77% of the laboratory populations, whereas protozoa made up 23% of the isolates. In addition, 9 genera were found in the breeding sites: 13 in the midguts of larvae, 6 in those of pupae, 9 in those of male midguts, and 10 in those of female midguts (Pereira et al., 2021).

Studies on the fungal consortium of mosquito larval guts provide new insight into the range of mosquito-fungal interactions. As ubiquitous forms, fungi are found in all mosquito habitats. Furthermore, water moulds consider the aquatic environment of mosquito larvae as a habitat. This contiguity leads to a myriad of interactions

between mosquitoes and watermolds. From the literature reviewed, about 158 fungi and 43 species water moulds were isolated from 149 mosquito species. About one-third of these species have been isolated from adult mosquitoes, exposing the considerable reduction in fungal sampling in adult mosquitoes compared to that in immature life stages. Two-thirds of the isolated fungi belong to the orders Blastocladiales, Eurotiales, Hypocreales, and Saccharomycetales. Most of the isolates either act as opportunistic, facultative, or obligate pathogens. A small proportion of the isolated water moulds are noted to be pathogenic (Tawidian et al., 2019).

Filamentous fungi and yeast are the prevailing fungal isolates present in mosquitoes' midgut and other tissues. Filamentous fungus incorporates some pathogenic forms of *Aspergillus* and *Penicillium* species and some genera of entomopathogenic fungi like *Beauveria* and *Metarhizium*. Several genera of yeast, like *Candida*, *Pichia*, and *Wickerhamomyces*, have been documented in *Aedes* and *Anopheles* mosquitoes by culture-dependent and culture-independent methods. Previous research on mosquito mycodiversity was based on these types of culture-dependent methods. For instance, *Wickerhamomyces anomalus* has been identified in the midgut and reproductive organ of *Anopheles stephensi*, a primary vector of malaria. In recent times, the advent of the high throughout sequencing (HTS) technique has widened our knowledge about mosquito mycobiome. This approach was used to analyse the mycobial composition of *Aedes triseriatus* and *Aedes japanicum*. The result revealed the presence of 21 distinct fungal OTUs, out of which 15 were common between these two species. The Ascomycota phylum remained the major fungal taxa in these two *Aedes* species. Though the presence of mycobiome is evident in mosquitoes, little is known about the tripartite interaction between vector, pathogen, and fungi (Jayakrishnan et al., 2018).

Ascomycota make up the majority of the mosquito-associated mycobiota (about 70-90%), followed by Basidiomycota (30-10%) in *Aedes* and *Culex* species. Several species of filamentous Ascomycota, such as *Aspergillus gracilis, Aspergillus puulaauensis*, *Phaeophleospora hymenocallidicola, Cladosporium* sp., and *Penicillium* sp. As well as different varieties of yeasts, *Aureobasidium pullulans*, *Candida parapsilosis,* and *Candida* sp. *Pichia burtonii* were also detected in more than 70% of the mosquito population. In terms of Basidiomycota, species associated with mosquitoes are primarily related to Agaricomycetes, Ustilaginomycetes, and Pucciniomycetes. Furthermore, a significant portion of the mosquito mycobiota is made up of yeasts or yeast-like fungi, which range from 19 to 47% on average and can even reach 84% in some *Aedes albopictus* populations (Badran & Aly, 1995; Muturi et al., 2016).

Normally, adult mosquitoes procure their gut mycobiota from water during their emergence or through blood meals or nectar while feeding, whereas mosquito larvae primarily get fungal entities from breeding water. A previous study pointed out that *Saccharomyces cerevisiae* and *Pseudozyma* sp. make up a microbial diet (that contains proteins, carbohydrates, amino acids, and B vitamins) that stimulates the building of energy reserves and the development of non-axenic *Aedes aegypti* larvae. Along with its nutritional role, yeast also creates gut hypoxia in mosquitoes. For e.g., *Saccharomyces cerevisiae* (yeast) has been demonstrated to cause gut hypoxia in *Aedes aegypti*, acting as a signal for growth and moulting (Bozic et al., 2017; Malassigne et al., 2020).

The presence of *Wickerhamomyces anomalus* in male and female reproduction organs in *Anopheles stephensi* indicates their potential role in mosquito reproduction and their likelihood of vertical transmission. Apart from this, some strains of

Wickerhamomyces anomalus residing in the midgut and gonads of *Anopheles* mosquitoes can produce lethal toxins with broad-spectrum anti-microbial activity and are frequently referred to as "killer yeasts." This killer mechanism is partially dependent on the exo-β-1,3-glucanase enzymatic activity of the toxins, which may shield *Anopheles* mosquitoes from infection by entomopathogenic fungi. These *Wickerhamomyces anomalus* effectively limit the development of *Plasmodium berghei* from gametocytes to ookinetes in females due to their presence in the midgut of mosquitoes (Ricci et al., 2011).

Previous records highlighted the existence of culturable yeasts in the wild larvae of *Culex pipiens* and *Culex theileri*. The strains of yeast were classified using restriction fragment length polymorphism (RFLP) analyses and identified by sequencing the D1/D2 region of the 26S rRNA gene. Representative strains of *Candida*, *Cryptococcus*, *Galactomyces*, *Hannaella*, *Meyerozyma*, *Pichia*, *Rhodosporidium*, *Rhodotorula*, *Trichosporon* and *Wickerhamomyces* were isolated. Representative strains of *Candida*, such as *Candida albicans*, *Candida glabrata*, *Candida pseudolambica*, *Metschnikowia bicuspidata*, *Saccharomyces cerevisiae*, and *Wickerhamomyces anomalus* were tested as sole feed during a 21-day feeding experiment to record the overall growth of *Culex pipiens* larvae. This study firmly establishes the nutritional value of yeast ingestion by mosquito larvae (Steyn et al., 2016).

Trichomycetes are a group of microfungi of the order Harpellales, an obligatory endosymbiont found in gut insect larvae. A well-known member of the Harpellales family is *Zancudomyces culisetae*, which resides in *Aedes aegypti* and in various dipteran hosts. Aquatic asexual fungal spores are ingested by larvae, which then extrude sporangiospores (initial fungal growth) that, in response to physiological

cues, attach to the lining of the digestive tract and grow in the hindgut by extracting nutrients from the lumen of the host's digestive lumen and excreting trichospores into the external environment. Although the nature of the fungal-host relationship is considered commensalistic, it can change to mutualism in response to environmental changes. Generally, host larvae develop through various developmental stages (instars), and during these transitions, shedding of fungi occurs with periodic molting. After each moulting period, subsequent larval instars are then recolonized. These morphological and physiological stressors caused by fungal colonisation may have an impact on larval bacterial communities, through environmental filtering or competitive exclusion of microbes unable to adapt to the modified conditions (Frankel-Bricker et al., 2020).

There is a deficit of studies on mosquito mycobiota when compared with those on mosquito bacterial microbiota. Jovana Bozic and colleagues worked on the identification of yeasts of clinical significance in laboratory-reared mosquitoes such as *Anopheles gambiae*, *Anopheles stephensi*, *Culex quinquefasciatus*, *Aedes albopictus*, and *Aedes aegypti*. Based on the sequenced region of the 18S rRNA gene, four fungal species were identified: *Candida parapsilosis*, *Meyerozyma guilliermondii*, the most repeatedly isolated species, *Rhodotorula glutinis*, and *Sporobolomyces cf. roseus* were detected rarely. *Meyerozyma guilliermondii* was isolated in all the mosquito species analysed. On the contrary, *Candida parapsilosis* was the most prevalent isolate from the female guts of *Anopheles gambiae*, but it was not detected in any of the other mosquitoes. Its presence in developmental stages and organs of different mosquito species was studied by PCR-specific assays on laboratory-reared species of *Anopheles gambiae*, *Anopheles stephensi*, *Culex quinquefasciatus*, *Aedes aegypti*, and *Aedes albopictus*, along with field-collected

individuals. Results justified that there was a stable association between *Candida parapsilosis* and reared mosquitoes during the entire life cycle and in adult male and female gut and gonads. A wide existence of *Candida parapsilosis* was also authenticated in varied populations of wild mosquitoes (Bozic et al., 2017).

The quality of breeding water plays a significant role in determining whether female mosquitoes will lay their eggs and if the mosquito larvae will successfully develop into adult mosquitoes. Physicochemical parameters such as temperature, salinity, conductivity, total dissolved solids (TDS), and pH have a significant role in mosquito species' distribution and larval abundance. Climatic parameters like temperature and moisture index significantly impact the distribution and abundance. Discernment about breeding habitats and vector surveillance may provide ideas for disease control programs. However, in terms of vector management, this area of research has been neglected. In a study, the physico-chemical parameters of mosquito breeding water were measured to investigate the correlation between water parameters and larval abundance. Sampling of water was done along with larval collection from a Yan Oya stream in Sri Lanka. Here, the larvae were identified as *Anopheles culicifacies* and as *Anopheles varuna* using the taxonomic key. Temperature, dissolved oxygen, pH, conductivity, TDS, alkalinity, ammonia nitrogen, nitrate nitrogen, calcium, magnesium, carbon dioxide, ferrous iron, phosphate, colour, and turbidity were measured. The relation between water parameters and mosquito larvae was analysed statistically using ANOVA. Among physicochemical parameters, *Anopheles culicifacies* was positively related only to temperature, and *Anopheles varuna* to calcium. Surprisingly, this study does not find any correlation with other physicochemical parameters (Piyaratne et al., 2005).

Recently, water quality parameters were examined in selected mosquito breeding sites in urban Accra, Ghana, with an abundance of *Anopheles* and *Culex* larvae. Physicochemical parameters showed up to 72% variance within the breeding sites and separated *Anopheles* and *Culex* habitats ($P< 0.05$). The larval abundances were generally influenced by water temperature**,** pH, nitrate, and total hardness, with contradictory impacts on the two mosquito species. However, total dissolved solids, biochemical oxygen demand (BOD), and alkalinity exclusively influenced *Anopheles* abundance, whereas total suspended solids, phosphate, sulphate, ammonium, and salinity were significant factors for *Culex* abundance (Kinga et al., 2022).

The relationship between the larval density of container-breeding mosquitoes and water parameters and their habitat was investigated by Gopalakrishnan and co-workers. The larvae and breeding water were collected both from natural and artificial habitats in semi-urban and rural areas from eight sites in Asom, India, during pre-monsoon, monsoon, and post-monsoon. *Aedes albopictus* (93.7%) was the dominant species in the container-breeding habitats, followed by *Culex quinquefasciatus* (2.77%), *Armigeres subalbatus* (2.26%), *Aedes aegypti* (0.76%), *Toxorhynchites* sp. (0.4%), and *Lutzia* sp. (0.11%). The water quality parameters, such as pH, conductivity, salinity, TDS, turbidity, and dissolved oxygen, were recorded. The data analysis was carried out using IBM SPSS 19 statistical software. The mosquito larval density in the container habitats showed a significant negative correlation with the conductivity of breeding water ($r = 0.89$; $p = 0.003$). Salinity, total dissolved solids, and turbidity of the water in the habitats were negatively correlated, whereas pH and dissolved oxygen were positively correlated with the larval density (Gopalakrishnan et al., 2013).

Recently, a study was designed to determine the abundance of culicine species communities in two rural and one urban breeding sites in Egypt and the physicochemical characteristics of these respective breeding sites. The study sites were Al-Beshlawy drainage canal (rural), El-Khartoum irrigation ditch (rural), and Tanta man-made ground hole (urban). The measured physicochemical parameters were temperature, pH, salinity, turbidity, dissolved oxygen, and nitrite. Diversity indices (Bray-Curtis dissimilarity index, Shannon-Wiener diversity index, dominance index) of mosquitoes were also calculated. The results showed the presence of *Culex pipiens*, *Culex univittatus*, *Culex antennatus*, *Culex quinqueifasciatus*, *Culex perexiguus*, and *Culiseta longiareolata* at three breeding sites. *Culex pipiens* showed eudominance in all three breeding sites. The Bray-Curtis-Curtisilarity index between culicine communities ranged from 0.03 to 0.12. This dissimilarity may be due to the absence of specific species in some sites, as well as the apparent differences in *Culex pipiens* and *Culiseta longiareolata* abundance between the breeding sites. The multiple regression analysis between the identified mosquito species and the physicochemical parameters reveals that salinity, nitrite content, turbidity, and pH are thought to be important variables to predict the presence of culicine species in water bodies (Elhawary et al., 2020).

In order to better comprehend the distribution of different mosquito larval species in El-Muqattam and Abu-Seir, two urban areas in the Cairo Governorate, certain physico-chemical characteristics such as temperature, pH, salinity, turbidity, dissolved oxygen (DO), and nitrite of mosquito breeding habitats were examined.The presence of *Culex pipiens*, *Culex perexiguus*, *Ochlerotatus caspius*, *Culex pusillus*, and *Culiseta longiareolata* was reported from these regions. Salinity and DO may be regarded as the predictive variables linked to the immature abundance based on the

significant association between the various parameters and the abundance of the two common larval species (*Culex pipiens* and *Culex perexiguus*). In terms of all mosquitoes, there is a shift from Muqattam planned-safe to unplanned-safe Abu-Seir habitats, primarily as a result of turbidity and nitrite (Kenawy et al., 2013).

A cross-sectional study was conducted in 13 villages in Tanzania's Muheza district between December 2015 and May 2016. From the sampling site, mosquito larvae were collected, and physicochemical factors like temperature, pH, conductivity, total dissolved solids, and salinity were analysed. The larvae of *Anopheles funestus* and *Anopheles gambiae* (s.I.) were identified using the sequencing technique. The study revealed the coexistence of *Anopheles* and *Culex* in breeding grounds. Data were evaluated using Stata 13 and R 3.3.0 statistical software. From the data analysis, it was found that, when compared to *Culex*, the abundance of *Anopheles* larvae was significantly higher (76.6%). The presence and higher densities were strongly correlated with the upper percentiles of salinity (OR = 7.05; 95% CI: 1.19–41.88, P = 0.032) and conductivity (OR = 5.47; 95% CI: 1.01-29.67, P = 0.056) (Emidi et al., 2017).

Prior to the development of DNA sequencing techniques, phylogenetic trees were solely used in systematics and taxonomy to describe linkages between species. Phylogenies are now used in practically all areas of biology. Phylogenies are used to describe relationships between paralogues in a gene family, histories of populations, the evolutionary and epidemiological dynamics of pathogens, the genealogical relationship of somatic cells during differentiation and cancer development, and the evolution of language, in addition to representing the relationships among species on the tree of life. A phylogeny is a tree composed of nodes that are connected by branches. Each node represents the beginning of a new genetic lineage, and each

branch indicates the persistence of a genetic lineage over time. For example, if the tree shows the relationship between a set of species, the nodes reflect speciation events. In other situations, interpretation can be different. For instance, in a tree of paralogous gene families, nodes in a gene tree of sequences taken from a population indicate gene duplication events, whereas nodes in a gene tree of sequences taken from a population represent birth events of people who are ancestral to the sample. Nowadays, molecular phylogeny has been used as a crucial tool for genome comparisons. Classifying metagenomic sequences, identifying genes, regulatory elements, and non-coding RNAs in newly sequenced genomes, interpreting both contemporary and historical individual genomes, and reconstructing human evolution are all examples of its usage in this regard (Marra et al., 2003; Salipante & Horwitz, 2006).

Yang and Rannala reviewed the types of phylogenetic reconstruction methods, i.e., distance-based or character-based. In distance matrix methods, the distance between each pair of sequences is determined, and the resulting distance matrix is used to reconstruct trees. For instance, neighbour joining (the distance matrix method) uses a cluster algorithm to apply the distance matrix to create a fully resolved phylogeny. Other two distance matrix methods include least squares, minimum evolution, and neighbourhood joining. Maximum Parsimony (MP), Maximum Likelihood (ML), and Bayesian inference techniques are three types of character-based reconstruction methods. These methods compare every sequence in the alignment simultaneously, scoring each tree by taking into account one character (a site in the alignment) at a time. The 'tree score' is the minimum number of changes for maximum parsimony, the log-likelihood value for maximum likelihood, and the posterior probability for Bayesian inference (Yang & Rannala, 2012).

For example, multiple isolates from each taxon taken from a collection of environmental enteric bacteria were used to construct a molecular phylogeny for seven taxa of enteric bacteria, including *Citrobacter freundii*, *Enterobacter cloacae*, *Escherichia coli, Hafnia alvei*, *Klebsiella oxytoca*, *Klebsiella pneumoniae,* and *Serratia plymuthica*. 16S rRNA gene and sequences from five housekeeping genes (gapA, groEL, gyrA, ompA, and pgi) were utilised to build individual gene trees and were concatenated to infer a composite molecular phylogeny for the species. There may be 'genotypic' clusters that correlate to conventional species classifications, as suggested by the tight species clusters created by isolates from each taxon in the individual gene trees. These sequence data, the resulting gene trees, and the consensus tree generate the first data set to evaluate the applicability of the recently proposed core genome hypothesis (CGH). The CGH offers a genetically based method for applying the biological species concept to bacteria (Wertz et al., 2003).

More than 3000 microbial (bacterial and archaeal) genomes have been made publicly accessible, allowing an unusual possibility to investigate evolutionary genomic trends and providing useful reference data for a number of other studies, including metagenomics. Understanding their phylogenetic relationships will greatly enhance the usefulness of these genome sequences. Therefore, Lang and co workers reconstructed the phylogeny of every available bacterial and archaeal genome. They identified 24 single-copy, ubiquitous genes that may be applicable to phylogenetic analysis. To aggregate the data for the 24 genes, they employed two methods. First, they concatenated the alignments of each gene into a single alignment, from which a ML tree was inferred using RAxML. Second, they applied a relatively recent method of merging gene data known as Bayesian Concordance Analysis (BCA), which is implemented in the BUCKy software and uses the outcomes of 24 single-gene

phylogenetic analyses to create a "primary concordance" tree. The comparison of these two methods provides similar results in terms of the phylogenetic tree inferred from 16S rRNA. They conclude that using a ML analysis of a concatenated alignment of conserved, single-copy genes is currently the best method for creating a single phylogenetic tree that can be used as a reference phylogeny for comparative analyses (Lang et al., 2013). Phylogeny can address several questions, like the origin and spread of viral infections, the demographic changes and migration patterns of species, the relationship between species and genes, etc. The advent of sequencing technologies has raised the standard of phylogenetic analysis to new heights. Phylogenies are present in nearly every field of biology and can be exploited for a number of research purposes.

GENERAL METHODOLOGY

LARVAL COLLECTION AND STUDY SITE

The mosquito larvae were collected during pre-monsoon, monsoon, and post-monsoon during 2018-2020 from three taluks (sites) of Thrissur district, namely Thrissur-site 1, Chavakad-site 2, and Mukundapuram-site 3. The collected larvae were brought to the laboratory (Communicable Disease Research Laboratory, CDRL, St. Joseph's College, Irinjalakuda) along with breeding water samples. The respective fourth instar larvae were identified as *Aedes aegypti*, *Culex quinquefasciatus*, and *Anopheles stephensi* using taxonomic keys (Barraud, 1934).

LARVAL GUT DISSECTION

After surface sterilisation of larvae using sodium hypochlorite (1%), sterile PBS, and 70% ethanol, the gut was carefully dissected under sterile conditions inside a laminar airflow cabinet. The posterior two fragments of the abdomen were gently and slowly pulled away from the abdomen's anterior portion. Later, the gut was used for further microbial analysis (Charles et al., 2011).

GUT MICROBIAL CULTURING (CULTURE DEPENDENT METHOD)

Bacterial culturing

The particular study focused on two gut entities of mosquito larvae, namely bacteria and fungi, by a culture-dependent method. For bacterial isolation, the gut suspension was initially cultured in sterile Luria-Bertani broth and incubated at 37±2°C for 18-24 hours until turbidity was observed in the tubes. The liquid culture was serially diluted to quantify the concentration of viable bacteria, and about 100 µl

of culture was streaked onto a sterile Luria-Bertani agar plate. Microbial growth was assessed based on the total number of CFU/ml.

Fungal culturing

To isolate gut fungal inhabitant, the gut suspension was cultured in potato dextrose broth. To avoid bacterial contamination the culture media was supplemented with broad spectrum antibiotic chloramphenicol. The culture was serially diluted until a desired countable concentration was obtained. CFU/ml was calculated for fungal quantification. The culture was incubated at 25± 1 °C in the dark for about 21 days. Depending on the type of fungus, this may change. The fungal spread plate was incubated and then plated on potato dextrose agar to obtain the fungal spread plate. Later, pure fungal culture was isolated from the spread plate.

GUT MICROBIAL ISOLATION & IDENTIFICATION

a) Bacterial isolation & identification

The four-quadrant streak method was used for isolating single colonies of bacterial isolates. For bacterial identification, morphological features were observed, and Gram staining (with crystal violet as the primary stain and safaranin as the counterstain) was performed. Biochemical tests such as starch hydrolysis tests, catalase tests, oxidase tests, IMViC tests, hydrogen sulphide production tests, and gelatin hydrolysis tests were carried out for the preliminary identification and characterization of bacteria based on their metabolic activities(Clarke & Cowan, 1952). Bacterial DNA (NucleoSpin® Tissue Kit method) was isolated, amplified, and sequenced as part of bacterial identification.

b) Fungal isolation & identification

The preliminary identification of the fungus was done by the direct observation of colonies under ambient sunlight. Lacto Phenol Cotton Blue (LPCB) staining was used to study colony morphologies such as septation of hyphae, conidiophore, and branching frequency of the fruiting body. Genus-level identification can be done by comparing fungal morphology with fungal atlases and later confirmed by molecular analysis (DNA isolation using the NucleoSpin® Tissue Nagel Kit method, amplification, and sequencing).

DIVERSITY INDICES

To quantify the microbial diversity between pre-monsoons, monsoon and post-monsoon diversity indices were used. Alpha diversity provides an insight into microbial diversity during each mosquito larvae season, whereas beta diversity measures the dissimilarity in microbial diversity within mosquito larvae between seasons. All the diversity indices were measured based on CFU/ml. The evenness between the seasons was calculated by using the Shannon Wiener evenness index. The Shannon-Weaver index places a greater weight on species richness, whereas the Simpson index considers species evenness more than species richness in its measurement. Shannon Wiener evenness, Shannon-Weaver index and Simpson index belongs to alpha diversity indices. The Bray-Curtis dissimilarity (beta diversity) analysis showed differences in microbiome composition between seasons. All the data were entered and systemized in a Microsoft Excel 2016 spreadsheet, and diversity indices were analysed using a formula.

PHYLOGENY CONSTRUCTION

A phylogenetic tree was constructed to trace the evolutionary relationship between gut-isolated microorganisms. By using Molecular Evolutionary Genetics Analysis (MEGA) software, 11 phylogenies of microbes were constructed. The Basic Local Alignment Search Tool (BLAST) was used to identify homologous DNA sequences to the sequence of interest. After aligning the sequence, the phylogenetic tree was constructed using the neighbour joining method.

ANALYSIS OF PHYSICOCHEMICAL PARAMETERS

The physicochemical parameters of the breeding water may influence the presence of larval gut content. About 1000 ml of breeding water sample were collected separately from the larval collection site from 2018–2020 from three sites during the pre-monsoon monsoon and post-monsoon periods, respectively. The physical parameters like pH measured by a pH metre, temperature in °C measured by a thermometer, and conductivity (mS) measured by the electrode method TDS (ppm) by TDS metre, total alkalinity (mg/l), total hardness (mg/l), DO (mg/l), BOD (mg/l), calcium (mg/l), magnesium (mg/l) by titrimetric method, nitrite (mg/l), and phosphate (mg/l) by colorimetric method.

STATISTICAL ANALYSIS

All data was entered and systemized in a Microsoft Excel 2016 spreadsheet. The mean percentage and ± standard deviation (SD) were calculated for each physicochemical parameter on a seasonal basis. The statistical significance between physicochemical parameters at different sites during different seasons was analysed by one-way ANOVA and larval abundance by Kruskal-Wallis test with graphical representation using SPSS version 19.0.

CHAPTER 1

Investigation of gut microbiota in *Aedes aegypti*,*Culex quinquefasciatus*, & *Anopheles stephensi* using culture-dependent methods. A focus on bacterial & fungal communities

1.1 INTRODUCTION

Mosquitoes are arthropod vectors' top priority, leading to epidemics or pandemics. *Aedes*, *Culex*, and *Anopheles* are three genera of mosquitoes with significant public health importance as they are involved in disease transmission. *Aedes aegypti* is one of the global vectors of arboviral diseases like dengue, chikungunya, etc. *Culex quinquefasciatus* was a cosmotropical mosquito belonging to the category of vectors. This abundant mosquito was responsible for lymphatic filariasis, West Nile virus, etc. *Anophles stephensi*, a representative from the genus *Anopheles*, was a significant vector for the spread of malaria (Murugan et al., 2015; Anoopkumar et al., 2017).

Control of arbovirus was challenging due to a number of factors, like a lack of appropriate vaccines, a deficit of antiviral drugs, and the development of resistance in vectors against insecticides, which escalated the situation. In this circumstance, it is imperative for public health to develop new approaches for rapid detection and to check the spread of arboviral diseases (Nasir et al., 2022). To curb this problem, a lot of things should be considered wisely. Bio control is one of the best elixirs, mainly due to its eco-friendly nature. Secondly, targeting mosquito larvae is an effective option. The compilation of these will be more productive in the field of vector management (Kumar & Hwang, 2006).

Like all other higher organisms, mosquitoes harbour microbes from their larval stage onward. At this stage of development, larvae hold a dynamic and diverse gut microbiota, which may be of transstadial inheritance or from their surrounding

environment. The larval gut consortium remains an ideal breeding place where the co-existence of bacteria, fungus, archea, protozoa, viruses, and algae occurs. The microbiota linked to mosquitoes may be mutualistic, commensal, or parasitic in nature. An insight into gut microbiota was beneficial to investigate their positive and negative impact on host survival and vector competence (Coon et al., 2014).

A lot of studies have concentrated on symbiotic bacteria in mosquitoes. The midgut is the site where most of the symbiotics are located when compared to other parts of the mosquito. In both larval and adult stages, Gram-negative bacteria were the dominant members. The prokaryotic microbiota comprises bacteria and protozoa, whereas eukaryotic members include fungi and yeast. Eukaryotic microbes such as fungi were overlooked as part of the mosquito's microbiota when compared to prokaryotes. Mosquito larvae also enclose non-pathogenic fungal microbiota; these fungi mostly colonise the mosquito midgut but can also be found in other tissues such as salivary glands and reproductive organs. The majority of symbiotic fungi within mosquitoes are yeasts like *Candida* and *Pichia*, yet little is known about their interaction with the host and other gut inhabitants (Tawidian et al., 2019).

By analysing the bacterial and fungal communities in the guts of mosquito larval species, we hope to better understand their gut microbiome. These findings may have implications for the development of new strategies for controlling mosquito-borne diseases. Therefore, the present objective was intended to investigate the gut content of three species of mosquito larvae: *Aedes aegypti*, *Culex quinquefasciatus*, and *Anopheles stephensi,* from three sites in Thrissur district.

1.2 MATERIALS AND METHODS

1.2.1 Study area

The study area included three localities of Thrissur (southwest of Kerala) district, namely site1: (Thrissur taluk 10° 31' 49.2420" N 76° 12' 53.0244" E), site 2: (Chavakkad taluk 10° 34' 59.91" N 76° 1' 7) and site 3: (Mukundapuram taluk 10° 19' 60.00" N 76° 13'60.00" E) based on the public health significance of the area with mosquito infestation (Figure 4.a). The larval sampling was conducted three times during the pre-monsoon, monsoon, and post -monsoon for a period of two years, from 2018 to 2020. The larvae were collected during the day using standard dippers from breeding sites, and immediately transferred to pre-sterilised plastic bottles along with breeding water, which were labelled and brought to CDRL, Irinjalakuda, Thrissur for further studies (Leake, 1994; Silver, 2008).

1.2.2 Larval identification

Preliminary identification of mosquito larvae were based on its resting and mobility pattern in water. If it laid horizontally to the water surface it belonged to genus *Anopheles*. If it inclined at an angle of 45° it would be *Aedes* mosquitoes and at an angle of 90° it would be *Culex*. For precise species level identification larvae were examined under microscope (Leica DM300). Different species of *Aedes*, *Culex* and *Anopheles* like *Aedes albopictus*, *Aedes walbus*, *Culex sinensis*, *Culex vishnui*, *Culex fascanus*, *Anophles culicifacies* and *Anophles subpictus* were also identified based on their taxonomic features (Christophers, 1933; Bar & Andrew, 2013). As the present study focussed on *Aedes aegypti*, *Culex quinquefasciatus* and *Anopheles stephensi* due importance was given to these larvae.

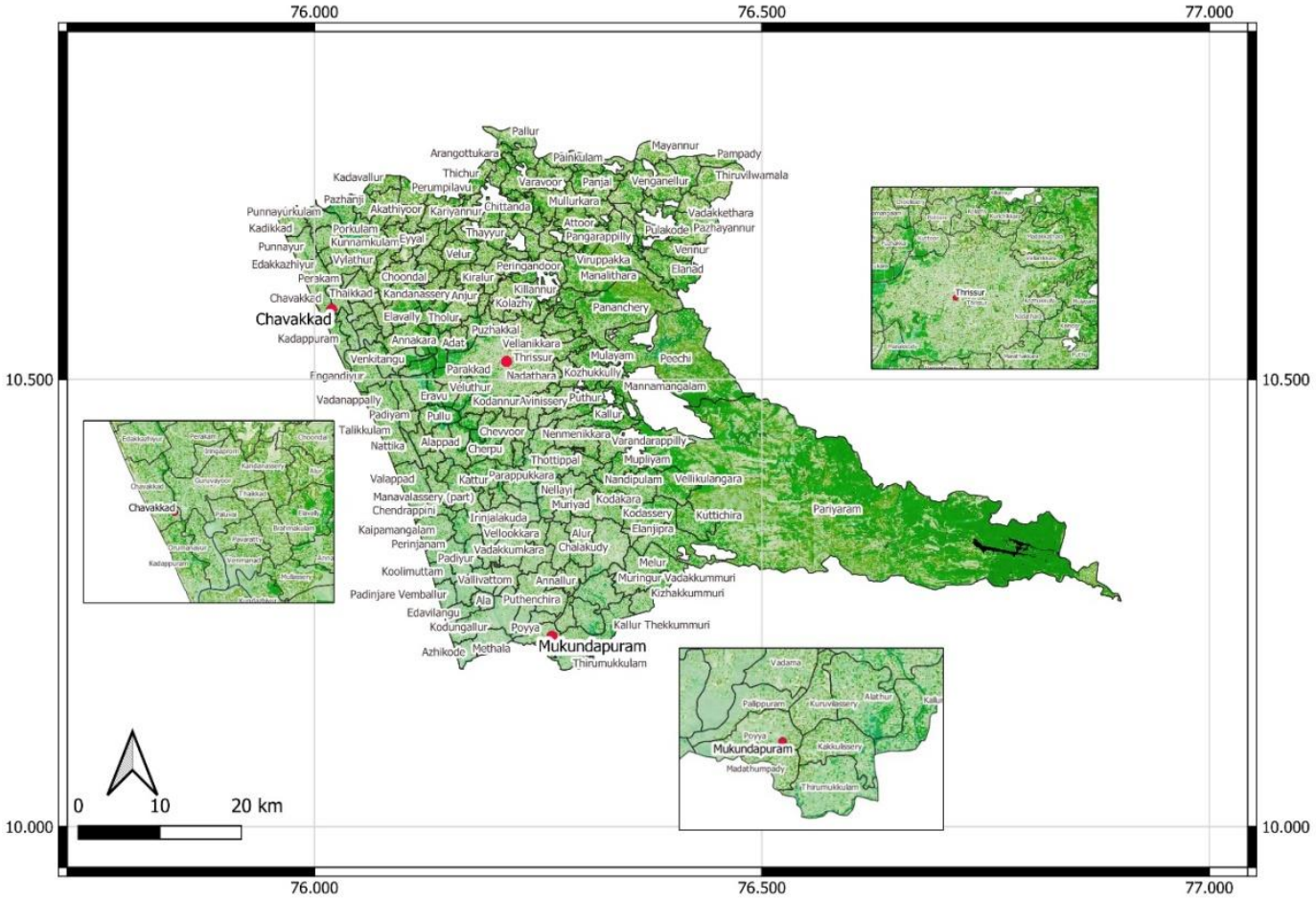

Figure 4.a: Geographical map representing the study area

Aedes aegypti (Linnaeus, 1762): The larval head was globular in shape. A pair of straight antennae and compound eyes were present on the lateral sides of the head beneath the antennae. Median brush and lateral brush are seen on the dorsal side of the head. On the ventral side, the mandible and maxillae were present. The larval neck connects the thorax with the head. The larval body had setate. The abdomen was long, cylindrical, dorsoventrally flattened, and VIII segmented. The number of lateral hair tufts that emerge from each abdominal segment varies. A row of comb spines was present on the VIIIth segment. Each comb spine had apical and sub-epical denticles and was pointed and curved at the tip. The comb scale, with a distinctive middle denticle along with lateral denticles, was a unique feature of *Aedes aegypti* larvae. The presence of a respiratory syphon with spiracles (respiratory openings) at its tip, surrounded by a perispiracular lobe, was found on the abdomen. At the terminal portion of the larval abdomen was the anal segment. It has a ventral brush with five pairs of variable-sized setae and four equally-sized anal papillae (translucent and boat-shaped) (Christophers, 1933; Bar & Andrew, 2013).

Anopheles stephensi (Liston, 1901): The larval head was longer than wide, heavily sclerotized and bearing a pair of antenna, mouthparts, and pairs of setae. The abdomen is narrower than the thorax and made up of X segments with abdominal setae. The main feature of *Anopheles* larvae was that the respiratory siphon is absent, and larvae obtain oxygen through a spiracular apparatus located at segment VIII of the abdomen. The presence of palmate setae on the dorsal surface of the abdomen assist them cling to the surface tension of the water as they laid parallel to water surface (Christophers, 1933; Rao, 1984).

Culex quinquefasciatus (Say, 1823): The larval head was short and stout darkening toward the base with enormous long and branched setae. A pair of antenna

and mouth parts were present on the head region. Setae present on the thorax are often solitary. The abdomen was divided into VIII segments, each with a unique setae pattern. The branch of seta 1 of abdominal segments III and IV is usually single; seta 1-III and IV are typically double, relatively short, barely reaching the margin of the following segment; seta V-VI is primarily double. Segment VII setae was branched and single. On the eighth abdominal segment, comb scales are uniformly fringed from the sides to the apex. The siphon was on the dorsal side of the abdomen, and in *Culex quinquefasciatus* it had several tufts of setae and was four times longer than it was broad. Four lengthy anal papillae protrude from the posterior end of the saddle, which is barrel-shaped and located on the ventral side of the abdomen (Christophers, 1933; Sirivanakarn & White, 1978 ; Dehghan et al., 2016).

1.2.3 Sample preparation

After identification, larvae were surface sterilised and then rinsed sequentially for one minute in the following solutions: sodium hypochlorite (1%), sterile PBS (81mM Na_2HPO_4, 19 mM NaH_2PO_4, 150 mM NaCl, pH 7.4), and ethanol (70%) to remove the surface bacteria. Prior to dissection, forceps and slides were cleaned in 70% ethyl alcohol to prevent bacterial contamination. The gut was then dissected carefully inside a laminar airflow cabinet by firmly pulling the thorax with blunt-ended sterile forceps. The posterior two fragments of the abdomen were gently and slowly pulled away from the abdomen's anterior portion. Later, the gut (10 numbers) was carefully transferred into a watch glass and ground in 300µl of PBS of pH 7.4 ± 0.2. Later, the suspension was equally divided for bacterial and fungal culturing (Garros et al., 2008 ; Charles et al., 2011).

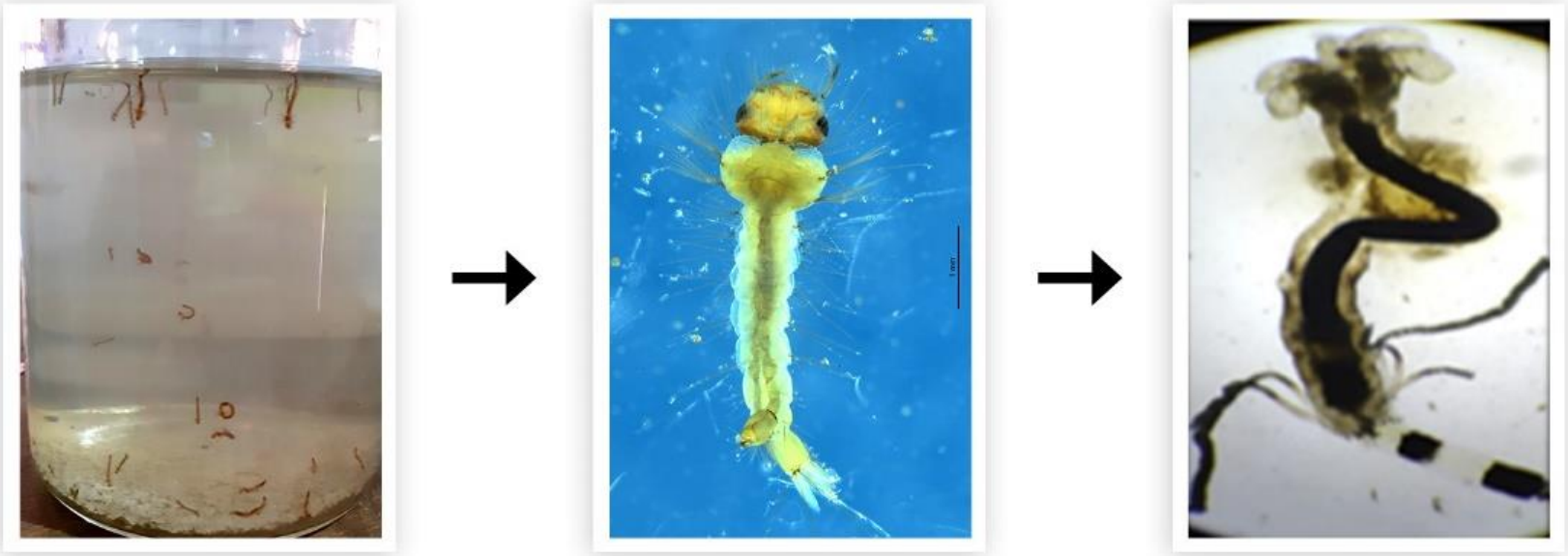

Figure 4.b: Sequential images showing mosquito larvae and larval gut (magnification 10 X)

1.2.4 Bacterial culturing and isolation

For bacterial culturing and isolation, the gut suspension was inoculated in liquid culture using Luria-Bertani broth (casein enzymic hydrolysate 10.000g/l; yeast extract 5.000g/l; NaCl 10.000g/l; final pH at 25ºC 7.5± 0.2) and incubated in a microbiological incubator at 37°C ± 2°C for 18-24 hours until turbidity was observed in the tubes. The liquid culture was serially diluted (1.0 $x10^5$) to decrease bacterial concentration. After serial dilution, the culture was streaked onto a sterile Luria-Bertani agar plate (casein enzymic hydrolysate 10.000g/l; yeast extract 5.000g/l; NaCl 10.000g/l; agar 15.000g/l; final pH at 25°C 7.5 ± 0.2) and incubated for 24-48hours at 37°C in a microbiological incubator under the aforementioned conditions until growth occurred on the plate.

A 100-µl volume from each dilution was plated on sterile plate count agar (PCA) and incubated at 37°C for 24-48 hours. Microbial growth was assessed based on the total number of CFU. Bacterial colonies were distinguished morphologically (i.e. shape, size, colour, margin, opacity and elevation). Morphologically distinct colonies were selected from primary plates for repeated subculture on nutrient agar plates until a pure colony was obtained.

$$\text{CFU/ml} = \frac{Number\ of\ colonies * dilution\ factor}{Volume\ of\ culture\ plate}$$

Following the incubation period, the isolates were picked up based on their apparent differences in colony morphology. The isolated isolates were then individually transferred to sterile Luria- Bertani agar plates using the streak plate method to obtain the pure cultures. The purified colonies were stored at 4°C for further analysis (Ruangpan & Tendencia, 2004).

1.2.5 Morphological characterisation using Gram staining

Staining technique and analysis of individual bacterial morphology were typically considered as the first stage to classify unidentified species into their befitting group. The Gram staining technique was used to analyze the bacterial morphology, endospores arrangement, and gram reactions. A sterile inoculation loop was used to transfer the specimen culture (2-4 loopful) onto a grease-free microscopic glass slide that had been cleaned with 70% alcohol (vol/vol) under aseptic conditions. A uniform thin smear was prepared and left to air dry in the laminar airflow hood. After being dried, the slides were heat fixed by waving them over a standard alcohol burner. Later, the slide was mounted on a staining bridge and flooded with Gram's crystal violet (primary stain) staining solution for 1 minute [crystal violet (Hucker's) Solution A:- 2.000 g of crystal violet in 20.000 ml of ethyl alcohol (95%). Solution B-ammonium oxalate 0.800 g in 80.000ml of distilled water). The smear should then be washed with tap water before being flooded with Gram's iodine for1 minute duration [Iodine 1.000 g and potassium iodide 2.000 g in 300.000 ml distilled water]. The slides were decolorized with Gram's decolorizer [50.0 ml of ethyl alcohol (95%) and 50.0 ml of acetone in a ratio of 1:1] until the blue colour stops flowing from the smear. Wash the smear with tap water and then the slide was counter-stained with 0.5% w/v safranin [0.500 gm of safranin O in 100.000 ml of ethyl alcohol (95%)]. Allow it to remain for 1 minute again, wash with tap water. The slides were air-dried and examined under oil immersion objective (Smith & Hussey, 2005).

1.2.6 Biochemical test for bacteria

Biochemical tests are performed to identify and characterize bacteria based on their metabolic activities. These tests aid in determining detailed properties (presence or absence of enzymes in metabolic pathway) concerning bacteria, which can be indicative of bacterial genus or species. Generally, these tests have been performed individually and selectively (Clarke & Cowan, 1952).

1.2.6.1 Starch hydrolysis test

The starch hydrolysis investigates whether bacteria are capable of producing the alpha-amylase enzyme. Here, starch agar (Himedia M107), a differential media was used for bacterial culturing. After incubation at a proper temperature of 37°C for 48 hours (2 days), the culture plate was flooded with Lugol's iodine (Himedia S019). Iodine reacts with starch to produce a blue-black colour. The appearance of a clear zone in the medium suggests a successful starch hydrolysis or positive test result because the test organism produces the exoenzyme amylase, which breaks down starch into mono and di-saccharides. Unhydrolyzed starch is indicated by a purple or blue zone, which is a negative test result (Clarke & Cowan, 1952).

1.2.6.2 Catalase test

The catalase test was used to determine the ability of bacteria to produce the catalase enzyme. During respiration, facultative anaerobic and aerobic bacteria produce hydrogen peroxide a harmful end product. These types of bacteria produce catalase enzymes that split hydrogen peroxide into oxygen and water. To test the presence of catalase, pure colonies were grown on Luria-Bertani agar slant. 3% hydrogen peroxide (Sigma Aldrich 88597) was added drop by drop onto the purified colony. If the test organism contained catalase enzyme, hydrogen peroxide will soon be converted to water and oxygen by the rapid generation of effervescences onto the

agar slant. If catalase is absent there will be a lack of bubble formation (Clarke & Cowan, 1952).

1.2.6.3 Oxidase test

This test was specifically used to identify aerobic bacteria, as the intracellular enzyme cytochrome oxidase reduces cytochrome to molecular oxygen, which was the terminal stage of the electron transport chain in aerobic respiration. The test organism was inoculated in a soya bean casein digest agar plate (Himedia M290) and incubated for 1-2days at a temperature of 37°C. Add 2 to 3 drops of Gordon McLeod Reagent (Himedia R026) onto a filter paper in a petri dish. Using an inoculated loop, smear the bacterial culture onto prepared filter paper. A positive oxidase test is indicated by the appearance of a deep blue-purple colour change on the test surface within 10 seconds. A negative oxidase test is indicated by the absence of colour change after the allotted time (Clarke & Cowan, 1952).

1.2.6.4 IMViC test

a) Indole production test

The indole test was a biochemical test used to determine the ability of an organism to produce indole, a compound derived from the amino acid tryptophan. This test is mainly used to differentiate the members of the Enterobacteriaceae family. Sterile 1% tryptone broth was prepared and using a sterile inoculating loop inoculated the broth with a pure culture of bacteria. A control tube without any inoculum serves as a negative control. The tubes were incubated at 37°C for 48 hours. After incubation added 3 drops of Kovac's reagent (Hi media R008) to the culture tubes. The tubes were shaken gently and were allowed to stand for a few minutes. A positive result was indicated by the appearance of a cherry-red colour at the interface between the culture

and the Kovac's reagent layer. This colour change indicates the presence of indole. A negative result shows no colour change (Clarke & Cowan, 1952).

b) Methyl-Red test

Inoculate sterile MR-VP (Hi media LQ082) broth with a pure bacteria culture and incubate at 37°C for 48 hours. After incubation added 5 drops of methyl red indicator (Hi media 1007). A positive result was indicated by the development of a stable red colour, indicating a low pH (acid production) due to glucose fermentation. Absence red colour indicate negative test result (Clarke & Cowan, 1952).

c) Voges- Proskauer test

Inoculate sterile MR-VP (Hi media LQ082) broth with a pure bacteria culture and incubate at 37°C for 48 hours. After incubation, an equal amount (10 drops) of VP reagent-A (Hi media R029) and VP reagent-B (Hi media R030) were added to the broth culture. The contents were mixed and allowed to stand for 15 to 30 minutes. The development of a pink to red colour in the upper layer of the broth, indicates a positive Voges-Proskauer test. The absence of pink colour in the upper layer of broth indicates negative test (Clarke & Cowan, 1952).

d) Citrate utilization test

This test was often performed on bacteria to differentiate between members of the Enterobacteriaceae family. Simmon's citrate agar (Himedia M099) slants were prepared and made sterile. Later agar slants were inoculated with pure culture bacteria. The tubes were then incubated at 37°C for 48 hours. Slant culture was observed for the growth and coloration of the medium. If the organism is citrate positive medium turns blue. The organism was citrate negative if there was no colour change (Clarke & Cowan, 1952).

1.2.6.5 Hydrogen sulphide production test

This test was used to determine an organism's ability to produce hydrogen sulfide gas during the metabolism of sulfur-containing amino acids. Sterilized SIM agar (Sulfide Indole Motility-Himedia M181) slants were prepared. Pure bacterial colonies were stab inoculated by using a sterile inoculating loop. The slants were incubated for 48 hours at 37°C. After incubation slants were examined for the presence or absence of black coloration along the stab line of inoculation. The presence of black colour indicates positive test and absence indicates negative test result (Clarke & Cowan, 1952).

1.2.6.6 Gelatin hydrolysis test

This hydrolysis test was used to determine an organism's ability to produce the enzyme gelatinase, which breaks down gelatin protein into its constituent amino acids. Prepare and sterilize gelatin agar slants using 500 ml of distilled water and 10 g of nutritional agar (Himedia M001) and 5 g of gelatin (Hi media GRM019). The slants were inoculated with a pure bacterial culture using a sterile inoculation loop and were then incubated at 37°C for 48 hours. After the incubation slants were gently tilted and observed for the presence or absence of solidification or liquefaction of the gelatin medium. If gelatin hydrolysis has occurred, the gelatin medium will remain liquid after incubation, indicating a positive result. If gelatin hydrolysis has not occurred it indicates a negative test result (Clarke & Cowan, 1952).

1.2.7 Genomic DNA isolation

Genomic DNA was isolated using NucleoSpin® Tissue Kit (Macherey-Nagel) following the manufacturer’s instructions. 1ml of pure bacterial culture was taken, centrifuged at 8000 ×g for five minutes, and the supernatant was discarded. By

pipetting up and down, the particle was resuspended in 180 µl Buffer T1. 25 µl Proteinase K was added, the mixture was vigorously vortexed, and the process was continued at 56°C until complete lysis was achieved and later incubated at 56°C until complete lysis was obtained. 200 µl of Buffer B3 was added, vortex thoroughly and incubated at 70°C for 10 minutes. 200 µl of 100% ethanol was added and mixed thoroughly by vortexing. The mixture was pipetted into a NucleoSpin® tissue column, placed in a 2 ml collection tube and centrifuged at 11000 ×g for 1 minute. The NucleoSpin® Tissue column was transferred to a new 2 ml tube and washed with 500 µl of BW buffer. The wash step was repeated using 600 µl of B5 buffer. After washing the NucleoSpin® tissue column was placed in a clean 1.5 ml micro centrifuge tube and DNA was eluted out using 50 µl of BE buffer.

1.2.8 Qualitative analysis of DNA

Using agarose gel electrophoresis, the DNA's quality was checked. To 5µl of the DNA sample added 1µl of 6X gel-loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH-8.0). 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 carried out with 0.5X TBE as electrophoresis buffer at 75 V until the bromophenol dye front migrated to the bottom of the gel. The gels were visualized in a UV transilluminator (Genei) under UV light using a gel documentation system (Bio-Rad).

1.2.9 PCR amplification of 16S rRNA gene

DNA amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems). To PCR tubes added 1 µl of DNA, 0.25 µl of forward primer named16S-RS-F (5’CAGGCCTAACACATGCAAGTC3’), 0.25 µl

of reverse primer named16S-RS-R (5'GGGCGGWGTGTACAAGGC3'), 5 µl 2X Phire master mix and 4 µl of distilled water make up to a final volume of 10.50 µl. The following conditions were used for the PCR cycling: 95°C for 5 minutes, followed by 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds 72°C for 60 seconds, before 7 minutes at 72°C. The quality of the DNA isolated was evaluated using agarose gel electrophoresis. To 5µl of DNA added 1µl of 6X gel-loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH-8.0). Later 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, the gel was run at 75 V until the bromophenol dye front has migrated to the bottom of the gel. The molecular standard used was a 2-log DNA ladder (NEB). The gels were visualized in a UV trans illuminator (Genei).

1.2.10 Sequencing

The sequencing was done at Rajiv Gandhi centre for Biotechnology Trivandrum by using Big Dye Terminator v3.1 cycle sequencing kit. They sequenced using the Sanger DNA sequencing technique in the ABI 3500 DNA analyzer (Applied Biosystems) following ExoSAP-IT treatment. The quality was checked by utilizing Applied Biosystems' sequence scanner software v1. Using Geneious Pro v5.1, sequence alignment and required editing of the obtained sequences were carried out (Bell, 2008).

1.2.11 Fungal culturing

The larval gut suspension was cultured on potato dextrose broth (PDB Himedia 3g in 100 ml distilled water) for fungal culturing. The broth was supplemented with 50 µg/ml chloramphenicol as a bacteriostatic agent. The culture

was incubated at 25± 1°C in the dark for about 21 days. Depending on the type of fungus, this may change. The liquid culture was serially diluted (1.0×10^5) to decrease fungal concentration. After serial dilution, the culture was streaked onto potato dextrose agar (PDA Himedia 3.9g in 100 ml distilled water) and incubated at 25± 1°C in the dark for about 21 days. A 100 µl volume from each dilution was plated on sterile PDA plates and incubated at 25± 1°C in the dark for about 21 days. Fungal growth was assessed based on the total number of CFU. Later, pure fungal culture was isolated from the spread plate (Ravimannan et al., 2014).

$$\text{CFU/ml} = \frac{Number\ of\ colonies * dilution\ factor}{volume\ of\ culture\ plate}$$

1.2.12 Preliminary identification of fungi

The preliminary identification of fungus was done by the direct observation of colonies under ambient sunlight. The wet mount method (LPCB staining) was used to study later colony morphologies such as septation of hyphae, conidiophore, and branching frequency of the fruiting body. The genus level identification can be done by comparing fungal morphology with fungal atlases (Sangeetha & Thangadurai, 2013).

1.2.13 Fungal staining

LPCB staining was a wet mount staining technique commonly used to visualize fungi by imparting blue colour to hyphae and spore. Under sterile environment a clean glass slides were taken and cleansed with 70% alcohol (vol/vol). Glass slides were then dried and added two drops of LPCB stain (Phenol crystals 20.000 mg, cotton blue 0.050 mg, lactic acid 20.000 ml, glycerol 20.000 ml, and

distilled water 20.00 ml). A Scotch tape was carefully placed over the fungal pure culture and then to the stain. The excess stain spread out and was wiped using tissue paper. It was kept as such for 5 minutes. The slides were observed first under low power (10X) and then switch to a higher power (40X) objective of a microscope(Leica DM300) for more detailed examination of spores and other structures (Sangeetha & Thangadurai, 2013).

1.2.14 Genomic DNA isolation

NucleoSpin® Plant II kit (Macherey-Nagel) method was used for fungal DNA extraction. The entire process was carried out under sterile conditions. 3 ml of fungal culture was centrifuged for 10 minutes at 5000 × g to isolate DNA. The cells were washed with 1 ml of 10 Mm EDTA (pH 8). After discarding the supernatant, pellets were centrifuged at 5000 ×g for 10 minutes. The pellet was resuspended in 600 µl sorbitol buffer (1.2 M sorbitol; 10 mM CaCl2; 0.1 M Tris/HCl pH 7.5; 35 mM ß-mercaptoethanol). 50 U lyticase was added to it and incubated at 30°C for 30 minutes. The mixture was centrifuged for 10 minutes at 2,000×g supernatant was removed, and the pellet was then resuspended in 180 µl buffer T1. To that added 25 µl Proteinase K solution and mixed vigorously. It was then incubated at 56°C until complete lysis was obtained. During the incubation period, it was vortexed occasionally. Added 200 µl buffer B3, again vortexed vigorously, and incubated at 70°C for 10 minutes. 210 µl ethanol (96%) was added to the sample and mixed. The sample was then loaded into the NucleoSpin® tissue column into the collection tube and centrifuged for 1 minute at 11000 × g. The column back was placed back into the collection tube after discarding the flow-through. For the first wash added 500 µl buffer (BW) and centrifuge for 1 minute at 11,000 ×g. Place the column back into the collection tube after discarding the flow-through. During the second wash added 600 µl buffer (B5)

to the column and centrifuged for 1 minute at 11000 × g. The column was placed back into the collection tube after discarding the flow-through. It was again centrifuged for 1 minute at 11000 ×g. To elute pure DNA the column was placed into a 1.5 ml microcentrifuge tube and added 100 µl of prewarmed buffer BE (70°C). Incubated at room temperature for 1 minute and then centrifuged for 1 minute at 11000 ×g.

1.2.15 Qualitative analysis of DNA

The quality of the DNA was checked using agarose gel electrophoresis. The samples (1µl) were loaded to 0.8% agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg/ml ethidium bromide. The gels were visualized in a UV transilluminator (Genei).

1.2.16 PCR amplification of ITS

Purified DNA samples were amplified in PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems). For PCR analysis added 5 µl of 2X Phire Master Mix, 0.25 µl forward primer (Universal ITS-1F TCCGTAGGTGAA CCTGCGG), 0.25 µl of reverse primer (Universal ITS-4R TCCTCCG CTTATTGATATGC), 4 µl of distilled water and 1 µl of sample DNA. The cycle conditions consisted of a single initial denaturation at 98°C for 30 seconds, then denaturation at 98°C for 5 seconds, annealing at 58°C for 10 seconds, extension at 72°C for 15 seconds, followed by 40 cycles with final extension at 72°C for 60 seconds and store at 4°C. The PCR products were checked in 1.2% agarose gels prepared in 0.5X TBE buffer containing 0.5 µg/ml ethidium bromide. The molecular standard used was a 2-log DNA ladder (NEB). The gels were visualized in a UV transilluminator (Genei). Later followed by ExoSAP-IT Treatment (GE Healthcare), 5 µl of PCR product is mixed with 0.5 µl of ExoSAP-IT and incubated at 37^{o}C for 15 minutes followed by enzyme inactivation at 85^{o}C for 5

minutes. For the removal of unwanted primers and dNTPs from a PCR product mixture. The sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the Big Dye terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). 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 (You et al., 2008).

1.2.17 Sequencing

The sequencing was done at Rajiv Gandhi Centre for Biotechnology Trivandrum by using Big Dye Terminator v3.1 cycle sequencing kit. They sequenced using the Sanger DNA sequencing technique in the ABI 3500 DNA analyzer (Applied Biosystems) following ExoSAP-IT treatment. The quality was checked by utilizing Applied Biosystems' sequence scanner software v1. Using Geneious Pro v5.1 sequence alignment and required editing of the obtained sequences were carried out (Bell, 2008).

1.2.18 Diversity indices

To quantify the number of species present in the larval gut during pre-monsoon, monsoon, and post-monsoon, diversity indices like Shannon Weaver diversity, Simpson's diversity, and Shannon evenness were determined. To compare the diversity between the seasons, the Sorensen index was calculated. These indices aid in assessing gut microbial diversity (bacterial and fungal species) obtained by the culture-dependent method. Species diversity and evenness were calculated for all the isolates obtained during different seasons. The presence of each isolate on the

respective culture plates was considered for the calculation of the indices. Shannon-Weaver and Simpson diversity indices provide more weight to community composition than species richness or evenness. These indices contemplate the relative abundances of different species. While considering relative abundances, a diversity index mainly focuses on both species richness and the evenness with which individuals are distributed among the different species. The Shannon-Weaver index prioritises species richness, although the Simpson index accounts for species evenness more than species richness in its measurement.

Simpson's diversity reflects the probability that two individuals taken randomly from the dataset are not of the same species. Values for Simpson's diversity range between 0 and 1, with larger values representing greater diversity.

Simpson diversity index $D = \frac{1 - \Sigma \, ni \, (ni-1)}{N \, (N-1)}$

Where, 'ni' is the number of the 'i' th species and 'N' is the total number of specimens in the studied habitat.

Shannon Weaver diversity is based on the uncertainty that randomly selected individuals from the dataset will be accurately identified as members of certain species. Larger values reflect greater uncertainty and, thus, greater diversity (Mohlmann et al., 2017).

$$\text{Shannon Weaver diversity index } H' = \sum_{i=1}^{S} pi \log (pi)$$

Where, 'S' is the total no of species in the community, 'Pi' is the proportion of individuals belonging to the 'i'th species in the community.

According to Shannon-Wiener's evenness scale, the values ranged from 0 to 1, with 1 denoting total evenness or the presence of all species in equal abundance (Mohlmann et al., 2017).

$$\text{Shannon – Wiener's evenness index } E = \frac{H'}{\ln(S)}$$

Where, 'S' is the total number of species in a habitat or in a geographical area. Values range from 0 to 1, with 1 denoting total evenness, or the abundance of all species (Mohlmann et al., 2017).

Bray Curtis' dissimilarity index (beta diversity) was apt to measure the heterogeneity between the communities or samples. It was normally used in microbiome studies used to express major comparisons between samples.

$$\text{Bray – Curtis dissimilarity index } BCd = \frac{\Sigma|x_i - x_j|}{\Sigma(x_i + x_j)}$$

Where, 'x' is the abundance of individual species. Subscripts 'i' and 'j' are used to differentiate between two samples. The value ranges from 0 to 1, where 0 indicates high similarity between samples and value close to 1 denotes higher dissimilarity between samples (Schroeder & Jenkins, 2018).

1.3 RESULTS

In this study, the culture dependent method (CDM) was used to investigate the gut microbial (bacteria and fungus) diversity associated with *Aedes aegypti*, *Culex quinquefasciatus,* and *Anopheles stephensi* from three sites, namely Site 1: Thrissur, Site 2: Chavakkad, and Site 3: Mukundapuram, during the pre-monsoon, monsoon, and post-monsoon. A total of 1265 isolates (1026 bacterial and 239 fungal isolates) were obtained from the larval guts of these three mosquito species, surveyed from

different sites during different seasons. Out of this, 63 bacterial isolates and 64 fungal isolates were selected based on their distinguished morphological features (Figure 23-36). The bacterial entities were then characterised using biochemical tests (Table 1–6) and further identified on the basis of 16S rRNA gene sequencing. Fungal characterization was based on ITS molecular approaches (Table 7-12). A total of 52 species (26 bacterial and fungal) were detected in the *Aedes aegypti* larval gut. The rationale of the study was the assessment and analysis of gut bacterial and fungal diversity according to seasonal changes.

Microbial communities from *Aedes aegypti* gut were mostly represented by phylum Pseudomonadota (66.67%), and phylum Bacillota (33.33%). Vast majority of bacteria associated with phylum Pseudomonadota were clustered into the order Enterobacterales (95.24%), and in the order Xanthomondales (4.76%) of the class Gamma-proteobacteria. While in the phylum Bacillota, two bacterial genera (*Enterococcus* and *Bacillus*) of class Bacilli were recognised. Members of the class Bacilli were classified into two different orders: Bacillaceae and Lactobacillales. All the bacterial species have been summarised in Table 13. On a family taxonomic scale, analysis of the most abundant phylotypes across samples from *Aedes aegypti* gut revealed taxa related to the family Enterobacteriaceae, tailed by Enterococcaceae, Yersiniaceae, Bacillaceae, and Xanothomonadales. Analysis of the bacterial community based on seasonal framework revealed a significant dominance of the genus *Enterobacter* with more than 13 bacterial strains. The other bacterial genera were represented by *Rahnella*, *Escherichia*, *Pseudomonas*, and *Serratia* in *Aedes aegypti*. While analysing the bacteria on a seasonal basis, the genus *Enterobacter* was exclusively dominant. The presence of *Stenotrophomonas maltophilia*, which belonged to the order Xanthomondales, was also reported. The total viable

count (TVC), or abundance of bacteria, was calculated using the total number of colonies isolated on Lauria Bertini medium plates. The TVC of microbes was calculated on a seasonal basis separately for each mosquito species. The pre-monsoon and monsoon seasons yielded the highest bacterial count for *Echerichia coli* (Figure 5 & 6). These counts were 2900000 CFU/ml and 31000000 CFU/ml, respectively. Conversely, during post-monsoon, *Stenotrophomonas maltophilia* displayed a maximum CFU value of 21000000 (Figure 7).

About 26 fungal strains were isolated by CDM from *Aedes aegypti* during the pre-monsoon, monsoon and post-monsoon. Division Ascomycota accounted for 96.15% of the fungal diversity across all the mycobial samples, while the remaining 3.85% was linked to division Mucoromycota. Among these divisions, two predominant classes (Eurotiomycetes and Dothideomycetes) in addition to other classes (Saccharomycetes, Zygomycetes, and Sordarimycetes) were recorded. Abundance according to seasons was shown in (Figure 8, 9 & 10).

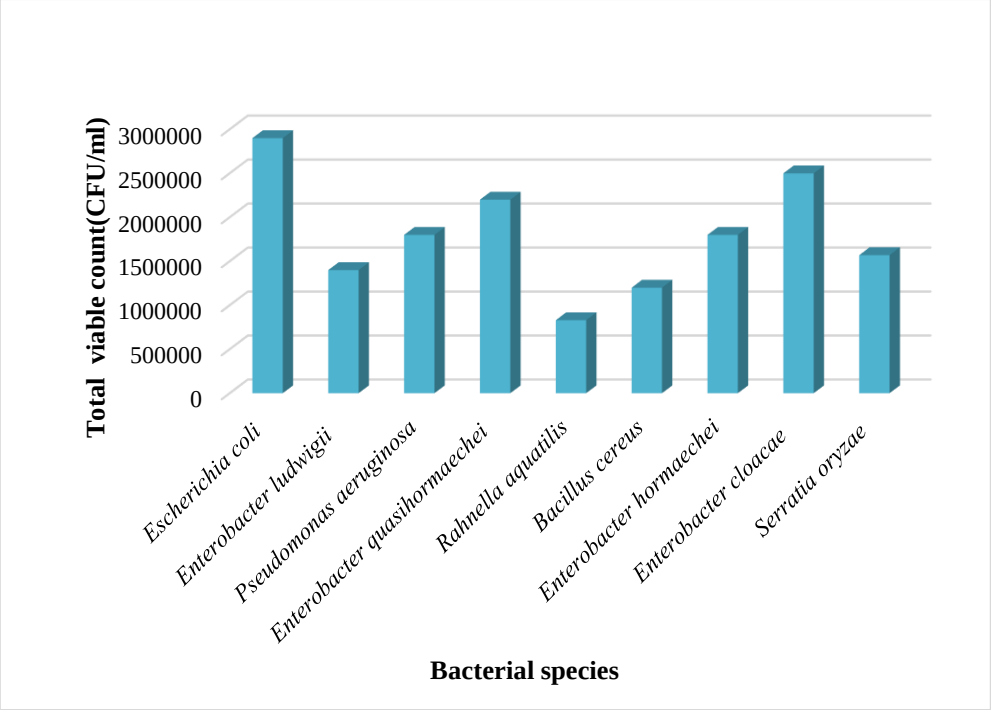

Figure 5. Total viable count of bacteria from *Aedes aegypti* larval gut during pre-monsoon

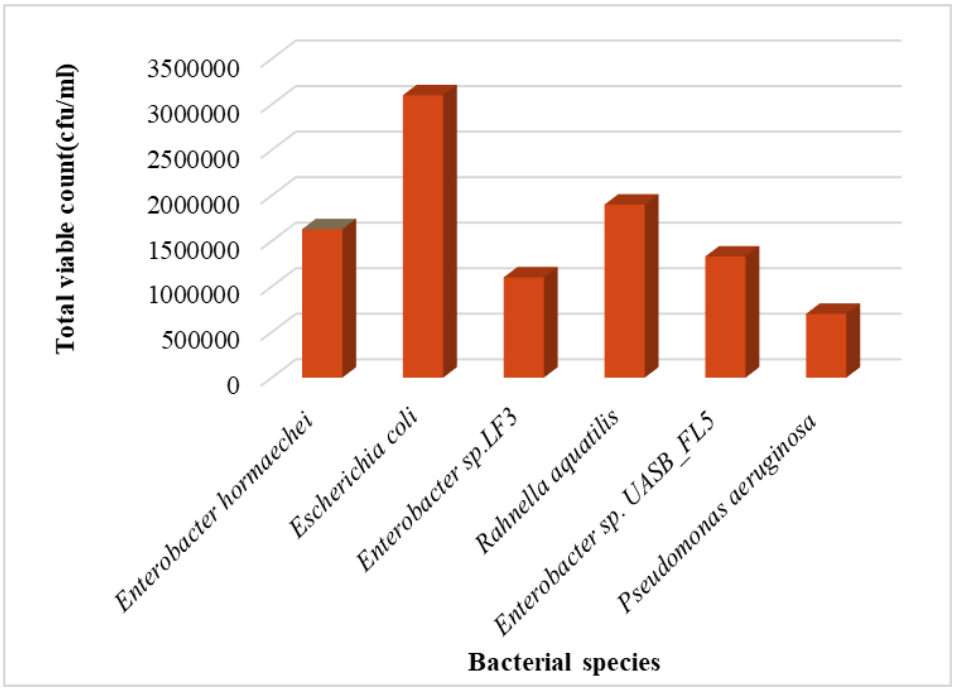

Figure 6. Total viable count of bacteria from *Aedes aegypti* larval gut during monsoon

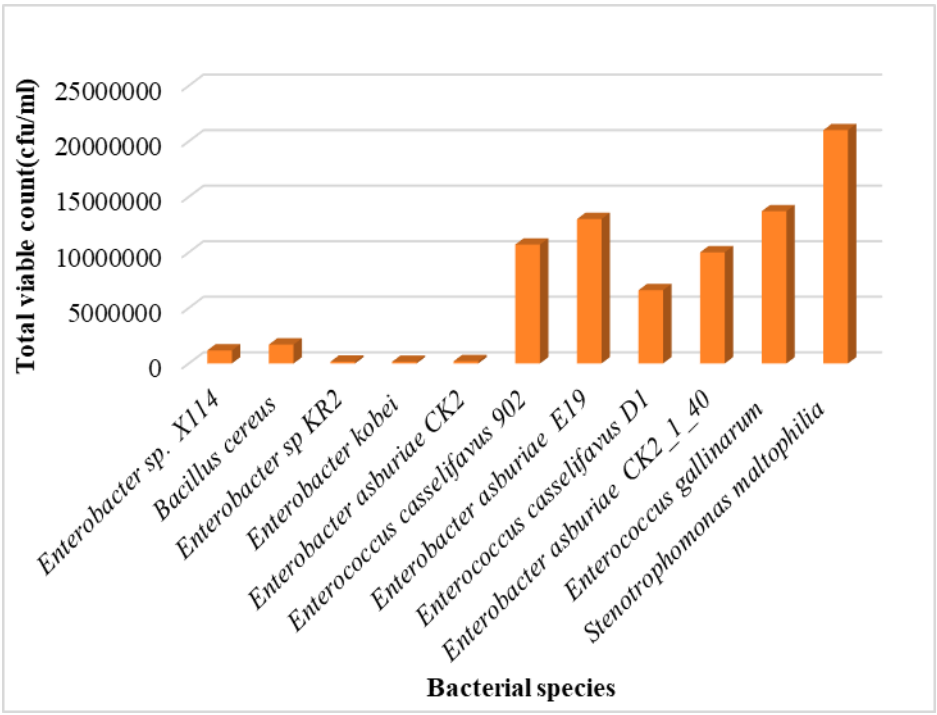

Figure 7. Total viable count of bacteria from *Aedes aegypti* larval gut during post-monsoon

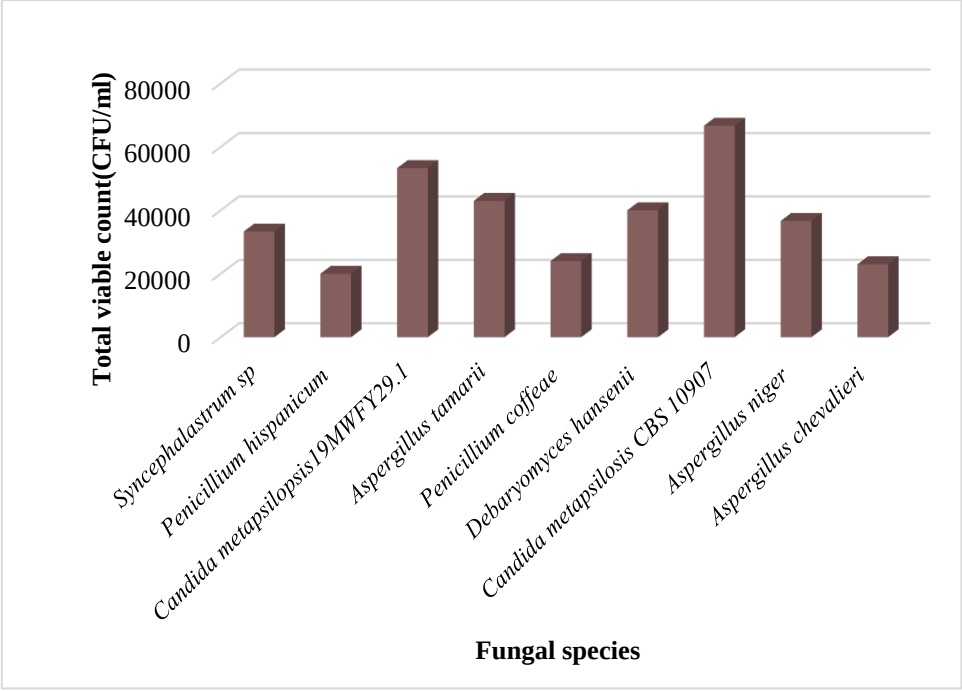

Figure 8. Total viable count of fungus from *Aedes aegypti* larval gut during pre-monsoon

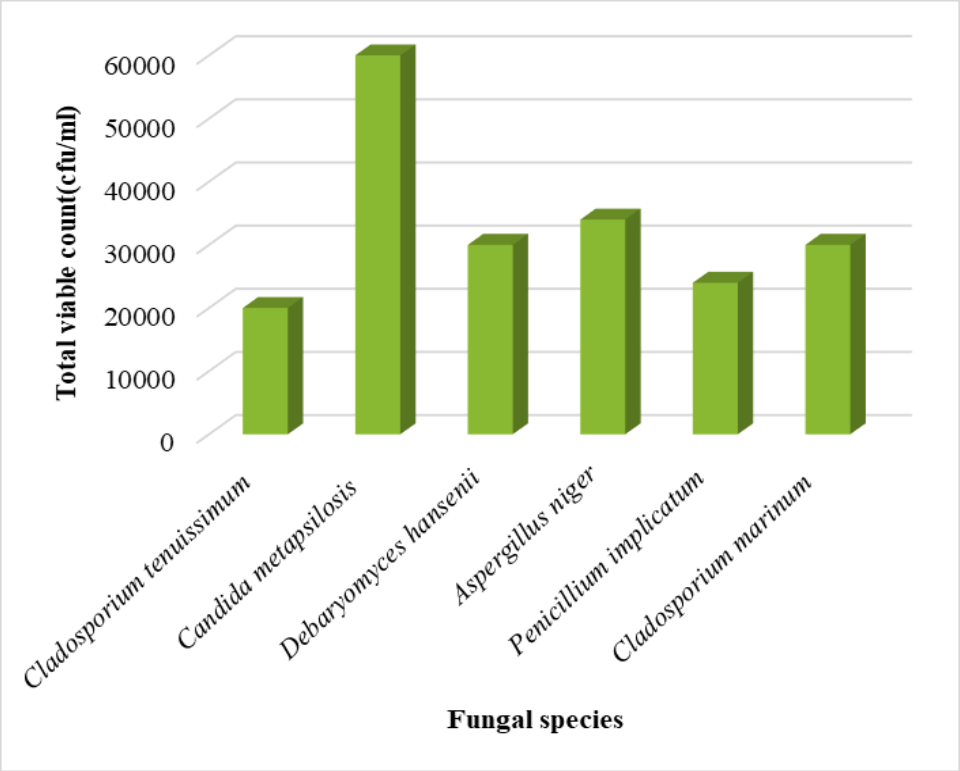

Figure 9. Total viable count of fungus from *Aedes aegypti* larval gut during monsoon

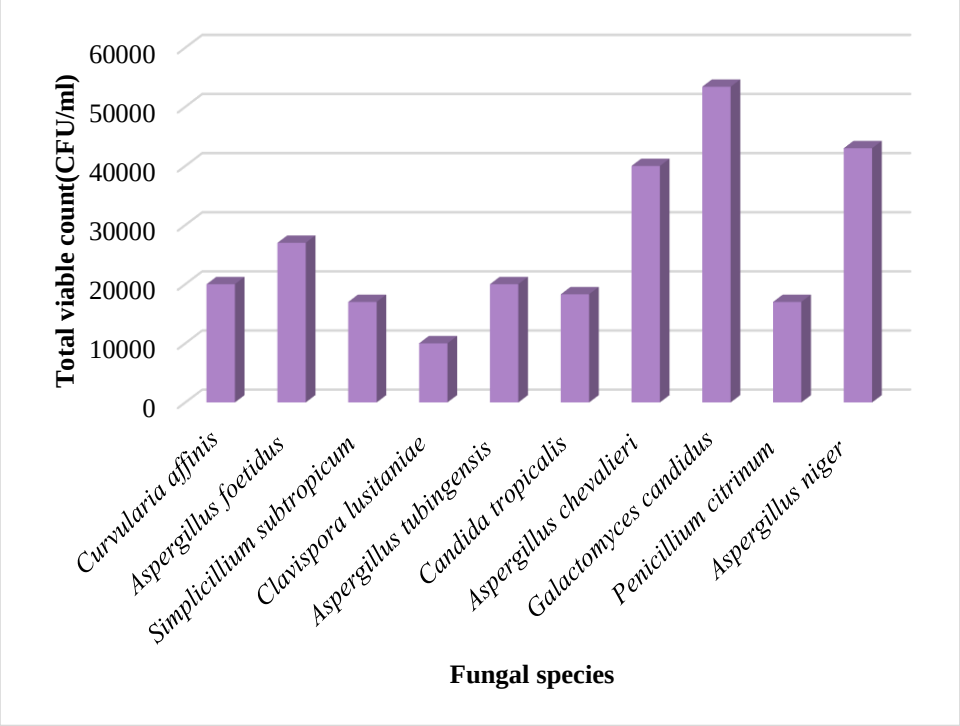

Figure 10. Total viable count of fungus from *Aedes aegypti* larval gut during post-monsoon

During pre-monsoon, *Candida metapsilosis* had the highest CFU/ml. *Candida metapsilosis* showed a greater abundance of 60000 CFU/ml in the monsoon season. During the post-monsoon period, *Galactomyces candidus* exhibited a greater abundance of 53400 CFU/ml. *Candida*, *Aspergillus*, and *Penicillum* were the prevalent taxa present in all seasons. *Syncephalastrum* sp. was solely recorded during the pre-monsoon. While two species of *Cladosporium* (*Cladosporium tenuissimum* and *Cladosporium marinum*) were procured during the monsoon season, several taxa were present during the post-monsoon season compared with the other two seasons. *Curvularia affinis*, *Simplicillum subtropicum*, and *Clavispora lusitaniae* were only documented during the post-monsoon season. The presence of *Syncephalstrum* species, *Cladosporium marinum*, *Cladosporium tenuissimum* and *Curvalaria affinis* were reported for the first time from mosquito larval gut.

The bacterial profiling of *Culex quinquefasciatus* revealed the preponderance of phylum Pseudomonadota (73.7%) as well as the presence of phylum Bacillota (26.3%). About seven bacterial genera (*Escherichia*, *Enterobacter*, *Acinetobacter*, *Rahnella*, *Serratia*, *Pseudomonas*, *Bacillus*) including 19 species from 3 orders (Enterobacterales, Moraxellales and Bacillales) scattered between two classes Gammaproteobacteria and Bacilli were detected from the larval gut (Table 14). Among these with the highest predominance was befitted to class Gammaproteobacteria than Bacilli. The exclusive presence of the order Enterobacterales marked the pre-monsoon bacterial inspection. A greater abundance or higher viability count was documented for *Escherichia coli* with a mean of 280000 (CFU/ml), while the presence of *Rahnella aquatilis* strain (MSSRFQS77, TW4), *Pseudomonas* sp., *Enterobacter* sp. and *Bacillus* sp. was reported during the pre-monsoon (Figure 11).

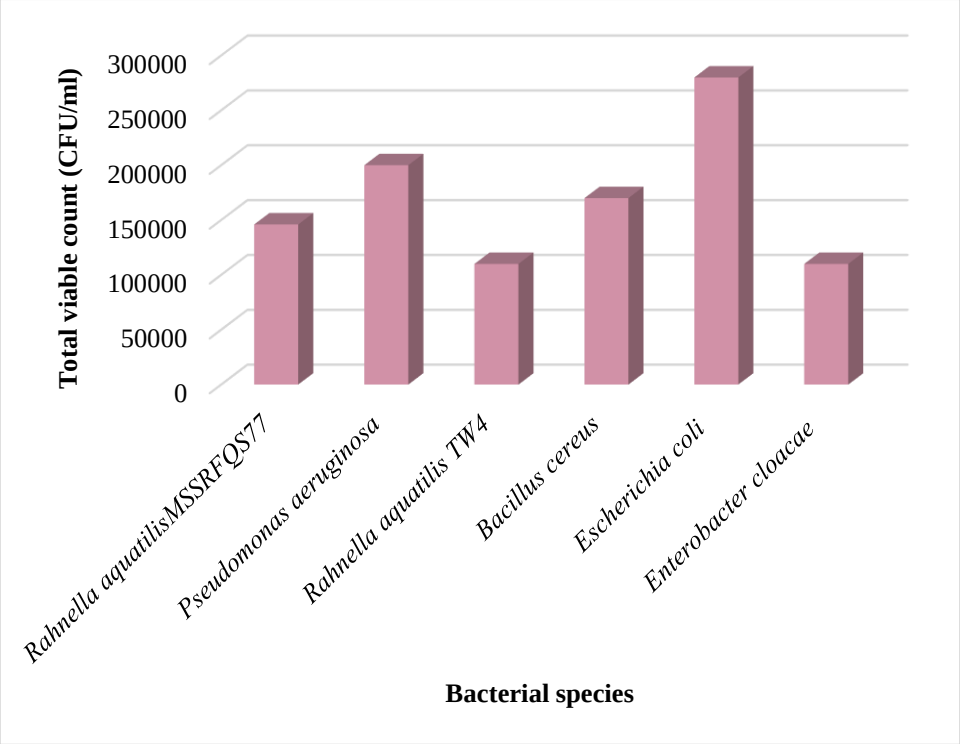

Figure 11. Total viable count of bacteria from *Culex quinquefasciatus* larval gut during pre-monsoon

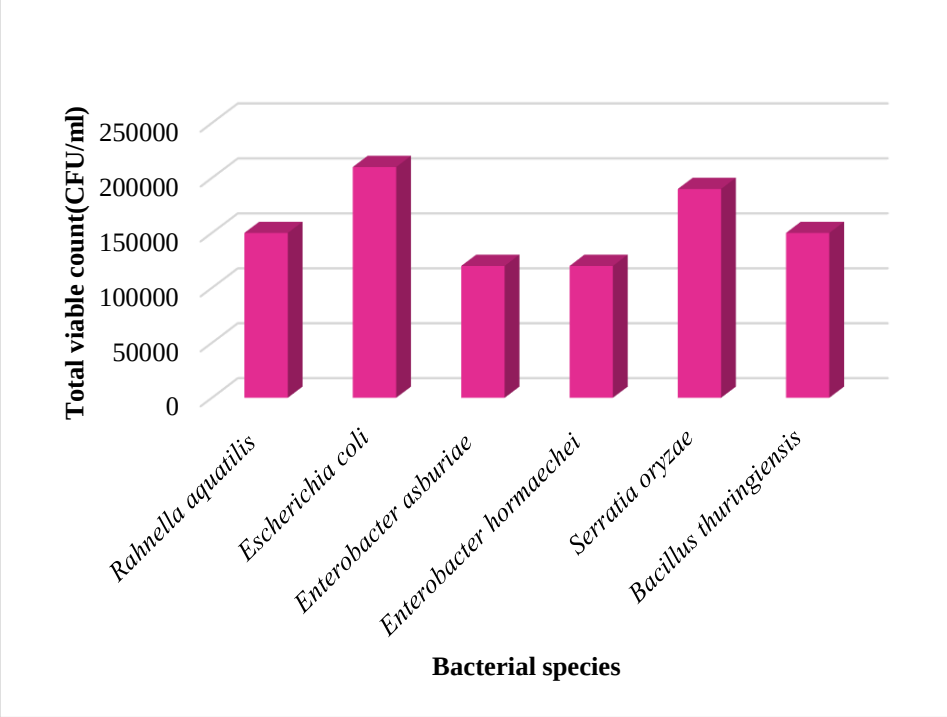

Figure 12. Total viable count of bacteria from *Culex quinquefasciatus* larval gut during monsoon

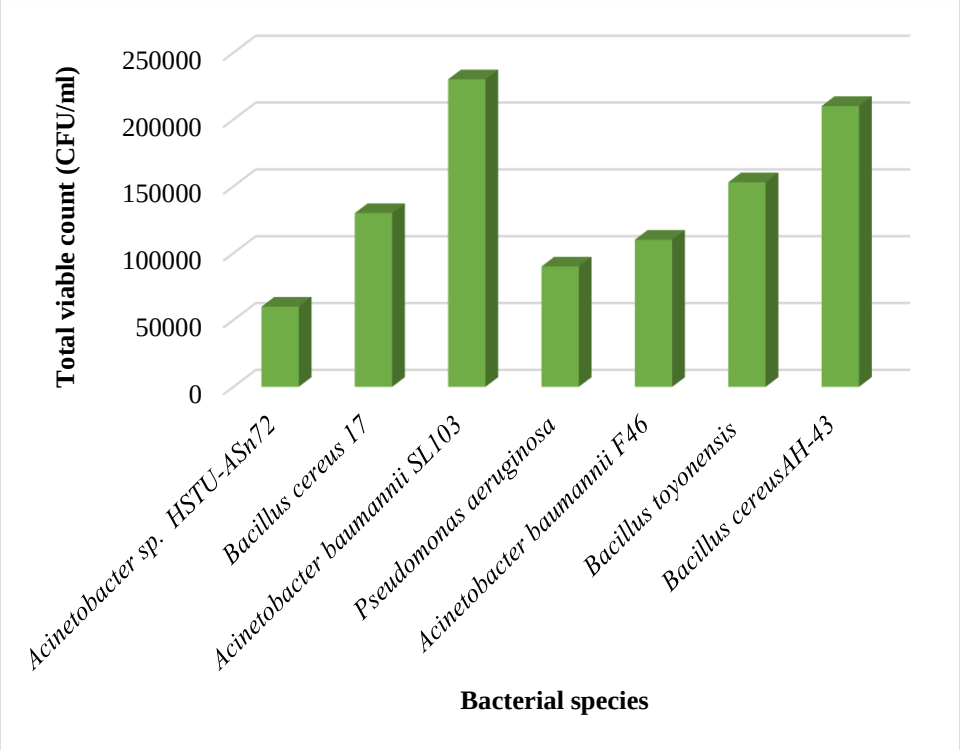

Figure 13. Total viable count of bacteria from *Culex quinquefasciatus* larval gut during post-monsoon

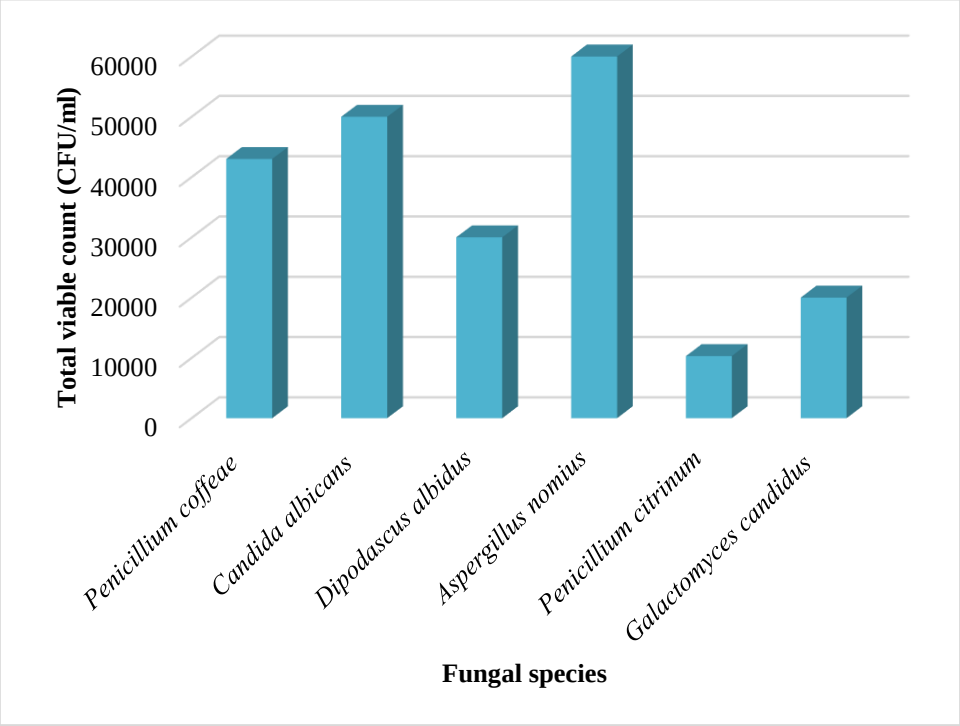

Figure14. Total viable count of fungus from *Culex quinquefasciatus* larval gut during pre-monsoon

The bacterial genera *Rahnella*, *Escherichia*, *Enterobacter*, *Serratia, and Bacillus* were isolated during the monsoon season. When comparing the bacterial abundance, *Escherichia coli* exhibited a greater value of 210000 (CFU/ml), whereas *Enterobacter asburiae* and *Enterbacter hormaechei* displayed a lower abundance of 120000 (CFU/ml). Other detected bacterial taxa comprise *Rahnella aquatilis*, *Serratia oryzae,* and *Bacillus thuringensis* (Figure 12).

When compared with pre-monsoon and monsoon genera, equal dominance was observed for *Bacillus* and *Acinetobacter* genera. Genus *Pseudomonas* was reported during the post-monsoon, and *Acinetobacter baumanni* was recorded as the most abundant taxa with 230000 CFU/ml. Surprisingly, *Acinetobacter* sp. was also reported as the least abundant taxa, with a value of 60000 CFU/ml. The details of bacterial species were summarised in (Figure13). Irrespective of seasonal variation, the genus *Bacillus* marked its presence in all seasons. While comparing the presence of *Rahnella aquatilis* and *Escherichia coli*, which was common during pre-monsoon and monsoon whereas *Pseudomonas areuoginosa* was common during pre and post-monsoon.

A total of 20 fungal communities were identified from *Culex quinquefasciatus* larvae. The gut fungal composition comprises Ascomycota (95%) and Mucromycota (5%), which were obtained as dominant divisions during the pre-monsoon, monsoon, and post-monsoon (Table 14). The classes found to be present across all samples included Eurotiomycetes (45%), Saccharomycetes (40%), Dothideomycetes (5%), class (5%) and Sordariomycetes (5%). The order Eurotiales (45%), Saccharomycetales (40%), Capnodiales (5%), Mucorales (5%), and Trichosphaeriales (5%) disseminated into five families: Trichocomaceae (15%), Aspergillaceae (20%),

Debaryomycetaceae (35%), Davidiellaceae (5%), and Trichospheriaceae (5%). Although the proportion of dominant fungal species varies in different seasons.

During pre-monsoon *Aspergillus nomius* displayed a greater abundance with 60000 (CFU/ml) followed by *Candida albicans*, *Penicillum coffeae*, *Dipodascus albidus*, *Galactomyces candidus* and *Penicillium citrinum* (Figure 14).

During monsoon season *Penicillium citrinum* showed greater abundance of 70000 (CFU/ml). The members of *Candida parapsilopsis* complex namely *Candida metapsilopsis* and *Candida orthopsilopsis* were obtained from gut. Other representatives includes *Geotrichum candidum*, *Aspergillus assiutensis*, *Aspergillus aculeatus* (Figure 15).

The fungal consortium from *Culex quinquefasciatus* during the post-monsoon was dominated by *Candida tropicalis* with 50000 CFU/ml. *Aspergillus foetidus*, *Candida metapsilopsis*, *Cunninghamella blakesleena*, *Cladosporium cladosporioides*, and *Nigrospora oryzae* were obtained. Genus *Candida* remains the most prominent fungal present in *Culex quinquefasciatus* (Figure 16).

A total of 18 distinct bacterial species were obtained from the of *Anopheles stephensi* larvae during different seasons. The isolates were categorised into major phyla such as Pseudomonadota (55.6 %) and Bacillota (44.4 %) assorted into two classes, Bacilli (44%) and Gammaproteobacteria (56%), then into three orders, namely Bacillales (39%) and Enterobacterales (56%), which diverged into 6 different families: Bacillaceae (28%), Enterococcaceae (6%), Paenibacillaceae (11%), Enterobacteriaceae (39%),Yersiniaceae (11%),Pseudomonadaceae (5%) (Table 15).

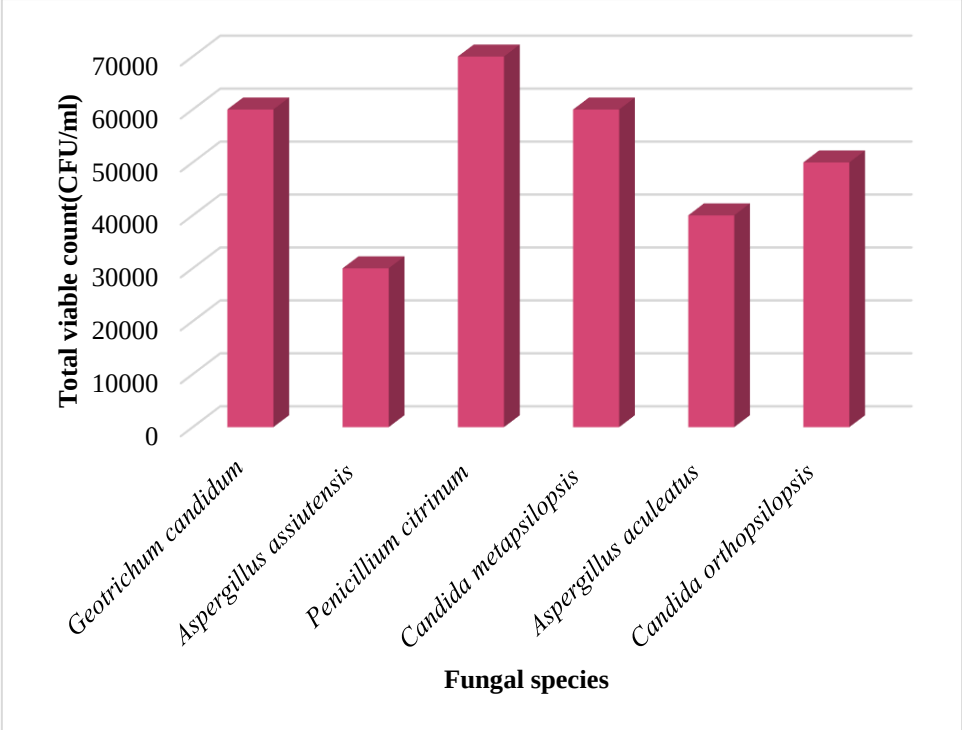

Figure15. Total viable count of fungus from *Culex quinquefasciatus* larval gut during monsoon

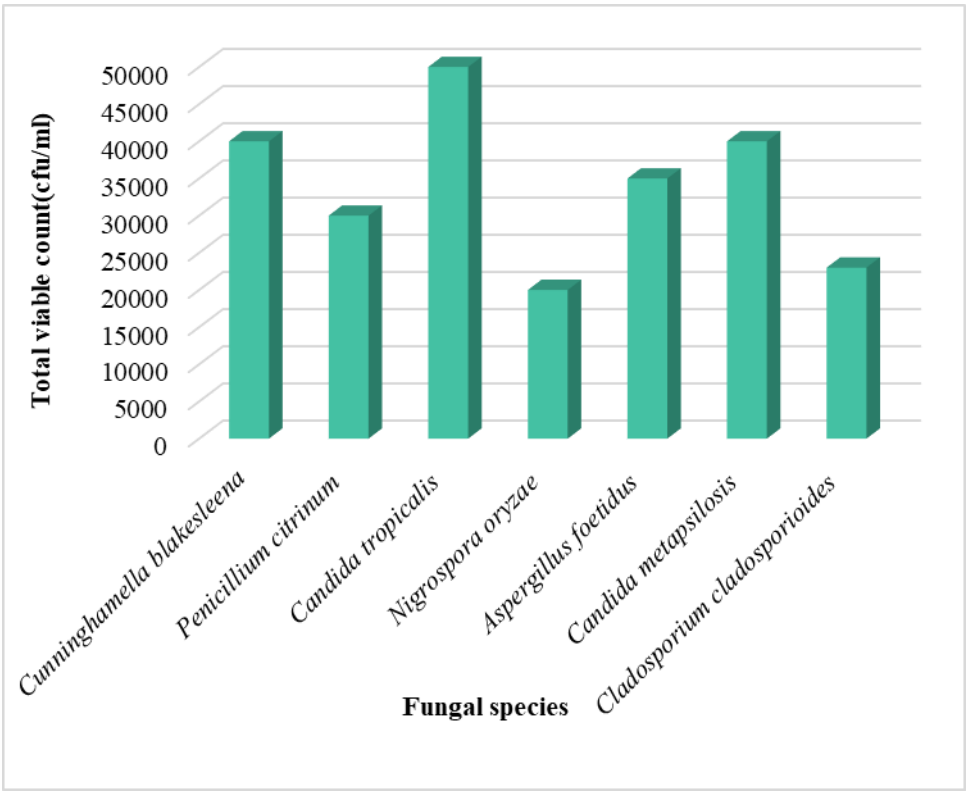

Figure16. Total viable count of fungus from *Culex quinquefasciatus* larval gut during post-monsoon

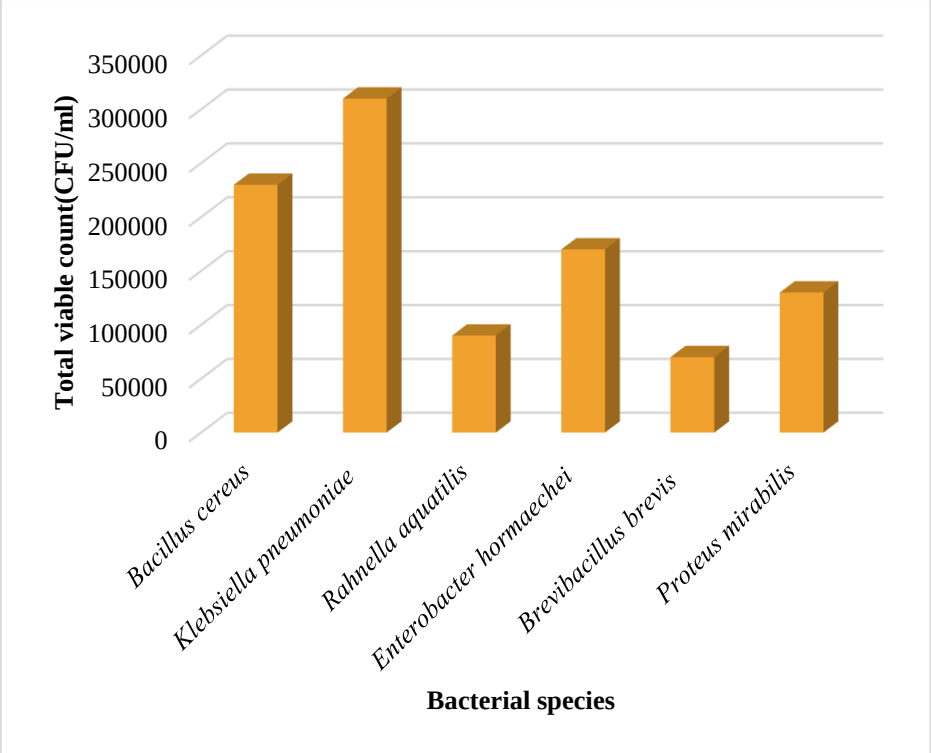

Figure17. Total viable count of bacteria from *Anopheles stephensi* larval gut during pre-monsoon

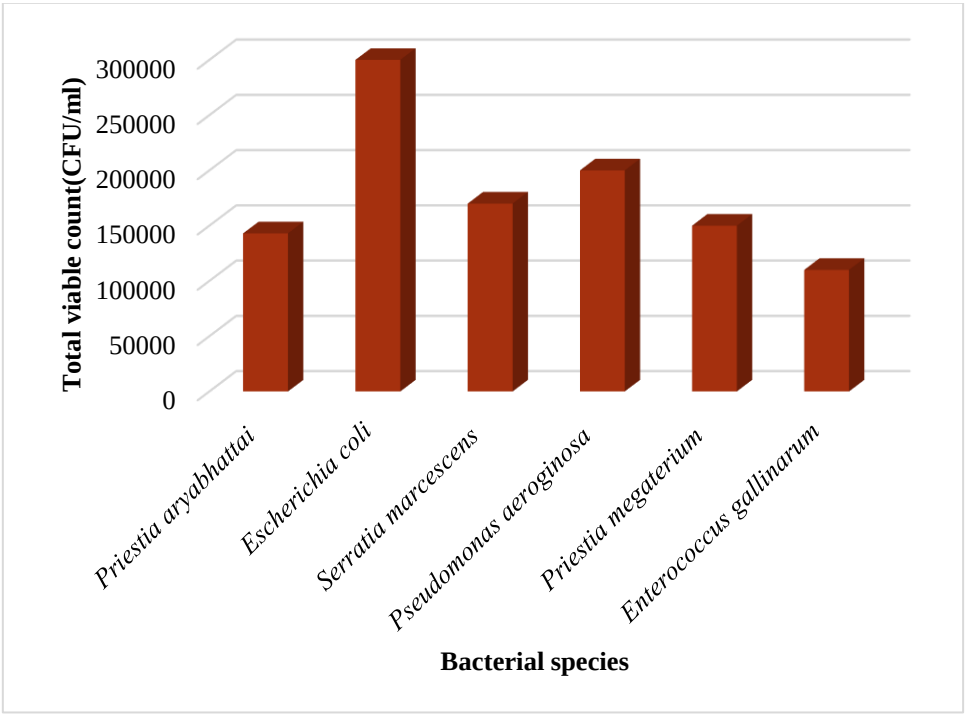

Figure 18. Total viable count of bacteria from *Anopheles stephensi* larval gut during monsoon

During the pre-monsoon, the most abundant and diverse members were gammaproteobacteria; among them, the highest abundance was observed for *Klebsiella pneumonia* with 310000 CFU/ml. The lowest abundance was observed for *Brevibacillus brevis* (Figure 17).

During the monsoon season, *Escherichia coli* remained the most abundant bacterial species, with 300,000 CFU/ml. Other identified bacterial gut inhabitants were *Pseudomonas aeroginosa*, *Serratia marcescens*, *Priestia megaterium*, *Priestia aryabhattai*, and *Enterococcus gallinarum* (Figure 18). *Priestia megaterium,* was reported for the first time from mosquito larval gut.

During the post-monsoon period, *Bacillus thuringensis* exhibited a higher abundance of 330000 CFU/ml. The other co-bacterial entities obtained from the larval gut were *Enterobacter cloacea*, *Bacillus cereus*, *Enterobacter bungandensis*, *Brevibacillus parabrevis,* and *Enterobacter ludwiggi* (Figure 19).

Around 18 fungal isolates were identified from the larval gut by ITS sequencing (Table 15). Here, all fungal taxa belonged to division Ascomycota and were included in two classes: Eurotiomycetes (56%), and Saccharomycetales (44%). Distributed among two orders, Eurotiales and Saccharomycetales, it is placed into two families, Trichocomaceae and Debaryomycetaceae.

During the pre-monsoon, *Dipodascus albidus* manifested a greater abundance of 30000 CFU/ml. However, a greater variety was displayed by the genera *Aspergillus* and *Penicillum* (Figure 20). It includes *Penicillum oxalicum* and *Penicillum rubens* in the case of the *Penicillium* genus. While that of the genus *Aspergillus* comprises the presence of *Aspergillus aculeatus* and *Aspergillus oryzae*.

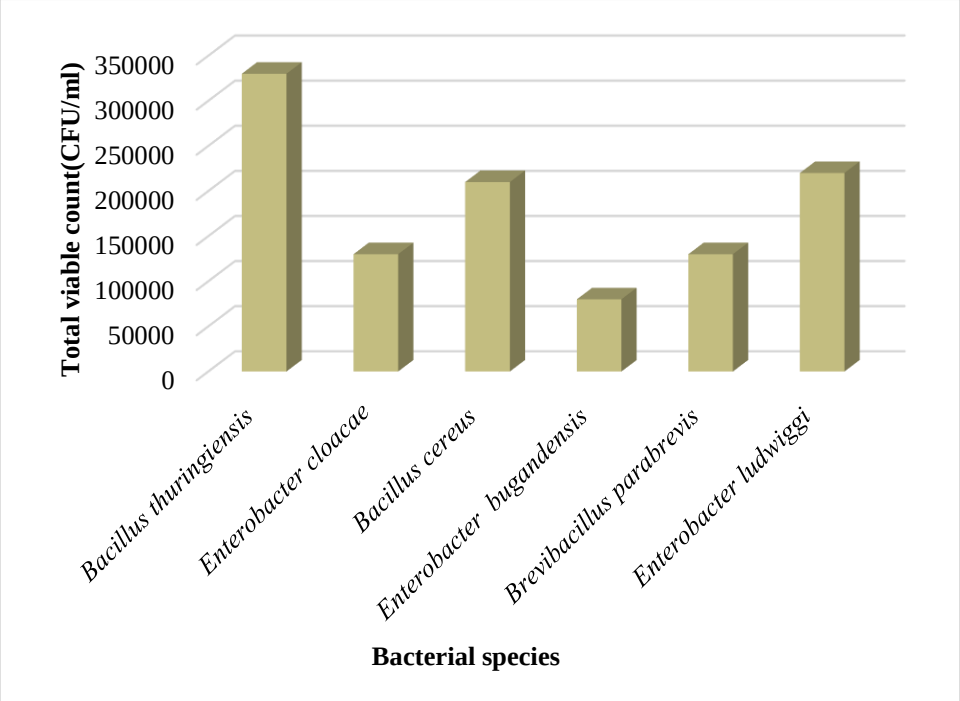

Figure 19. Total viable count of bacteria from *Anopheles stephensi* larval gut during post- monsoon

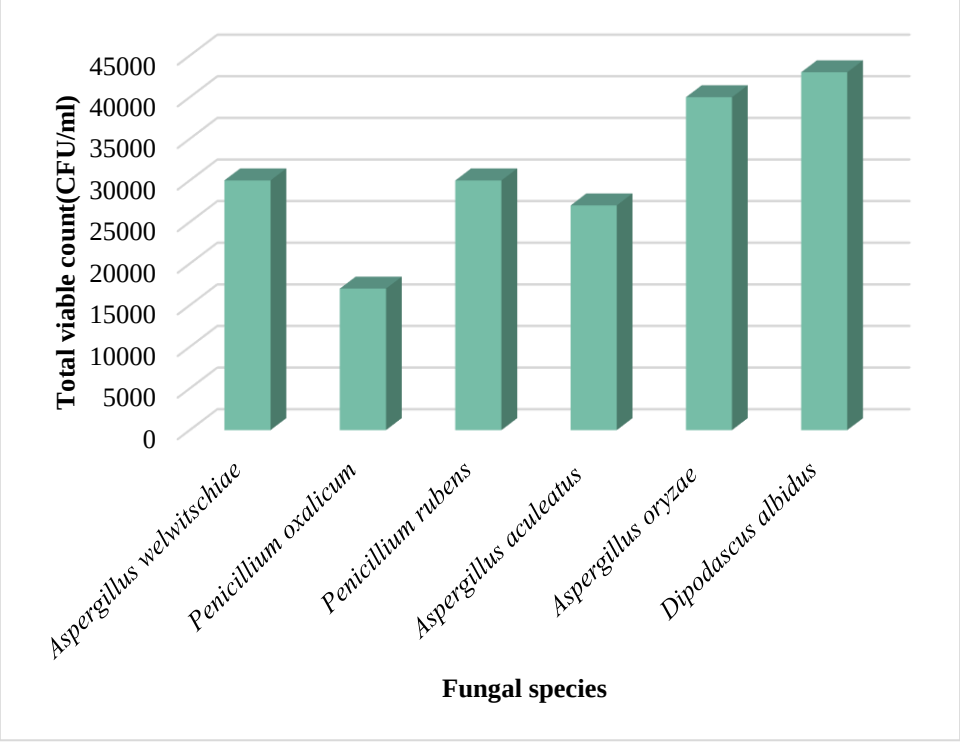

Figure 20. Total viable count of fungus from *Anopheles stephensi* larval gut during pre-monsoon

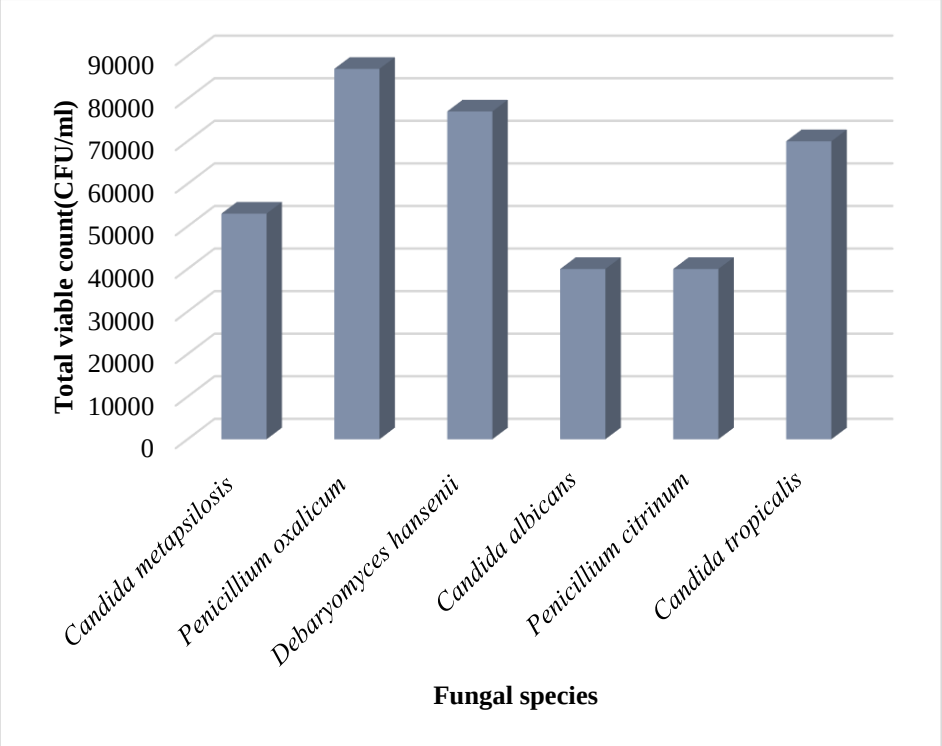

Figure 21. Total viable count of fungus from *Anopheles stephensi* larval gut during monsoon

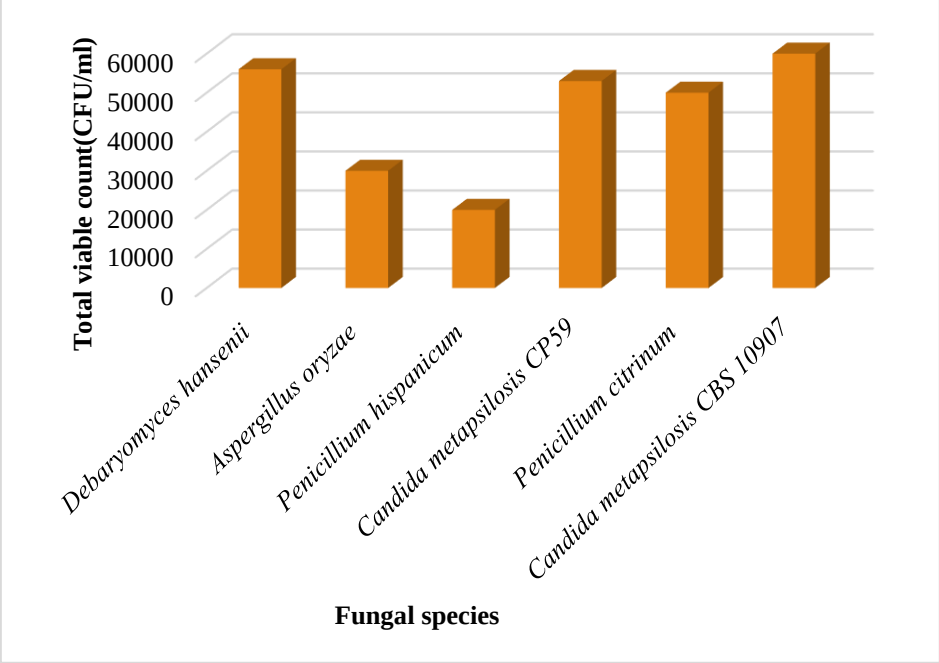

Figure 22. Total viable count of fungus from *Anopheles stephensi* larval gut during post-monsoon

The monsoon season was marked by the dominance of genus *Candida*, like *Candida metapsilosis, Candida albicans,* and *Candida tropicalis*. This was followed by the genus *Penicillium*. Even though genus *Candida* marked its dominance, the highest abundance of 87,000 CFU/ml (Figure 21) was scored by *Penicillum oxalicum* among fungal isolates.

During the post-monsoon period, a greater presence of *Candida* sp. was noticed in the *Anopheles stephensi* larval gut. Different strains of *Candida metapsilopsis* (CBS 10907, CP59) were reported to have a higher abundance of *Candida metapsilosis* (60000 CFU/ml) (Figure 22). The other fungal gut entities were *Aspergillus oryzae* and *Penicillum hispanicum*.

Table1: Biochemical characteristics of bacterial species isolated from *Aedes aegypti* larval gut

Starch hydrolysis	Catalase	Oxidase	Indole production	Methyl red	Voges Proskauer	Citrate	Hydrogen sulphide	Gelatin hydrolysis	Specimen code
−	+	−	+	+	−	−	−	−	BPRAS1A
−	+	−	−	−	+	+	+	−	BPRAS1B
−	+	+	−	−	+	+	−	+	BPRAS1C
−	+	−	−	+	+	+	−	−	BPRAS2A
−	+	+	−	+	+	+	−	−	BPRAS2B
+	+	−	−	−	+	+	−	−	BPRAS2C
−	+	−	−	+	+	+	−	−	BPRAS3A
+	+	−	−	−	+	+	−	−	BPRAS3B
−	+	−	+	+	−	+	−	+	BPRAS3C
−	+	−	−	+	+	+	−	−	BMOAS1A
−	+	−	+	+	−	−	−	−	BMOAS1B
−	+	−	−	−	+	+	−	+	BMOAS2A
−	+	+	−	+	+	+	−	−	BMOAS2B
−	+	−	−	−	+	+	+	+	BMOAS3A
−	+	+	−	−	+	+	−	+	BMOAS3B
−	+	−	−	−	+	+	+	+	BPMAS1A
+	+	−	−	−	+	+	−	−	BPMAS1B
−	+	−	−	−	+	+	+	+	BPMAS2A
−	+	−	−	+	+	+	−	−	BPMAS2B
+	+	−	−	+	−	+	−	−	BPMAS2C
−	−	−	−	−	−	+	−	−	BPMAS3A
+	+	−	−	+	−	+	−	−	BPMAS3B
−	−	−	−	−	−	+	−	−	BPMAS3C
+	+	−	−	+	−	+	−	−	BPMAS3D
−	−	−	−	−	−	−	−	−	BPMAS3E
−	+	−	−	−	−	+	−	+	BPMAS3F

Table 2: Morphological and staining characteristics of bacterial species isolated from *Aedes aegypti* larval gut

Specimen code	Bacteria	Gram staining	Morphology
BPRAS1A	*Escherichia coli*	Gram –ve	Rod shaped
BPRAS1B	*Enterobacter ludwigii* VVS01-S1	Gram –ve	Rod shaped
BPRAS1C	*Pseudomonas aeruginosa*	Gram –ve	Rod shaped
BPRAS2A	*Enterobacter quasihormaechei*	Gram –ve	Rod shaped
BPRAS2B	*Rahnella aquatilis*	Gram –ve	Rod shaped
BPRAS2C	*Bacillus cereus*	Gram+ve	Rod shaped
BPRAS3A	*Enterobacter hormaechei* E26	Gram –ve	Rod shaped
BPRAS3B	*Enterobactercloacae* HNXY160623	Gram –ve	Rod shaped
BPRAS3C	*Serratia oryzae*	Gram -ve	Rod shaped
BMOAS1A	*Enterobacter hormaechei*	Gram –ve	Rod shaped
BMOAS1B	*Escherichia coli*	Gram –ve	Rod shaped
BMOAS2A	*Enterobacter*	Gram –ve	Rod shaped
BMOAS2B	*Rahnella aquatilis*	Gram –ve	Rod shaped
BMOAS3A	*Enterobacter* sp. UASB_FL5	Gram –ve	Rod shaped
BMOAS3B	*Pseudomonas aeruginosa* KOKG	Gram –ve	Rod shaped
BPMAS1A	*Enterobacter* sp. X114	Gram –ve	Rod shaped
BPMAS1B	*Bacillus cereus* AQ-105	Gram+ve	Rod shaped
BPMAS2A	*Enterobacter* sp. KR2	Gram –ve	Rod shaped
BPMAS2B	*Enterobacter kobei* NPKC3	Gram –ve	Rod shaped
BPMAS2C	*Enterobacter asburiae* CK2	Gram –ve	Rod shaped
BPMAS3A	*Enterococcus casselifavus* 902	Gram + ve	Coccus shaped
BPMAS3B	*Enterobacter asburiae* E19	Gram –ve	Rod shaped
BPMAS3C	*Enterococcus casselifavus* D1	Gram+ ve	Coccus shaped
BPMAS3D	*Enterobacter asburiae* CK2_1_40	Gram –ve	Rod shaped
BPMAS3E	*Enterococcus gallinarum* ClaCZ15	Gram+ ve	Coccus shaped
BPMAS3F	*Stenotrophomonas maltophilia* PEG-305	Gram– ve	Rod shaped

Table 3: Biochemical characteristics of bacterial species isolated from *Culex quinquefasciatus* larval gut

Starch hydrolysis	Catalase	Oxidase	Indole production	Methyl Red	Voges Proskauer	Citrate	Hydrogen sulphide	Gelatin hydrolysis	Specimen code
−	+	−	−	+	+	+	−	−	BPRCS1A
−	+	+	−	−	−	+	−	+	BPRCS1B
−	+	−	−	+	+	+	−	−	BPRCS2A
+	+	−	−	−	+	+	−	−	BPRCS2B
−	+	−	+	+	−	_	−	−	BPRCS3A
+	+	−	−	−	+	+	−	−	BPRCS3B
−	+	−	−	+	+	+	−	−	BMOCS1A
−	+	−	+	+	−	_	−	−	BMOCS1B
+	+	−	−	+	−	+	−	−	BMOCS2A
−	+	−	−	+	+	+	−	−	BMOCS2B
−	+	−	+	+	−	+	−	+	BMOCS3A
+	+	−	−	−	+	+	−	−	BMOCS3B
−	+	−	−	−	−	+	−	−	BPMCS1A
−	+	−	−	−	+	+	−	−	BPMCS1B
−	+	−	−	−	−	+	−	−	BPMCS2A
−	+	+	−	−	−	+	−	+	BPMCS2B
−	+	−	−	−	−	+	−	−	BPMCS3A
+	+	−	−	+	−	+	−	−	BPMCS3B
+	+	−	−	−	+	+	−	−	BPMCS3C

Table 4: Morphological and staining characteristics of bacterial species isolated from *Culex quinquefasciatus* larval gut

Specimen code	**Bacteria**	**Gram Staining**	**Morphology**
BPRCS1A	*Rahnella aquatilis* MSSRFQS77	Gram –ve	Rod shaped
BPRCS1B	*Pseudomonas aeruginosa*	Gram –ve	Rod shaped
BPRCS2A	*Rahnella aquatilis* TW4	Gram –ve	Rod shaped
BPRCS2B	*Bacillus cereus*	Gram+ve	Rod shaped
BPRCS3A	*Escherichia coli* XJ141J-125-NF1	Gram –ve	Rod shaped
BPRCS3B	*Enterobacter cloacae* CSBB_493	Gram –ve	Rod shaped
BMOCS1A	*Rahnella aquatilis* MSSRFQS77	Gram –ve	Rod shaped
BMOCS1B	*Escherichia coli* XJ141J-125-NF1	Gram –ve	Rod shaped
BMOCS2A	*Enterobacter asburiae* HAOB_L21	Gram –ve	Rod shaped
BMOCS2B	*Enterobacter hormaechei* EHo5	Gram –ve	Rod shaped
BMOCS3A	*Serratia oryzae* J046	Gram –ve	Rod shaped
BMOCS3B	*Bacillus thuringiensis*	Gram+ve	Rod shaped
BPMCS1A	*Acinetobacter baumannii*	Gram –ve	Cocco bacillus
BPMCS1B	*Pseudomonas aeruginosa*	Gram –ve	Rod shaped
BPMCS2A	*Acinetobacter baumanni*	Gram –ve	Cocco bacillus
BPMCS2B	*Pseudomonas aeruginosa*	Gram –ve	Rod shaped
BPMCS3A	*Acinetobacter baumannii*	Gram –ve	Cocco bacillus
BPMCS3B	*Bacillus toyonensis*	Gram+ve	Rod shaped
BPMCS3C	*Bacillus cereus*	Gram+ve	Rod shaped

Table 5: Biochemical characteristics of bacterial species isolated from *Anopheles stephensi* larval gut

Starch hydrolysis	Catalase	Oxidase	Indole production	Methyl Red	Voges Proskauer	Citrate	Hydrogen sulphide	Gelatin hydrolysis	Specimen code
+	+	–	–	–	+	+	–	–	BPRSS1A
–	+	–	–	–	+	+	–	–	BPRSS1B
–	+	–	–	+	+	+	–	–	BPRSS2A
–	+	–	–	+	+	+	–	–	BPRSS2B
–	+	–	–	–	–	–	–	+	BPRSS3A
–	+	–	–	+	–	+	+	+	BPRSS3B
+	+	–	–	+	–	+	–	+	BMOSS1A
–	+	–	+	+	–	–	–	–	BMOSS1B
+	+	–	–	–	+	+	–	+	BMOSS2A
–	+	+	–	–	+	+	–	+	BMOSS2B
+	+	–	–	+	–	+	+	+	BMOSS3A
–	–	–	–	–	–	–	–	–	BMOSS3B
+	+	–	–	–	+	+	–	–	BPMSS1A
+	+	–	–	–	+	+	–	–	BPMSS1B
+	+	–	–	–	+	+	–	–	BPMSS2A
–	+	–	–	+	+	+	–	–	BPMSS2B
+	+	+	–	–	–	–	–	+	BPMSS3A
–	+	–	–	–	+	+	–	–	BPMSS3B

Table 6: Morphological and staining characteristics of bacterial species isolated from *Anopheles stephensi* larval gut

Specimen code	Bacteria	Gram Staining	Morphology
BPRSS1A	*Bacillus cereus* NA5	Gram+ ve	Rod shaped
BPRSS1B	*Klebsiella pneumoniae* IP2	Gram-ve	Rod shaped
BPRSS2A	*Rahnella aquatilis* TW5	Gram-ve	Rod shaped
BPRSS2B	*Enterobacter hormaechei* 9	Gram-ve	Rod shaped
BPRSS3A	*Brevibacillus brevis* DZBY12	Gram+ ve	Rod shaped
BPRSS3B	*Proteus mirabilis* MGPBL14	Gram-ve	Rod shaped
BMOSS1A	*Priestia aryabhattai* X14	Gram+ ve	Rod shaped
BMOSS1B	*Escherchia coli* TUM13773	Gram-ve	Rod shaped
BMOSS2A	*Serratia marcescens* MICUL	Gram-ve	Rod shaped
BMOSS2B	*Pseudomonas aeroginosa*	Gram-ve	Rod shaped
BMOSS3A	*Priestia megaterium* 240315GAR19S11	Gram+ ve	Rod shaped
BMOSS3B	*Enterococcus gallinarum* 1880	Gram-ve	Coccus shaped
BPMSS1A	*Bacillus thuringiensis* NSK-KAU	Gram+ ve	Rod shaped
BPMSS1B	*Enterobacter cloacae* 07	Gram-ve	Rod shaped
BPMSS2A	*Bacillus cereus* EB61	Gram+ ve	Rod shaped
BPMSS2B	*Enterobacter bugandensis* KPC863	Gram-ve	Rod shaped
BPMSS3A	*Brevibacillus parabrevis* PS12	Gram+ ve	Rod shaped
BPMSS3B	*Enterobacter ludwiggi* GN091	Gram-ve	Rod shaped

Table 7: Details of identified bacterial and fungal species from *Aedes aegypti* larval gut

	Pre-monsoon	**Monsoon**	**Post-monsoon**
Bacteria	1. *Escherchia coli* strain TUM13773 2. *Enterobacter ludwigii* strain VVS01-S1 3. *Pseudomonas aeruginosa* 4. *Enterobacter quasihormaechei* strain WCHEs 5. *Rahnella aquatilis* strain TW4 6. *Bacillus cereus* 7. *Enterobacter hormaechei* strain E26 8. *Enterobacter cloacae* strain HNXY160623 9. *Serratia oryzae* strain J046	10. *Enterobacter hormaechei* strain SH19PTE2 11. *Escherchia coli* 12. *Enterobacter sp.* strain LF3 13. *Rahnella aquatilis* 14. *Enterobacter sp.*strain UASB_FL5 15. *Pseudomonas aeruginosa* strain KOKG	16. *Enterobacter* sp. strain X114 17. *Bacillus cereus* strain AQ-105 18. *Enterobacter* sp.strainKR2 19. *Enterobacter kobei* strain NPKC3 20. *Enterobacter asburiae* strain CK2 21. *Enterococcus casselifavus* strain 902 22. *Enterobacter asburiae* strain E19 23. *Enterococcus casselifavus* strain D1 24. *Enterobacter asburiae* strain CK2_1_40 25. *Enterococcus gallinarum* strain ClaCZ15 26. *Stenotrophomonas maltophilia* strain PEG-305
Fungus	27. *Syncephalastrum sp.* isolate kBR1-1 28. *Penicillium hispanicum* isolate D06 29. *Candida metapsilopsis* strain 19MWFY29.1 30. *Aspergillus tamarii* isolate 18 31. *Penicillium coffeae* strain SCAU032 32. *Debaryomyces hansenii* strain HBLC10 33. *Candida metapsilosis* strain CBS 10907 34. *Aspergillus niger* isolate KL3 35. *Aspergillus chevalieri* strain CBS	36. *Cladosporium tenuissimum* strain SFC20230103-M83 37. *Candida metapsilosis* 38. *Debaryomyces hansenii* isolate HBLC10 39. *Aspergillus niger* strain KL3 40. *Penicillium implicatum* isolate M1 41. *Cladosporium marinum* strain SFC20230103-M34	42. *Curvularia affinis* 43. *Aspergillus foetidus* isolate N1 44. *Simplicillium subtropicum* strain SC55A01 45. *Clavispora lusitaniae* IFM 56973 46. *Aspergillus tubingensis* isolate SACCR 110743 47. *Candida tropicalis* isolate Hb39 48. *Aspergillus chevalieri* strain CBS 522.65 49. *Galactomyces candidus* isolate HNMF055 50. *Penicillium citrinum* isolate ED2 51. *Aspergillus niger*

Table 8: Details of identified bacterial and fungal species from *Culex quinquefasciatus* larval gut

	Pre-monsoon	**Monsoon**	**Post-monsoon**
Bacteria	1. *Rahnella aquatilis* strain MSSRFQS77 2. *Pseudomonas aeruginosa* 3. *Rahnella aquatilis* 4. *Bacillus cereus* strain 17 5. *Escherichia coli* strain XJ141J-125-NF1 6. *Enterobacter cloacae* strain CSBB_493	7. *Rahnella aquatilis* strain H_6_2_1910140577 8. *Escherichia coli* 9. *Enterobacter asburiae* strain HAOB_L21 10. *Enterobacter hormaechei* strain EHo5 11. *Serratia oryzae* strain J046 12. *Bacillus thuringiensis* strain NSK-KAU	13. *Acinetobacter* sp. strain HSTU-ASn72 14. *Bacillus cereus* 15. *Acinetobacter baumannii* strain SL103 16. *Pseudomonas aeruginosa* 17. *Acinetobacter baumannii* strain F46 18. *Bacillus toyonensis* strain VITSN1505 19. *Bacillus cereus* strain AH-43
Fungus	20. *Penicillium coffeae* strain SCAU032 21. *Candida albicans* 22. *Dipodascus albidus* 23. *Aspergillus nomius* strain OKra-A 24. *Penicillium citrinum* isolate ED2 25. *Galactomyces candidus* isolate HNMF055	26. *Geotrichum candidum* isolate AY06 27. *Aspergillus assiutensis* CBS 132773 28. *Penicillium citrinum* isolate ED2 29. *Candida metapsilopsis* 30. *Aspergillus aculeatus* strain YLA2 31. *Candida orthopsilopsis isolate ilia39*	32. *Cunninghamella blakesleena* strain A-11 33. *Penicillium citrinum* strain PeciHA25A01 34. *Candida tropicalis* isolate Hb39 35. *Nigrospora oryzae* strain MPL-S7L2A 36. *Aspergillus foetidus* isolate N1 37. *Candida metapsilosis* strain CBS 10907 38. *Cladosporium cladosporioides* strain DBR-4

Table 9: Details of identified bacterial and fungal species from *Anopheles stephensi* larval gut

	Pre-monsoon	**Monsoon**	**Post-monsoon**
Bacteria	1. *Bacillus cereus* strain NA5 2. *Klebsiella pneumoniae* strain IP2 3. *Rahnella aquatilis* strain TW5 4. *Enterobacter hormaechei* strain 9 5. *Brevibacillus brevis* strain DZBY12 6. *Proteus mirabilis* strain MGPBL14	7. *Priestia aryabhattai* strain X14 8. *Escherichia coli* 9. *Serratia marcescens* strain MICUL 10. *Pseudomonas aeruginosa* 11. *Priestia megaterium* strain 240315GAR19S11 12. *Enterococcus gallinarum* strain 1880	13. *Bacillus thuringiensis* strain NSK-KAU 14. *Enterobacter cloacae* strain 07 15. *Bacillus cereus* strain EB61 16. *Enterobacter bugandensis* strain KPC863 17. *Brevibacillus parabrevis* strain PS12 18. *Enterobacter ludwiggi* strain GN091
Fungus	19. *Aspergillus welwitschiae* strain LayoO1 20. *Penicillium oxalicum* isolate Ting Ho 4 21. *Penicillium rubens* isolate 2010F5 22. *Aspergillus aculeatus* YLA2 23. *Aspergillus oryzae* strain DJL-FUNG03B 24. *Dipodascus albidus*	25. *Candida metapsilosis* isolate CP59 26. *Penicillium oxalicum* isolate Ting Ho 4 27. *Debaryomyces hansenii* isolate HBLC10 28. *Candida albicans* 29. *Penicillium citrinum* strain PeciHA25A01 30. *Candida tropicalis*	31. *Debaryomyces hansenii* 32. *Aspergillus oryzae* strain DJL-FUNG03B 33. *Penicillium hispanicum* isolate D06 34. *Candida metapsilosis* isolate CP59 35. *Penicillium citrinum* strain PeciHA25A01 36. *Candida metapsilosis* strain CBS 10907

Table 10: Systematic list of microbial isolates from *Aedes aegypti* larval gut

Bacteria	Fungus
Phylum : **Pseudomonadota** Class : Gammaproteobacteria Order : Enterobacterales Family : Enterobacteriaceae 1. *Escherichia coli* strain TUM13773 (BPRAS1A, BMOAS1B) 2. *Enterobacter ludwigii* strain VVS01-S (BPRAS1B) 3. *Enterobacter quasihormaechei* strain WCHEs (BPRAS2A) 4. *Enterobacter hormaechei* strain E26 (BPRAS3A) 5. *Enterobacter cloacae* strain HNXY160623 (BPRAS3B) 6. *Enterobacter hormaechei* strain SH19PTE2 (BMOAS1A) 7. *Enterobacter* sp. strain LF3 (BMOAS2A) 8. *Enterobacter* sp. strain UASB_FL5 (BMOAS3A) 9. *Enterobacter* sp. strain X114 (BPMAS1A) 10. *Enterobacter* sp.strain KR2 (BPMAS2A) 11. *Enterobacter* kobei strain NPKC3 (BPMAS2B) 12. *Enterobacter asburiae* strain CK2 (BPMAS2B) 13. *Enterobacter asburiae* strain E19 (BPMAS3C) 14. *Enterobacter asburiae* strain CK2_1_40 (BPMAS3D)	Division : **Ascomycota** Class : Eurotiomycetes Order : Eurotiales Family : Trichocomaceae 1. *Penicillium hispanicum* isolate D06 (FPRAS1B) 2. *Penicillium coffeae* strain SCAU032 (FPRAS2B) 3. *Penicillium implicatum* isolate M1 (FMOAS3A) 4. *Penicillium citrinum* isolate ED2 (FPMAS2C) Division : **Ascomycota** Class : Eurotiomycetes Order : Eurotiales Family : Aspergillaceae 1. *Aspergillus tamarii* isolate 18 (FPRAS2A) 2. *Aspergillus niger* isolate (FPRAS3B,FMOAS2B & FPMAS3A) 3. *Aspergillus foetidus* isolate N1(FPMAS1B) 4. *Aspergillus tubingensis* isolate SACCR 110743 (FPMAS1E) 5. *Aspergillus chevalieri* strain CBS 522.65 (FPMAS2A & FPRAS3C)
Phylum : **Pseudomonadota** Class : Gammaproteobacteria Order : Enterobacterales Family : Yersiniaceae 1. *Rahnella aquatilis* strain TW4 (BPRAS2B) 2. *Rahnella aquatilis* (BMOAS2B)	Division : **Ascomycota** Class : Saccharomycetes Order : Saccharomycetales Family : Debaryomycetaceae 1. *Candida metapsilosis* (FPRAS1C,FPRAS3A & FMOAS1B) 2. *Candida tropicalis* (FPMASIF, FPMAS3B) 3. *Debaryomyces hansenii* (FPRAS2C& FMOAS2A) 4. *Galactomyces candidus* isolate HNMF055 (FPMAS2B) 5. *Clavispora lusitaniae* IFM 56973 (FPMAS1D)
Phylum : **Pseudomonadota** Class : Gammaproteobacteria Order : Enterobacterales Family : Pseudomonadaceae **1.** *Pseudomonas aeruginosa* (BPRAS1C,BMOAS3B)	

Phylum : **Pseudomonadota** Class : Gammaproteobacteria Order : Xanthomonadales Family : Xanthomonadaceae 1. *Stenotrophomonas maltophilia* strain PEG-305 (BPMAS3F)	Division : **Ascomycota** Class : Dothideomycetes Order : Capnodiales Family : Davidiellaceae 1. *Cladosporium marinum* strain SFC20230103-M34 (FMOAS3A) 2. *Cladosporium tenuissimum* strain SFC20230103-M83 (FMOAS1A)
Phylum : **Pseudomonadota** Class : Gammaproteobacteria Order : Enterobacterales Family : Yersiniaceae *1.* *Serratia oryzae* strain J06 (BPRAS3C)	Division : **Ascomycota** Class : Dothideomycetes Order : Pleosporales Family : Pleosporaceae *1.* *Curvularia affinis*(FPMAS1A)
Phylum : **Bacillota** Class : Bacilli Order : Bacillales Family : Bacillaceae 1. *Bacillus cereus* 2. strain AQ-105(BPMAS1B) 3. *Bacillus cereus* 4. strain AH-43(BPRAS2C)	**Division : Ascomycota** Class : Sordarimycetes Order : Hyporeales Family : Cordycipitacea 1. *Simplicillium subtropicum* strain SC55A01(FPMAS1C)
Phylum : **Bacillota** Class : Bacilli Order : Lactobacillales Family : Enterococcaceae 1. *Enterococcus casselifavus* strain 902(BPMAS3A) 2. *Enterococcus casselifavus* strain D1(BPMAS3C) 3. *Enterococcus gallinarum* strain ClaCZ15(BPMAS3E)	Division : **Mucromycota** Class : Mucoromycetes Order : Mucorales Family : Syncephalstraceae 1. *Syncephalastrum* sp. isolate kBR1-1(FPRAS1A)

Table 11: Systematic list of microbial isolates from *Culex quinquefasciatus* larval gut

Bacteria	Fungus
Phylum : **Pseudomonadota** Class : Gammaproteobacteria Order : Enterobacterales Family : Enterobacteriaceae 1. *Escherichia coli* (BPRCS3A & BMOCS1B) 2. *Enterobacter cloacae* 3. strain CSBB_493 (BPRCS3B) 4. *Enterobacter asburiae* strain HAOB_L21 (BMOCS2A) 5. *Enterobacter hormaechei* strain EHo5 (BMOCS2B)	Division : **Ascomycota** Class : Eurotiomycetes Order : Eurotiales Family : Trichocomaceae 1. *Penicillium coffeae* strain SCAU032 (FPRCS1A) 2. *Penicillium citrinum* isolate ED2 (FPRCS3A & FMOCS2A) 3. *Penicillium citrinum* strain PeciHA25A01 (FPMCS1B)
Phylum : **Pseudomonadota** Class : Gammaproteobacteria Order : Moraxellales Family : Moraxellaceae 1. *Acinetobacter* sp. strain HSTU-ASn72 (BPMCS1A) 2. *Acinetobacter baumannii* strain SL103 (BPMCS2A) 3. *Acinetobacter baumannii* strain F46 (BPMCS3A)	Division : **Ascomycota** Class : Eurotiomycetes Order : Eurotiales Family : Aspergillaceae 1. *Aspergillus nomius* strain OKra-A (FPRCS2B) 2. *Aspergillus assiutensis* strain CBS 132773 (FMOCS1B) 3. *Aspergillus aculeatus* strainYLA2 (FMOCS3A) 4. *Aspergillus foetidus* isolate N1 (FPMCS2A & FPMCS3B)
Phylum : **Pseudomonadota** Class : Gammaproteobacteria Order : Enterobacterales Family : Yersiniaceae 1. *Rahnella aquatilis* strain MSSRFQS77 (BPRCS1A) 2. *Rahnella aquatilis* strain TW4 (BPRCS2A) 3. *Rahnella aquatilis* strain_H_6_2_1910140577(BMOCS1A) 4. *Serratia oryzae* strain J046 (BMOCS3A)	Division : **Ascomycota** Class : Saccharomycetes Order : Saccharomycetales Family : Debaryomycetaceae 1. *Candida albicans* (FPRCS1B) 2. *Dipodascus albidus* (FPRCS2A) 3. *Geotrichum candidum* isolate AY06 (FMOCS1A) 4. *Candida tropicalis* isolate Hb39 (FPMCS1C) 5. *Candida metapsilosis* strain CBS 10907(FPMCS2B & FMOCS2B) 6. *Galactomyces candidus* isolate HNMF055(FPRCS3B) **7.** *Candida orthopsilopsis* isolate ilia39 (FMOCS3B)
Phylum : **Pseudomonadota** Class : Gammaproteobacteria Order : Enterobacterales Family : Pseudomonadaceae 1. *Pseudomonas aeruginosa* (BPRCS1B & BPMCS2B)	

Phylum : **Bacillota** Class : Bacilli Order : Bacillales Family : Bacillaceae 2. *Bacillus cereus* (BPRCS2B & BPMCS1B) 3. *Bacillus toyonensis* strain VITSN1505(BPMCS3B) 4. *Bacillus cereus* strain AH-43(BPMCS3C) 5. *Bacillus thuringiensis* strain NSK-KAU(BMOCS3B)	Division : **Ascomycota** Class : Dothideomycetes Order : Capnodiales Family : Davidiellaceae 1. *Cladosporium cladosporioides* strain DBR-4(FPMCS3A) Division : **Ascomycota** Class : Sordariomycetes Order : Trichosphaeriales Family : Trichosphaeriaceae 1. *Nigrospora oryzae* strain MPL-S7L2A (FPMCS1D) Division : **Mucoromycota** Class : Mucoromycetes Order : Mucorales Family : Cunninghamellaceae 1. *Cunninghamella blakesleena* strain A-11 (FPMCS1A)

Table 12: Systematic list of microbial isolates from *Anopheles stephensi* larva gut

Bacteria	Fungus
Phylum : **Bacillota** Class : Bacilli Order : Bacillales Family : Bacillaceae 1. *Bacillus cereus* strain NA5(BPRSS1A) 2. *Bacillus thuringiensis* strain NSK-KAU (BPMSS1A) 3. *Bacillus cereus* strain EB61(BPMSS2A)	Division : **Ascomycota** Class : Eurotiomycetes Order : Eurotiales Family : Trichocomaceae 1. *Penicillium oxalicum* isolate Ting Ho 4 (FPRSS1B & FMOSS1B) 2. *Penicillium rubens* isolate 2010F5(FPRSS2A) 3. *Penicillium citrinum* strain PeciHA25A01(FMOSS2A & FMOSS3A) 4. *Penicillium hispanicum* isolate D06 (FPMSS2A)
Phylum : **Bacillota** Class : Bacilli Order : Lactobacillales Family : Enterococcaceae 1. *Enterococcus gallinarum* strain 1880 (BMOSS3B)	Division : **Ascomycota** Class : Eurotiomycetes Order : Eurotiales Family : Aspergillaceae 1. *Aspergillus welwitschiae* strain LayoO1(FPRSS1A) 2. *Aspergillus aculeatus* strain YLA2 (FPRSS2B) 3. *Aspergillus oryzae* strain DJL-FUNG03B (FPRSS3A & FPMSS1B)
Phylum : **Bacillota** Class : Bacilli Order : Bacillales Family : Paenibacillaceae 1. *Brevibacillus brevis* strain DZBY12 (BPRSS3A) 2. *Brevibacillus parabrevis* strain PS12 (BPMSS3A)	**Division** : **Ascomycota** Class : Saccharomycetes Order : Saccharomycetales Family : Debaryomycetaceae 1. *Dipodascus albidus* (FPRSS3B) 2. *Debaryomyces hansenii* isolate HBLC10 (FPMSS1A & FMOSS2A) 3. *Candida metapsilosis* isolate CP59 (FMOSS1A & FPMSS2B) 4. *Candida metapsilosis* strain CBS 10907 (FPMSS3B) 5. *Candida tropicalis* (FMOSS3B) 6. *Candida albicans* (FMOSS2B)
Phylum : **Bacillota** Class : Bacilli Order : Bacillales Family : Bacillaceae 1. *Priestia megaterium* strain 240315GAR19S11(BMOSS3A) 2. *Priestia aryabhattai* strain X14 (BMOSS1A)	

Phylum : **Pseudomonadota** Class : Gammaproteobacteria Order : Enterobacterales Family : Enterobacteriaceae 1. *Enterobacter hormaechei* strain 9 (BPRSS2B) 2. *Enterobacter cloacae* strain 07 (BPMSS1B) 3. *Enterobacter bugandensis* strain KPC863 (BPMSS2B) 4. *Escherichia coli* (BMOSS1B) Phylum : **Pseudomonadota** Class : Gammaproteobacteria Order : Enterobacterales Family : Yersiniaceae 1. *Rahnella aquatilis* strain TW5 (BPRSS2A) Phylum : **Pseudomonadota** Class : Gammaproteobacteria Order : Enterobacterales Family : Enterobacteriaceae 2. *Klebsiella pneumoniae* strain IP2(BPRSS1B) 3. *Klebsiella pneumoniae* strain CSMCRI-22 (BPMSS3B) 4. *Proteus mirabilis* strain MGPBL14 (BPRSS3B) Phylum : **Pseudomonadota** Class : Gammaproteobacteria Order : Enterobacterales Family : Yersiniaceae 1. *Serratia marcescens* strain MICUL (BMOSS2A)	

Table 13: Summarized details of microbiota obtained from the larval gut of *Aedes aegypti*

Bacteria						**Fungus**					
Specimen code	**Microbial species**	**Strain**	**Accession Number**	**CFU/ ml**	**Mode to calculate indices**	**Specimen code**	**Microbial species**	**Strain**	**Accession Number**	**CFU/ ml**	**Mode to calculate indices**
BPRAS1A	*Escherichia coli*	TUM13773	OR361757	2900000	2900000	FPRAS1A	*Syncephalastrum* sp	kBR1-1	OR352253	33300	33300
BPRAS1B	*Enterobacter ludwigii*	VVS01-S1	OR053799	1400000	1400000	FPRAS1B	*Penicillium hispanicum*	D06	OR352208	20000	20000
BPRAS1C	*Pseudomonas aeruginosa*	KOKG	OR053798	1800000	1800000	FPRAS1C	*Candida metapsilopsis*	19MWFY29.1	OR352309	53300	53300
BPRAS2A	*Enterobacter quasihormaechei*	WCHEs	OR053825	2200000	2200000	FPRAS2A	*Aspergillus tamarii*	18	OP752702	43000	43000
BPRAS2B	*Rahnella aquatilis*	TW4	OR083429	830000	830000	FPRAS2B	*Penicillium coffeae*	SCAU032	OR361746	24000	24000
BPRAS2C	*Bacillus cereus*	AH-43	ON514441	1200000	1200000	FPRAS2C	*Debaryomyces hansenii*	HBLC10	OR352207	40000	40000
BPRAS3A	*Enterobacter hormaechei*	E26	OM534659	1800000	1800000	FPRAS3A	*Candida metapsilosis*	CBS 10907	OR352206	66666	66666
BPRAS3B	*Enterobacter cloacae*	HNXY160623	OR077103	2500000	2500000	FPRAS3B	*Aspergillus niger*	KL3	OR361748	36667	36667
BPRAS3C	*Serratia oryzae*	J046	OR083434	1570000	1570000	FPRAS3C	*Aspergillus chevalieri*	CBS522.65	OR352306	23000	23000
BMOAS1A	*Enterobacter hormaechei*	SH19PTE2	OR053795	1630000	1630000	FMOAS1A	*Cladosporium tenuissimum*	SFC20230103-M83	OR352368	20000	20000
BMOAS1B	*Escherichia coli*		OR361757	3100000	3100000	FMOAS1B	*Candida metapsilopsis*		OR352309	60000	60000
BMOAS2A	*Enterobacter* sp.	LF3	OR053796	1100000	1100000	FMOAS2A	*Debaryomyces hansenii*		OR352207	30000	30000

BMOAS2B	*Rahnella aquatilis*		OR067094	1900000	1900000	FMOAS2B	*Aspergillus niger*		OR361748	34000	34000
BMOAS3A	*Enterobacter* sp.	UASB_FL5	OR053797	1330000	1330000	FMOAS3A	*Penicillium implicatum*	M1	OR352251	24000	24000
BMOAS3B	*Pseudomonas aeruginosa*		OR053798	700000	700000	FMOAS3B	*Cladosporium marinum*	SFC20230103-M34	OR352301	30000	30000
BPMAS1A	*Enterobacter* sp.	X114	OM534655	1170000	1170000	FPMAS1A	*Curvularia affinis*	88F	OR352304	20000	20000
BPMAS1B	*Bacillus cereus*	AQ-105	OM534657	1700000	1700000	FPMAS1B	*Aspergillus foetidus*	N1	OR352254	27000	27000
BPMAS2A	*Enterobacter* sp.	KR2	OR053724	163000	163000	FPMAS1C	*Simplicillium subtropicum*	SC55A01	OR396889	17000	17000
BPMAS2B	*Enterobacter kobei*	NPKC3	OM434410	150000	150000	FPMAS1D	*Clavispora lusitaniae*	IFM56973	OR352379	10000	10000
BPMAS2C	*Enterobacter asburiae*	CK2	OM436004	210000	210000	FPMAS1E	*Aspergillus tubingensis*	SACCR 110743	OP578190	20000	20000
BPMAS3A	*Enterococcus casselifavus*	902	OM348508	10700000	10700000	FPMAS1F	*Candida tropicalis*	Hb39	OP579181	26600	26600
BPMAS3B	*Enterobacter asburiae*	E19	OM534644	13000000	13000000	FPMAS2A	*Aspergillus chevalieri*		OR352306	40000	40000
BPMAS3C	*Enterococcus casselifavus*	D1	OM534646	6600000	6600000	FPMAS2B	*Galactomyces candidus*	HNMF055	LR983935	53400	53400
BPMAS3D	*Enterobacter asburiae*	CK2_1_40	OM534648	10000000	10000000	FPMAS2C	*Penicillium citrinum*	ED2	OR352163	17000	17000
BPMAS3E	*Enterococcus gallinarum*	ClaCZ15	OM534649	13700000	13700000	FPMAS3A	*Aspergillus niger*	KL3	OR361748	43000	43000
BPMAS3F	*Stenotrophomonas maltophilia*	PEG-305	OR053793	21000000	21000000	FPMAS3B	*Candida tropicalis*		OP579181	10000	10000

Table14: Summarized details of microbiota obtained from the larval gut of *Culex quinquefasciatus*

Bacteria						**Fungus**					
Specimen code	**Microbial species**	**Strain**	**Accession Number**	**CFU/ ml**	**Mode to calculate indices**	**Specimen code**	**Microbial species**	**Strain**	**Accession Number**	**CFU/ ml**	**Mode to calculate indices**
BPRCS1A	*Rahnella aquatilis*	MSSRFQS77	OR083430	146000	146000	FPRCS1A	*Penicillium coffeae*		OR361746	43000	43000
BPRCS1B	*Pseudomonas aeruginosa*		OR053798	200000	200000	FPRCS1B	*Candida albicans*		NR125332	50000	50000
BPRCS2A	*Rahnella aquatilis*		OR083429	110000	110000	FPRCS2A	*Dipodascus albidus*		OR352378	30000	30000
BPRCS2B	*Bacillus cereus*	17	ON514443	170000	170000	FPRCS2B	*Aspergillus nomius*	Okra-A	MF510822	60000	60000
BPRCS3A	*Escherichia coli*	XJ141J-125-NF1	OR083588	280000	280000	FPRCS3A	*Penicillium citrinum*		OR352163	10300	10300
BPRCS3B	*Enterobacter cloacae*	CSBB_493	ON514444	110000	110000	FPRCS3B	*Galactomyces candidus*		LR983935	20000	20000
BMOCS1A	*Rahnella aquatilis*	_H_6_2_1910140577	OR083435	150000	150000	FMOCS1A	*Geotrichum candidum*	AY06	OR361747	60000	60000
BMOCS1B	*Escherichia coli*	XJ141J-125-NF1	OR083588	210000	210000	FMOCS1B	*Aspergillus assiutensis*	CBS132773	OR352311	30000	30000
BMOCS2A	*Enterobacter asburiae*	HAOB_L21	OR083586	120000	120000	FMOCS2A	*Penicillium citrinum*		OR352163	70000	70000
BMOCS2B	*Enterobacter hormaechei*	EHo5	OR083432	120000	120000	FMOCS2B	*Candida metapsilopsis*		OR352309	60000	60000
BMOCS3A	*Serratia oryzae*		OR083434	190000	190000	FMOCS3A	*Aspergillus aculeatus*	YLA2	OR352362	40000	40000
BMOCS3B	*Bacillus thuringiensis*	NSK-KAU	ON427760	150000	150000	FMOCS3B	*Candida orthopsilopsis*	ilia39	OP753092	50000	50000

BPMCS1A	*Acinetobacter sp.*	HSTU-ASn72	ON514442	60000	60000	FPMCS1A	*Cunninghamella blakesleena*	A-11	OR372495	40000	40000
BPMCS1B	*Bacillus cereus*	17	ON514443	130000	130000	FPMCS1B	*Penicillium citrinum*	PeciHA25A01	OR352367	30000	30000
BPMCS2A	*Acinetobacter baumannii*	SL103	ON428668	230000	230000	FPMCS1C	*Candida tropicalis*	Hb39	OP579181	50000	50000
BPMCS2B	*Pseudomonas aeruginosa*		OR053798	90000	90000	FPMCS1D	*Nigrospora oryzae*	MPL-S7L2A	OR352365	20000	20000
BPMCS3A	*Acinetobacter baumannii*	F46	ON514404	110000	110000	FPMCS2A	*Aspergillus foetidus*		OR352254	30000	30000
BPMCS3B	*Bacillus toyonensis*	VITSN1505	ON514440	153000	153000	FPMCS2B	*Candida metapsilosis*	CBS 10907	OR352206	40000	40000
BPMCS3C	*Bacillus cereus*		ON514441	210000	210000	FPMCS3A	*Cladosporium cladosporioides*	DBR-4	OR352369	23000	23000
						FPMCS3B	*Aspergillus foetidus*		OR352254	40000	40000

Table 15 : Summarized details of microbioata obtained from the larval gut of *Anopheles stephensi*

Bacteria						Fungus					
Specimen Code	**Microbial species**	**Strain**	**Accession Number**	**CFU/ ml**	**Mode to calculate indices**	**Specimen Code**	**Microbial species**	**Strain**	**Accession Number**	**CFU/ ml**	**Mode to calculate indices**
BPRSS1A	*Bacillus cereus*	NA5	ON427829	230000	230000	FPRSS1A	*Aspergillus welwitschiae*	LayoO1	OR352363	30000	30000
BPRSS1B	*Klebsiella pneumoniae*	IP2	OR084103	310000	310000	FPRSS1B	*Penicillium oxalicum*	Ting Ho 4	OR352364	17000	17000
BPRSS2A	*Rahnella aquatilis*	TW5	OR084096	90000	90000	FPRSS2A	*Penicillium rubens*	2010F5	OP579174	30000	30000
BPRSS2B	*Enterobacter hormaechei*	9	OR084095	170000	170000	FPRSS2B	*Aspergillus aculeatus*	YLA2	OR352362	27000	27000
BPRSS3A	*Brevibacillus brevis*	DZBY12	OR083652	70000	70000	FPRSS3A	*Aspergillus oryzae*	DJL-FUNG03B	OR352361	40000	40000
BPRSS3B	*Proteus mirabilis*	MGPBL14	OR083653	130000	130000	FPRSS3B	*Dipodascus albidus*		OR352378	43000	43000
BMOSS1A	*Priestia aryabhattai*	X14	ON453889	143000	143000	FMOSS1A	*Candida metapsilosis*	CP59	OP753139	53000	53000
BMOSS1B	*Escherichia coli*		OR361757	300000	300000	FMOSS1B	*Penicillium oxalicum*		OR352364	87000	87000
BMOSS2A	*Serratia marcescens*	MICUL	OR083651	170000	170000	FMOSS2A	*Debaryomyces hansenii*	HBLC10	OR352207	77000	77000
BMOSS3B	*Pseudomonas aeroginosa*		OR053798	200000	200000	FMOSS2B	*Candida albicans*		NR125332	40000	40000
BMOSS3A	*Priestia megaterium*	240315GAR19 S11	ON427474	150000	150000	FMOSS3A	*Penicillium citrinum*	PeciHA25A01	OR352367	40000	40000
BMOSS3B	*Enterococcus gallinarum*	1880	ON427712	110000	110000	FMOSS3B	*Candida tropicalis*	Hb39	OP579181	70000	70000
BPMSS1A	*Bacillus thuringiensis*	NSK-KAU	ON427760	330000	330000	FPMSS1A	*Debaryomyces hansenii*	HBLC10	OR352207	56000	56000
BPMSS1B	*Enterobacter cloacae*	07	ON427819	130000	130000	FPMSS1B	*Aspergillus oryzae*	DJL-FUNG03B	OR352361	30000	30000
BPMSS2A	*Bacillus cereus*	EB61	OM838310	210000	210000	FPMSS2A	*Penicillium hispanicum*	D06	OR352208	20000	20000
BPMSS2B	*Enterobacter bugandensis*	KPC863	ON402851	80000	80000	FPMSS2B	*Candida metapsilosis*	CP59	OP753139	53000	53000
BPMSS3A	*Brevibacillus parabrevis*	PS12	OR083650	130000	130000	FPMSS3A	*Penicillium citrinum*		OR352367	50000	50000
BPMSS3B	*Enterobacter ludwiggi*	GN091	OR362736	220000	220000	FPMSS3B	*Candida metapsilosis*	CBS 10907	OR352206	60000	60000

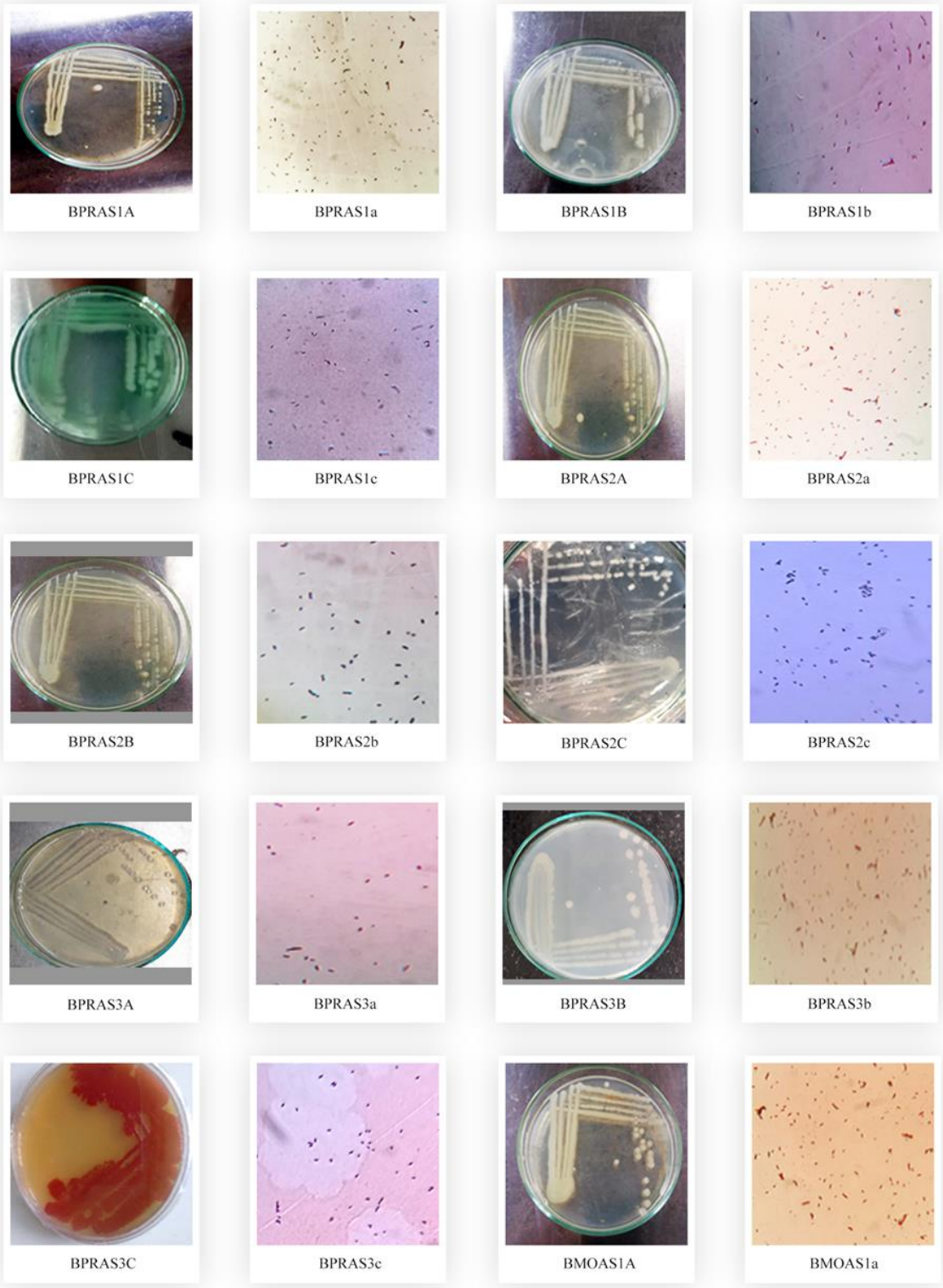

Figure 23. Culture plates and stained image of bacteria isolated from *Aedes aegypti* larval gut during pre-monsoon, monsoon, and post-monsoon

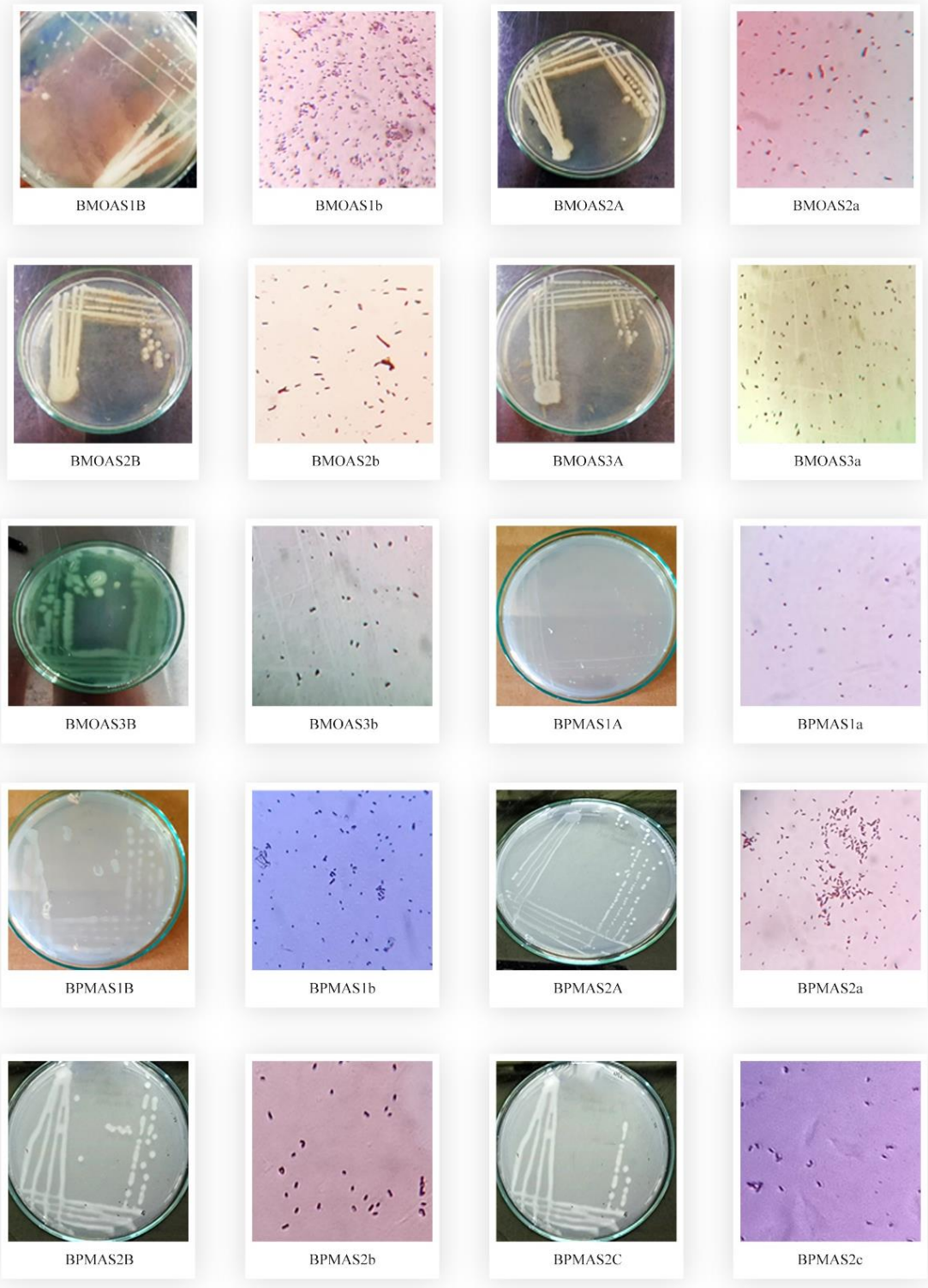

Figure 24. Culture plates and stained image of bacteria isolated from *Aedes aegypti* larval gut during pre-monsoon, monsoon, and post-monsoon

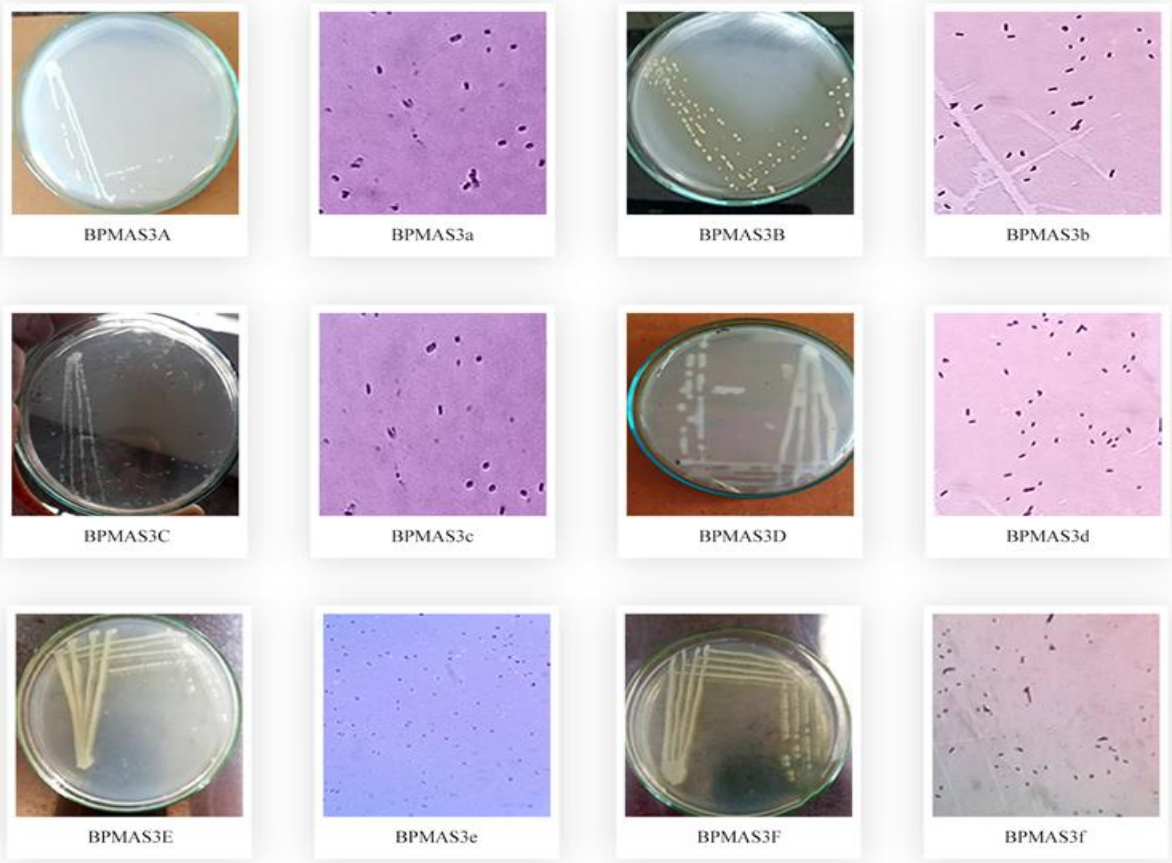

Figure 25. Culture plates and stained image of bacteria isolated from *Aedes aegypti* larval gut during pre-monsoon, monsoon, and post-monsoon

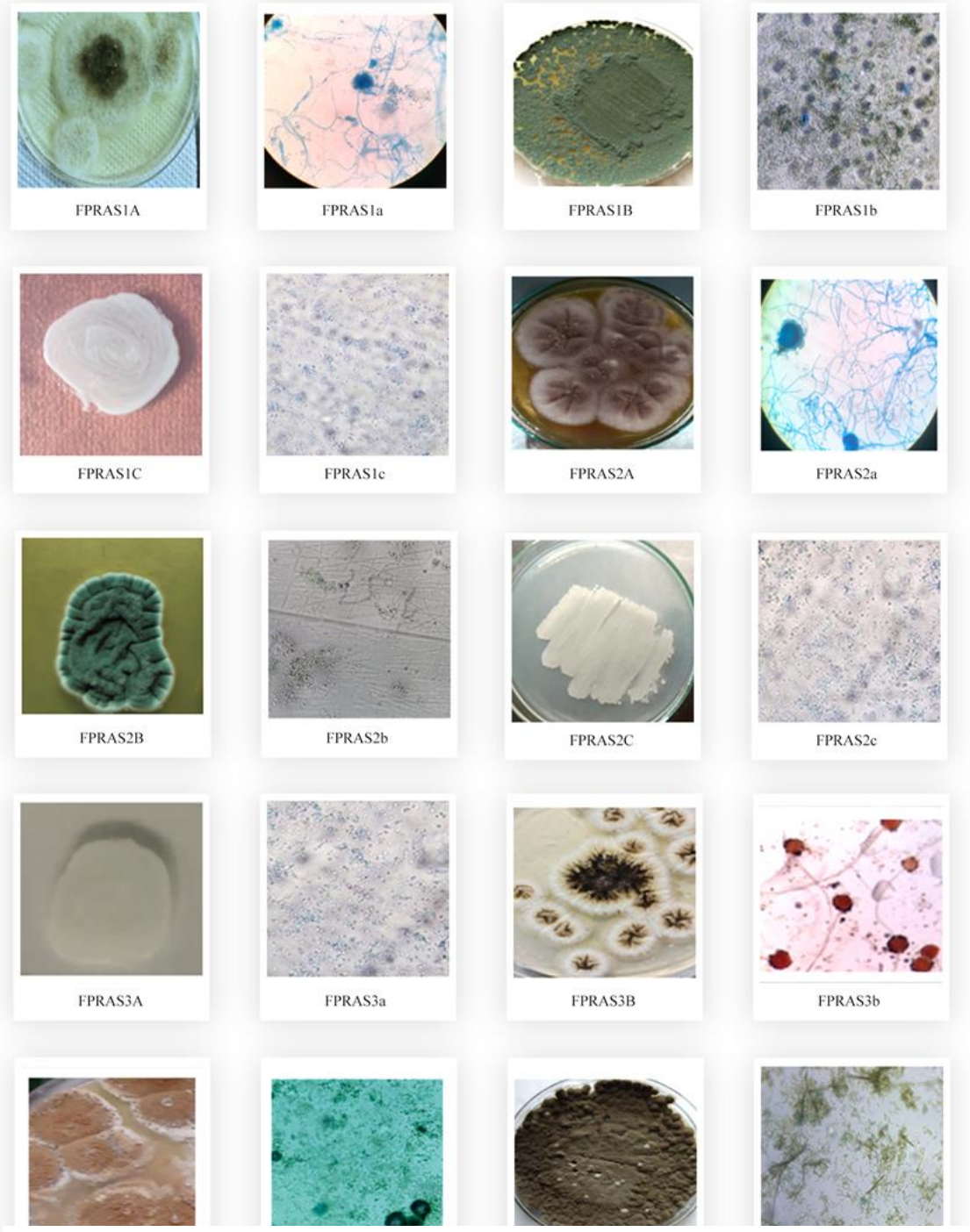

Figure 26. Culture plates and stained image of fungi isolated from *Aedes aegypti* larval gut during pre-monsoon, monsoon, and post-monsoon

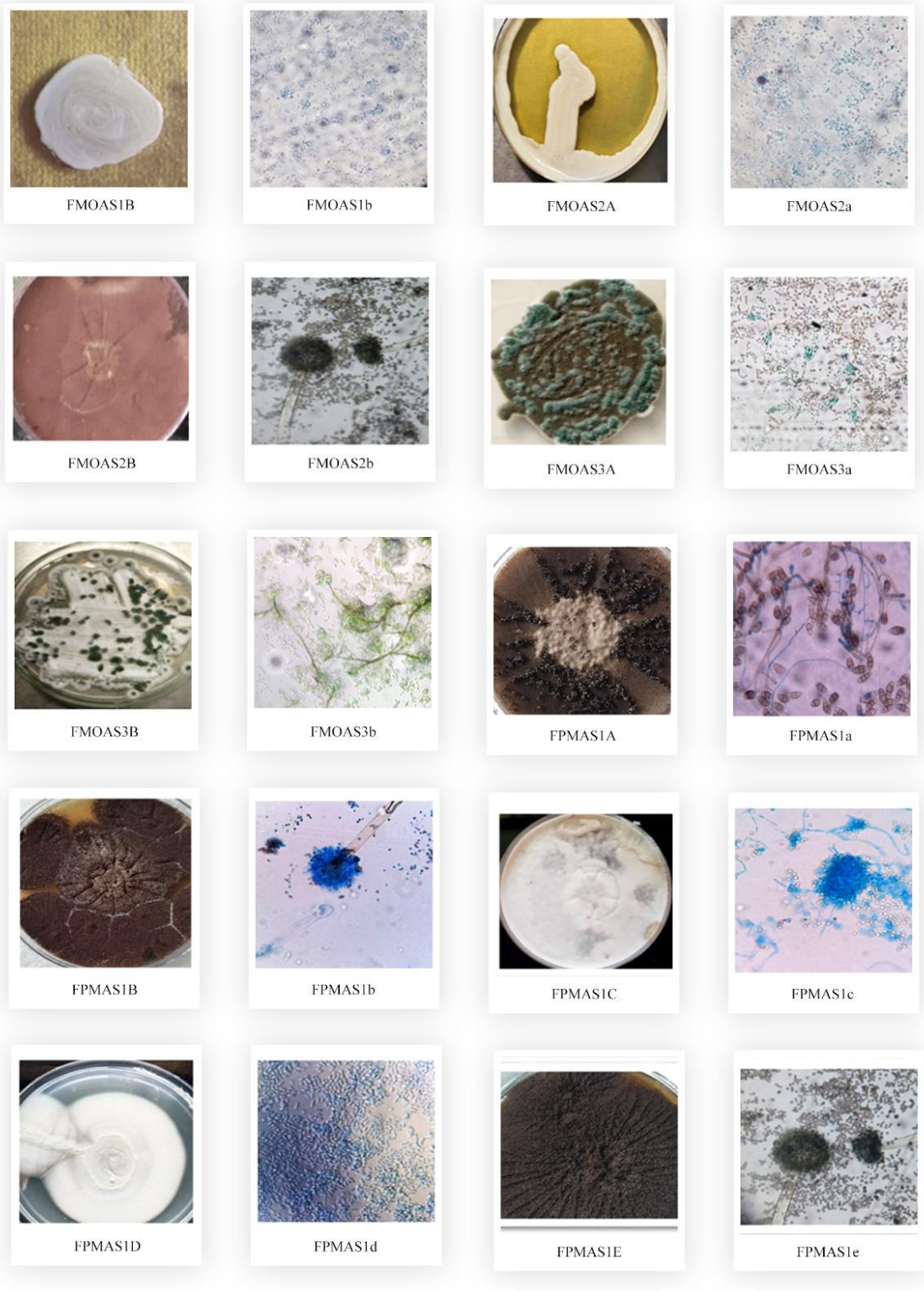

Figure 27. Culture plates and stained image of fungi isolated from *Aedes aegypti* larval gut during pre-monsoon, monsoon, and post-monsoon

Figure 28. Culture plates and stained image of fungi isolated from *Aedes aegypti* larval gut during pre-monsoon, monsoon, and post-monsoon

Figure 29. Culture plates and stained image of bacteria isolated from *Culex quinquefasciatus* during pre-monsoon, monsoon, and post-monsoon

Figure 30. Culture plates and stained image of bacteria isolated from *Culex quinquefasciatus* larval gut during pre-monsoon, monsoon, and post-monsoon

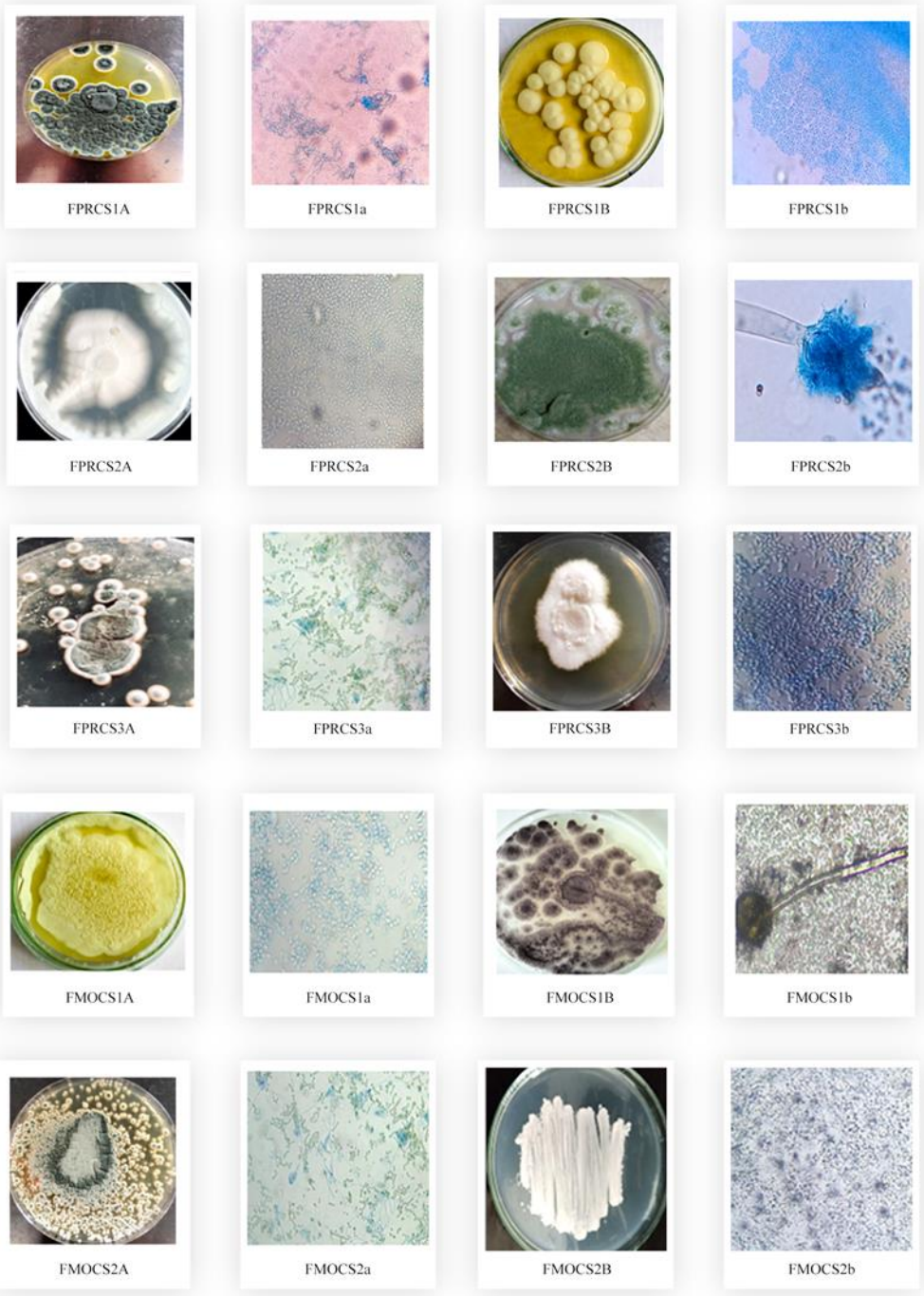

Figure 31. Culture plates and stained image of fungi isolated from *Culex quinquefasciatus* larval gut during pre-monsoon, monsoon, and post-monsoon

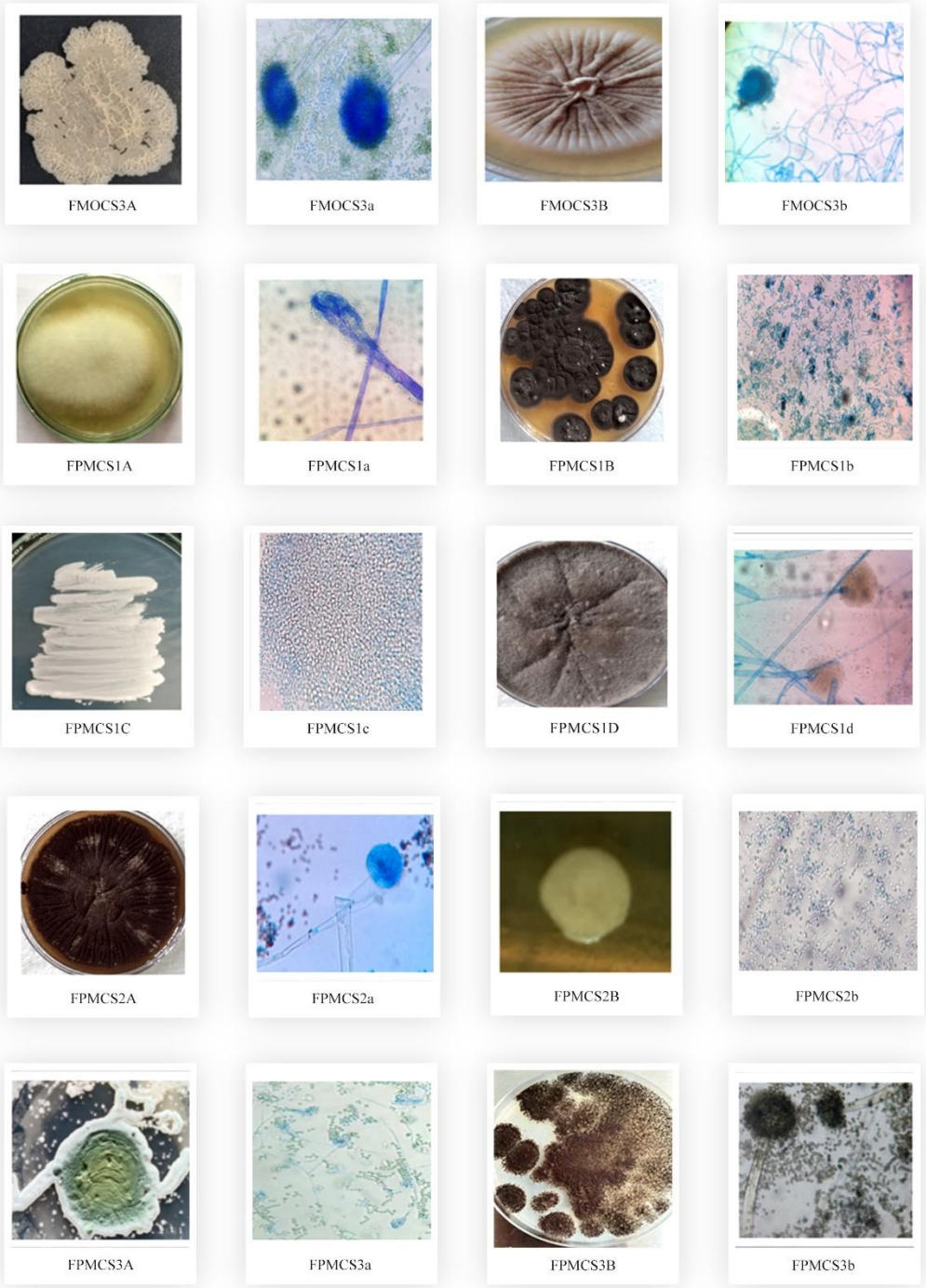

Figure 32. Culture plates and stained image of fungi isolated from *Culex quinquefasciatus* larval gut during pre-monsoon, monsoon, and post-monsoon

Figure 33. Culture plates and stained image of bacteria isolated from *Anopheles stephensi* larval gut during pre-monsoon, monsoon, and post-monsoon

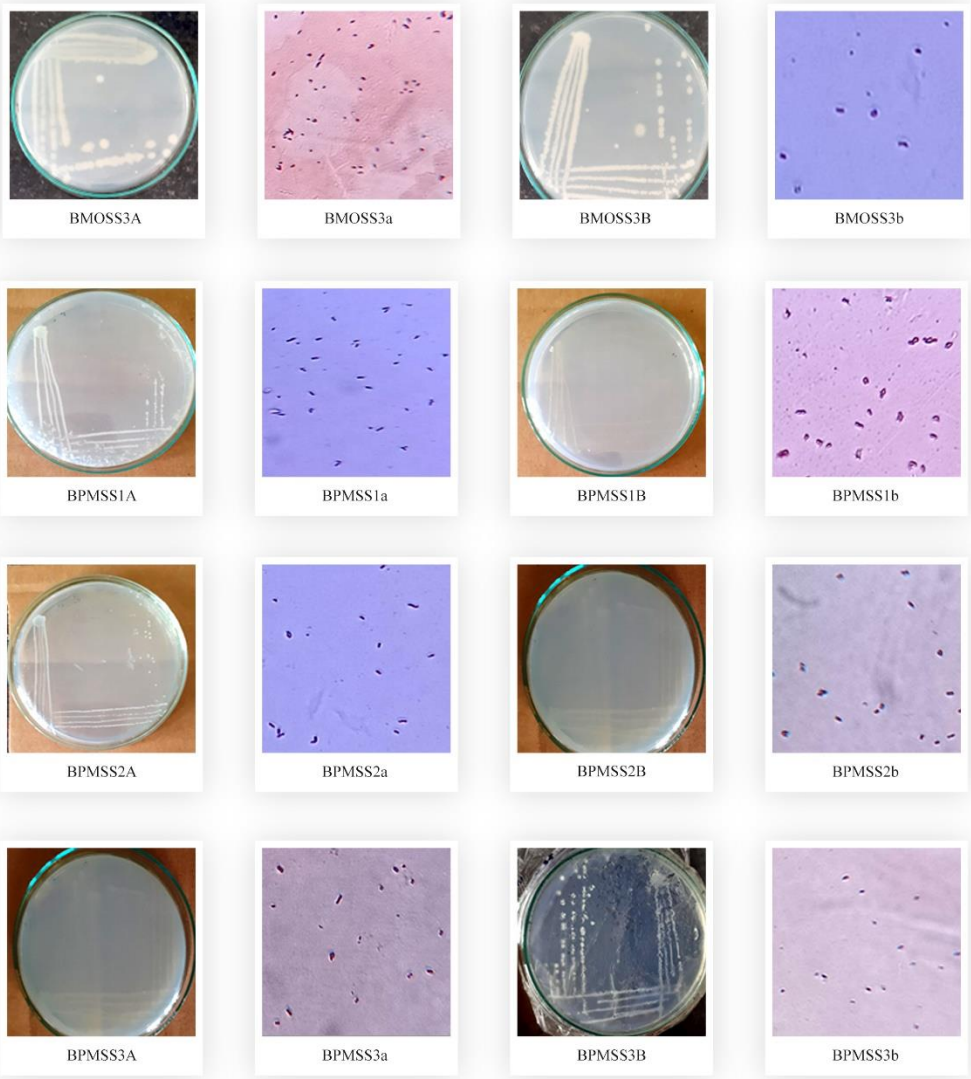

Figure 34. Culture plates and stained image of bacteria isolated from *Anopheles stephensi* larval gut during pre-monsoon, monsoon, and post-monsoon

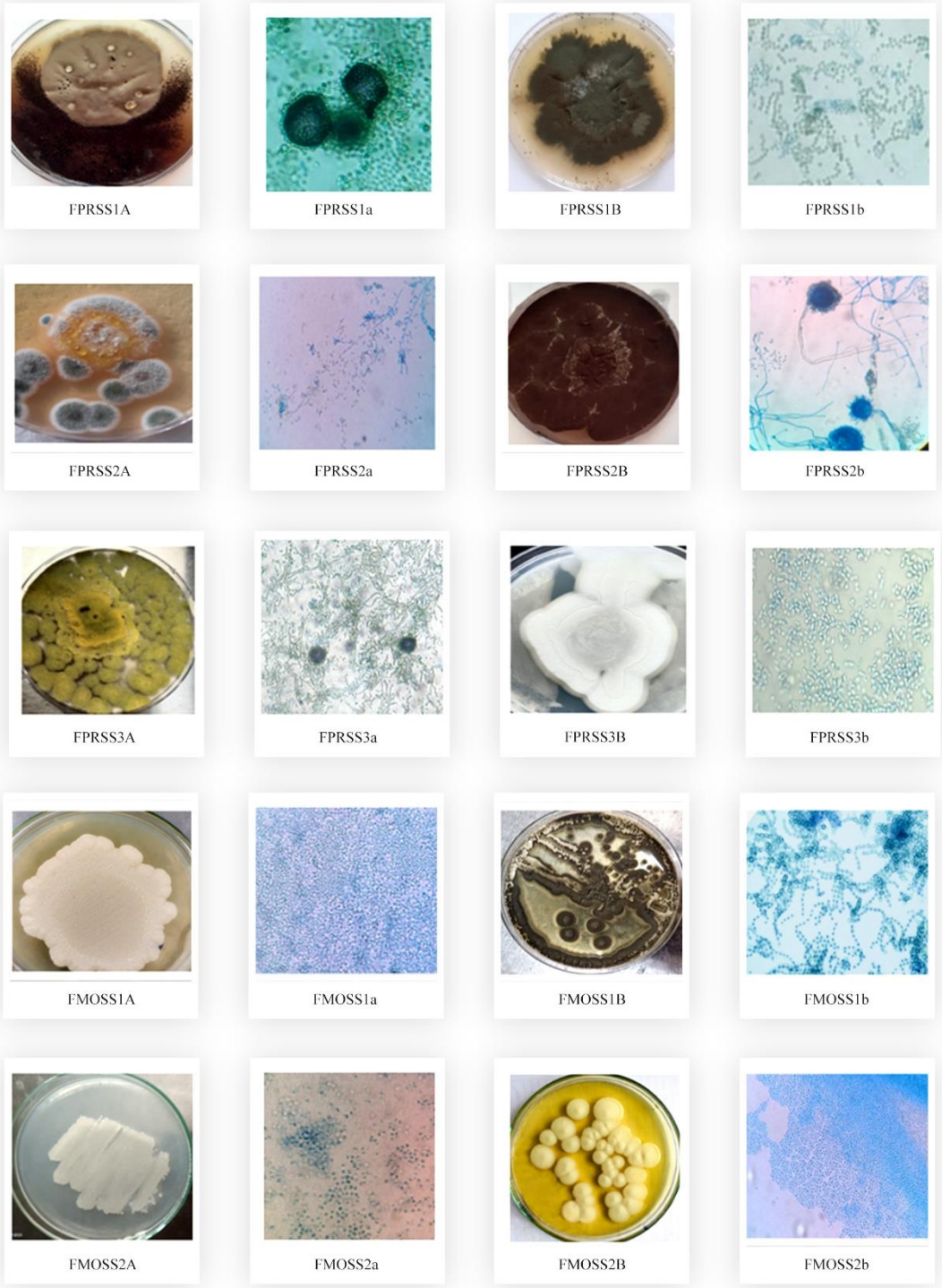

Figure 35. Culture plates and stained image of fungi isolated from *Anopheles stephensi* larval gut during pre-monsoon, monsoon, and post-monsoon

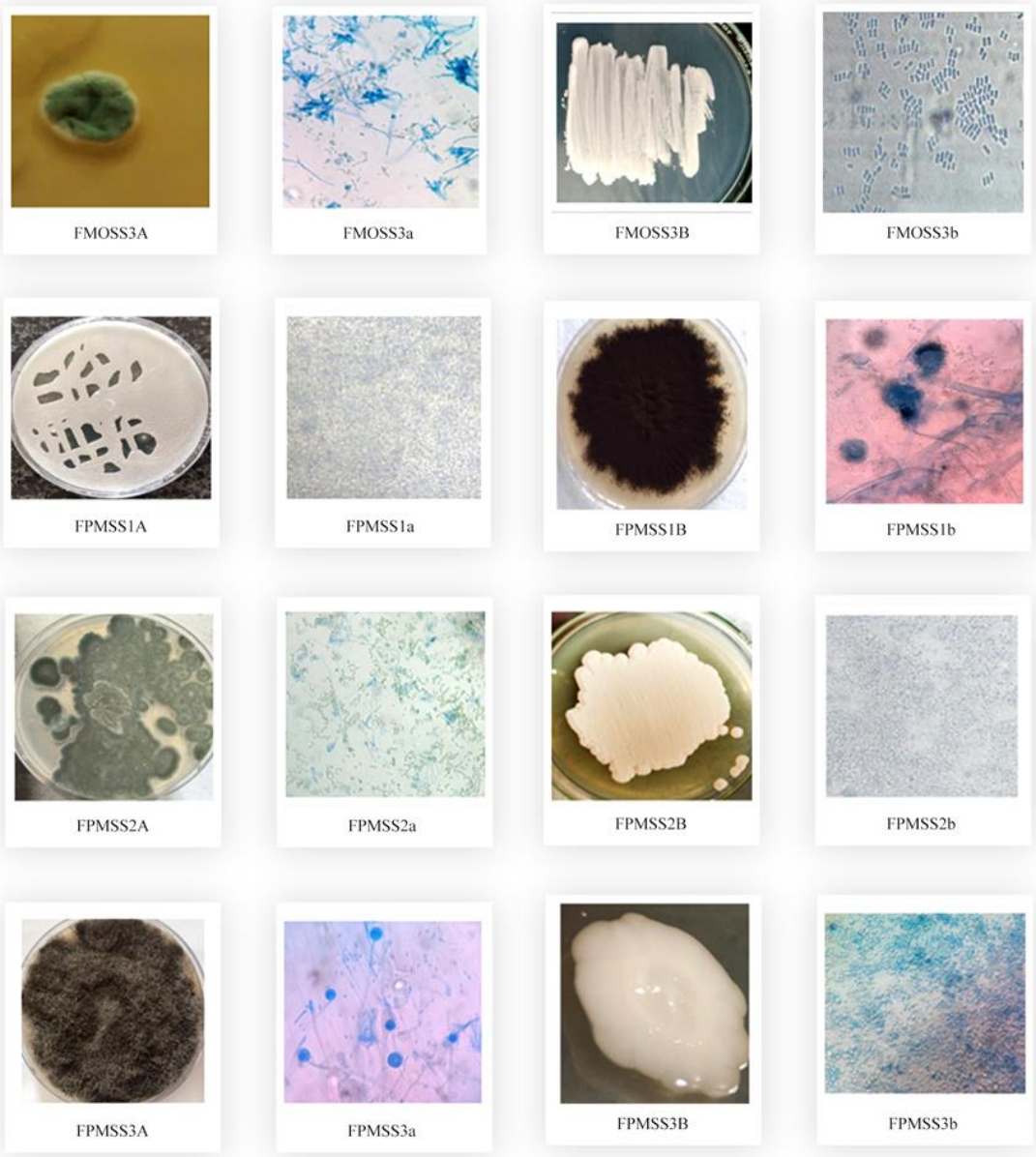

Figure 36. Culture plates and stained image of fungi isolated from *Anopheles stephensi* larval gut during pre-monsoon, monsoon, and post-monsoon

DIVERSITY INDICES OF BACTERIAL AND FUNGAL ISOLATES FROM MOSQUITO LARVAE DURING DIFFERENT SEASONS

In the present study, alpha and beta diversity indices were used to quantify microbial diversity. To estimate dominance, species richness, abundance, and eveness during different seasons, the Simpson diversity index, Shannon-Weiner index, and Shannon-Wiener eveness index were used as part of alpha diversity indices. On the other hand, the difference in species composition between seasons was measured using the Bray Curtis dissimilarity index (Beta diversity).

In *Aedes aegypti*, the value of the Simpson diversity index was observed to range between 0.80-0.88 for bacteria and 0.81-0.87 for fungi across the seasons. i.e., during the premonsoon it was 0.88 for bacteria and 0.87 for fungi; during the monsoon it was 0.80 for bacteria and 0.81 for fungi; and during the postmonsoon the Simpson index was 0.83 for bacteria and 0.87 for fungi. When taking *Culex quinquefasciatus* into account, the Simpson index for fungus was found to be 0.79, 0.82, and 0.85 for pre-monsoon, monsoon, and post-monsoon, while for bacteria it was found to be (pre-monsoon: 0.81; monsoon: 0.83; and post-monsoon: 0.83). For *Anopheles stephensi*, the pre-monsoon and post-monsoon Simpson diversity indices are 0.79, 0.81, and 0.80 for bacteria, respectively. A value of 0.82 was recorded during the pre-monsoon, monsoon, and post-monsoon.

In the case of *Aedes aegypti,* a higher value for the Simpson index was reported during the pre-monsoon, considering both gut entities under study. For *Culex quinquefasciatus,* a greater value for the Simpson diversity index was recorded

during the post-monsoon. While for *Anopheles stephensi,* the monsoon season scored greater value for both bacteria and fungi. In all cases, a value falling approximately

between 0.80 and 0.88 indicated a moderate to high level of diversity (Table 16). As observed, seasonality may have an impact on the diversity of microbiota found in various mosquito species. While taking into account of specific mosquito larval species, the observed diversity is more or less consistent across seasons, indicating species-specific preferences or differences in microbial communities.

The high fluctuation of Shannon Weaver was observed in the case of *Aedes aegypti* and *Culex quinquefasciatus*, suggests the uneven distribution of genera. For bacteria, it was 2.14 during the pre-monsoon, 1.69 during the monsoon, and 1.89 during the post-monsoon. For *Aedes aegypti*, it was 2.13 during the pre-monsoon, 1.72 during the monsoon, and 2.18 during the post-monsoon. For bacteria, it was about 1.73, 1.77, and 1.86 during the pre-monsoon, monsoon, and post-monsoon seasons, respectively, in *Culex quinquefasciatus*, and for fungi, it was 1.66 during the pre-monsoon, 1.76 during the monsoon, and 1.87 during the post-monsoon. In contrast, fungal genera in *Anopheles stephensi* are widely distributed across the seasons. But in the case of bacteria, there are seasonal variations (pre-monsoon : 1.67, monsoon : 1.74, and post-monsoon : 1.60), resulting in an uneven distribution among microbial populations (Table 16).

With regards to the evenness of microbial communities, the Shannon evenness for gut bacterial entities in *Aedes aegypti* was 0.94 during the pre-monsoon, 0.90 during the monsoon, and 0.60 during the post-monsoon. For fungal entities, a value of 0.93 was obtained for fungi during the pre-monsoon and monsoon seasons and 0.89 during the post-monsoon season.

Table 16: Alpha diversity indices of larval gut microbiota during pre-monsoon, monsoon and post-monsoon

Pre -Monsoon						
Alpha Diversity Indices	***Aedes aegypti***		***Culex quinquefasciatus***		***Anopheles stephensi***	
	Bacteria	Fungus	Bacteria	Fungus	Bacteria	Fungus
Simpson Diversity Index	0.88	0.87	0.81	0.79	0.79	0.82
Shannon-Weaver Diversity Index	2.14	2.13	1.73	1.66	1.67	1.75
Shannon - Wiener Eveness Index	0.94	0.93	0.94	0.88	0.89	0.96
Monsoon						
Alpha Diversity Indices	***Aedes aegypti***		***Culex quinquefasciatus***		***Anopheles stephensi***	
	Bacteria	Fungus	Bacteria	Fungus	Bacteria	Fungus
Simpson Diversity Index	0.80	0.81	0.83	0.82	0.81	0.82
Shannon-Weaver Diversity Index	1.69	1.72	1.77	1.76	1.74	1.75
Shannon -Wiener Eveness Index	0.90	0.93	0.98	0.97	0.95	0.96
Post-Monsoon						
Alpha Diversity Indices	***Aedes aegypti***		***Culex quinquefasciatus***		***Anopheles stephensi***	
	Bacteria	Fungus	Bacteria	Fungus	Bacteria	Fungus
Simpson Diversity Index	0.83	0.87	0.83	0.85	0.80	0.82
Shannon-Weaver Diversity Index	1.89	2.18	1.86	1.87	1.60	1.73
Shannon-Wiener Eveness Index	0.60	0.89	0.92	0.96	0.91	0.94

For *Culex quinquefasciatus*, larvae Shannon evenness values were 0.94 during the pre-monsoon, 0.98 during the monsoon, and 0.92 during the post-monsoon for bacteria, and 0.88, 0.97, and 0.96 were obtained for fungi during the pre-monsoon, monsoon, and post-monsoon, respectively. In the case of *Anopheles stephensi* for bacteria, the evenss value was 0.89 during the pre-monsoon, 0.95 during the monsoon, and 0.91 during the post-monsoon. While a value of 0.96 was obtained for both pre-monsoon and monsoon for fungi and a value of 0.94 during the post-monsoon. As the values were closer to 1.0, it indicated a higher diversity (Table16).

In the present study Bray Curtis dissimilarity index was used to accesss the difference in gut microbiota (bacteria and fungus) of *Aedes aegypti*, *Culex quinquefascuatus* and *Anopheles stephensi* based on seasonal changes.

When comparing the gut bacterial composition of *Aedes aegypti,* it was observed that a higher value of 1.0 was observed during the post-monsoon compared to the pre-monsoon comparison. A notably low value of 0.29 was observed when comparing the monsoon and postmonsoon seasons. A moderate value of 0.7 was obtained upon comparing the pre-monsoon and monsoon seasons. Regarding fungi, greater values of 0.94 and 0.90 were found when comparing the monsoon to the post-monsoon and the post-monsoon to the pre-monsoon. The value of 0.77 was obtained during the pre-monsoon and monsoon.

The Bray Curtis dissimilarity index revealed a moderate-high variation in gut bacterial composition of 0.82 (pre-monsoon to monsoon), 1.0 (monsoon to post-monsoon), and 0.86 (post-monsoon to pre-monsoon). On the other hand, fungi demonstrated some uniform values of 0.93, 0.94, and 0.96 for pre-monsoon to monsoon, monsoon to post-monsoon, and post-monsoon to pre-monsoon

comparisons. While analysing *Anopheles stephensi*, pre-monsoon to monsoon and monsoon to post-monsoon exhibit the highest value of 1.0. For the post-monsoon to pre-monsoon bacterial comparison, a value of 0.91 was found. Regarding fungi, a value of 0.97 was obtained during pre-monsoon to monsoon, a value of 0.84 during monsoon to post-monsoon, and a value of 0.92 during post-monsoon to pre-monsoon comparisons (Table 17).

Table 17: Beta diversity index of larval gut microbiota during pre-monsoon, monsoon and post-monsoon

Beta Diversity						
Bray Curtis Dissimilarity Index	***Aedes aegypti***		***Culex quinquefasciatus***		***Anopheles stephensi***	
	Bacteria	Fungus	Bacteria	Fungus	Bacteria	Fungus
Pre-monsoon -Monsoon	0.70	0.77	0.82	0.93	1.0	0.97
Monsoon - Post-monsoon	0.29	0.94	1.0	0.94	1.0	0.84
Post-monsoon-Pre-monsoon	1.0	0.90	0.86	0.96	0.91	0.92

1.4 DISCUSSION

The present study provided a comprehensive, culture-dependent approach for the characterisation of the gut microbiome of vector larval species of *Aedes aegypti*, *Culex quinquefasciatus*, and *Anopheles stephensi*. Despite increasing knowledge about the composition and diversity of bacteria linked to mosquitoes in the adult stage, relatively little data pertaining to the larval composition is available (Wu et al., 2019). To fill this gap, the present study focused on the bacterial and fungal communities of these larvae sampled from three different sites in Thrissur district across pre-monsoon, monsoon, and post-monsoon. The study reported here is the first of its kind, where comparative and simultaneous analysis of bacterial and fungal gut content and clonization has been carried out in these mosquito species on a seasonal basis. In the present study, through the examination of 16S rRNA sequencing, 1026 bacterial gut isolates and 239 fungal isolates were obtained by ITS sequencing from the larval gut of these vector mosquitoes. To date, only a few studies have explored the mycobiota in the larval gut across mosquito species. In that respect, the coexistence of fungal and bacterial communities with larval guts was largely understudied. So, this knowledge provides a thorough insight into mosquito-associated bacterial and fungal communities.

It was found that bacterial taxa from *Aedes aegypti* during pre-monsoon include *Escherichia coli*, *Enterobacter ludwigii* strain VVSO1-S1, *Pseudomonas aeruoginosa*, *Enterobacter quasihormaechei* strain WCHE, *Rahnella aquatilis* strain TW4, *Bacillus cereus*, *Enterobacter hormaechei* strain E26, *Enterobacter cloacae* strain HNXY160623, and *Serratia oryzae*. During monsoon season the presence of *Enterobacter hormaechei strain* SH19PTE2**,** *Enterobacter* sp. strain LF3, *Rahnella aquatilis*, *Enterobacter* sp. strain UASB_FL5, *Pseudomonas aeruginosa* strain

KOKG, and *Escherchia coli was* observed. Whereas during post-monsoon, *Enterobacter* sp. strain X114, *Bacillus cereus* strain AQ-105, *Enterobacter* sp. strain KR2, *Enterobacter kobei* strain NPKC3, *Enterobacter asburiae* strain CK2, *Enterococcus casselifavus* strain 902, *Enterobacter asburiae* strain E19, *Enterococcus casselifavus* strain D1, *Enterobacter asburiae* strain CK2_1_40, *Enterococcus gallinarum* strain ClaCZ15, and *Stenotrophomonas maltophilia* strain PEG-305 Twenty-six bacterial species representing seven genera such as *Enterobacter*, *Escherichia, Pseudomonas, Bacillus*, *Rahnella, Serratia,* and *Stenotropmonas* characterized in this study corroborate the findings of other workers that have already documented these bacterial genera in some mosquito species (Gusmao et al., 2010; Coon et al., 2014; Ranasinghe et al., 2020). While on seasonal cross-taxon comparison, the most abundant bacteria grouped into the class Gammaproteobacteria, order Enterobacterales, family Enterobacteriacea, and the common isolates belonged to the genus *Enterobacter* were found in the gut of *Aedes aegypti,* suggesting stable association within the gut lumen. However, on the contrary, there is no evidence for the occurrence of this particular genus *Enterobacter* as a core microbiota in mosquito larval gut (Cirimotich et al., 2011). Its presence may in turn be regulated due to larval feeding behaviour as well as habitat preference of mosquito species, or rather, the environment, which dominates genetics in shaping the gut microbiota (Strand, 2018).

Interestingly, most of the identified bacteria from *Aedes aegypti* larvae were primarily composed of Gram-negative aerobes and anaerobes (Boissière et al., 2012; Osei-Poku et al., 2012). The supremacy of Gram-negative bacteria was their ability to invade and proliferate within hosts more than other bacterial groups (Engel & Moran, 2013). Meanwhile, in the present investigation, notable variability was observed

between sites during different seasons, and commonality was identified in gut bacterial taxa within the same site during different seasons in mosquito larvae. Several studies have suggested that factors like environment, species and sex are responsible for bacterial overlap and variability within the mosquito gut microbiota (Coon et al., 2014).

Very sparse knowledge was available about fungal communities from mosquito larvae. So, the present study provides an overview of fungal isolates from mosquito larval gut. It was found that division Ascomycota (89%) was the most abundant division across all seasons, even though the presence of division Mucromycota (11%) found in *Aedes aegypti*. Within the division Ascomycota, genera *Aspergillus*, *Penicillium,* and *Candida* were more prevalent in the gut across larvae. This prevalence could be attributed to the fact that these fungi thrive as saprophytes in decaying vegetation, soil, organic matter, and dead plant materials present in the breeding water. Pereira and co-workers reported the presence of *Aspergillus* species and *Geotrichum* species in larval breeding water. These results demonstrate the exposure of mosquito larvae to these fungi and the chances of their acquisition into the gut (Pereira et al., 2005).

Earlier investigations on fungi from mosquito breeding containers documented the presence of different species of *Aspergillus, Penicillum,* and *Candida*, suggesting greater chances of interaction between fungi and culicidae larvae (Wasinpiyamongkol & Kanchanaphum, 2019). In general, *Candida* species was reported as the most abundant form of fungus in mosquito larvae during all seasons (Figure 8, 9 & 10), suggesting its significance in mosquito larvae. Earlier works have described the role of *Candida* species as a chief nutrient supplement provider for synthesising essential amino acids, vitamins, minerals, and trace elements

(Malassigne et al., 2020). Apart from nutritional requirements, yeast can be implicated in promoting gut hypoxia in mosquitoes, effectively serving as a signal for growth and moulting (Valzania et al., 2018). It was considered as core micro biome in *Aedes albopictus* (Luis et al., 2019). Along with these fungi, the teleomorph form *Clavispora lusitaniae* of *Candida lusitaniae* was part of the gut consortium. In addition, the larvicidal properties of endophytic *Penicillum* species and *Aspergillus* species, especially *Aspergillus tamari*, were proven to be effective against *Aedes aegypti* and *Culex quinquefasciatus* by the studies of (Maketon et al., 2014 ; Baskar et al., 2020).

The presence of *Cladosporium tenuissimum* and *Cladosporium marinum* was reported for the first time from the larval gut of *Aedes aegypti*. Earlier, the presence of *Cladosporium* was reported from the guts of larvae, pupae, and adults of the sap beetle *Brachypeplus glaber* (Cline et al., 2014). This endophytic fungi was also known its insecticidal potential against the insect *Spodoptera litura* (Singh et al., 2016). The endophytic fungus *Curvularia affini*s was also reported for the first time from mosquito larval gut; this particular genus was reported earlier from Sea Anemone (Khiralla et al., 2019). This dematiaceous filamentous fungi contain several secondary metabolites which possess beneficial implications like anti-microbial, anti-inflammatory properties(Mehta et al., 2022). Several studies have quoted the presence of entomopathogenic fungi *Simplicillum* species from both adult males, adult females, and larvae of sand fly *Phlebotomus perniciosus* soil, freshwater, marine and terrene environments (Martin et al., 2018; Wei et al., 2019). The filamentous fungi *Syncephalastrum* sp. was present in soil (Barcoto et al., 2017).

On the other hand, most of the bacterial genera recovered from *Culex quinquefasciatus* during the present study include *Escherichia coli*, *Enterobacter*, *Acinetobacter*, *Rahnella*, *Serratia*, and *Bacillus* during the pre-monsoon and

monsoon. During the post-monsoon, the genera *Acinetobacter* and *Bacillus* were prevalent. The presence of the genus *Pseudomonas* was reported during the pre-monsoon and post-monsoon. Similarly, the genus *Serratia* marked its presence during monsoons and post-monsoons. Interestingly, in the present study, the presence of the genus *Bacillus* was found in *Culex quinquefasciatus* during all seasons. The present study confirmed the presence of these genera in *Culex quinquefasciatus* larvae, and the results are frequent and consistent, as suggested by previous work of (Vasanthi & Hoti, 1992; Chandel et al., 2013; Pennington et al., 2016).

More over, as stated in *Aedes aegypti*, the study concerned with *Culex quinquefasciatus reports* the colonisation of *Penicillium* sp., *Candida* sp., and *Aspergillus* sp. from the gut of mosquito larvae during different seasons. As per the earlier reports, the fungal microbiome communities are primarily composed of the division Ascomycota, with Basidiomycota present in smaller proportions. Consequently, in the present work, in line with these findings, it was observed that fungal diversity and distribution were predominately characterised by the prevalence of Ascomycota fungi, and the remaining belonged to Mucoromycota (Flores et al., 2022). During the pre-monsoon, the highest CFU/ml was observed for *Aspergillus nomius*, while during the monsoon and post-monsoon, the highest abundance was observed for *Penicillum citrinum* and *Candida tropicalis,* respectively. These species have previously been documented in culicidae. In a previous survey, *Penicillum citrinum* isolated from mosquito breeding water was found to have the potential to control mosquitoes biologically. This fungus has been found to induce mortalities in *Culex quinquefasciatus* (Wasinpiyamongkol & Kanchanaphum, 2019) due to the presence of patulina metabolite secreted from *Penicillum citrinum*.

Another study under taken by (Vasanthi & Hoti, 1992) assessed the microbial flora in gut of *Culex quinquefasciatus* breeding, in the cess pits. The genus *Aspergillus* and *Streptomyces* were more frequently encountered fungi from gut and discussing their potential in larval mortality. According to their findings, the exposure to the *Aspergillus* genus results in over 50% mortality in larvae. In the present study the majority of these fungi belonged to the genus *Aspergillus*.

Furthermore, saprobic *Cladosporium* sp. was found in the gut of *Culex quinquefasciatus* larvae. This filamentous ascomycete was found in vectors like *Culex pipiens* and *Aedes albopictus*, which may suggest its existence in mosquito habitats, as mentioned by (Chandler et al., 2015). This genera was frequently found in indoor environment, and under certain circumstance some become dominant, as noted by (Zhan et al., 2018). It's likely that *Culex quinquefasciatus* acquired *Cladosporium* species from its natural habitat and later passed them on to its progeny. A further fungal species, *Cunninghamella blakesleena,* which belonged to the Mucromycota division, was also detected in the mycobiome of the *Culex quinquefasciatus* larval gut. The presence of these fungi, along with *Fusarium roseum*, *Mucor plumbeus*, *Gliocladium* sp., *Cladosporium cladosporioides*, *Aspergillus* sp., *Mortierella isabellina*, *Rhizopus arrhizus*, and *Trichothecium roseum*, was found on the substrates of mosquito breeding sites. This strongly suggests its acquisition from breeding sites (Luz et al., 2007). *Nigrospora oryzae*, a saprophytic fungus, has been found in mosquito larvae. It's possible that larval feeding caused this fungus to colonise in larval gut (Li et al., 2023).

A multitude of cultivable bacteria were isolated from the *Anopheles stephensi* larval gut. These include *Bacillus cereus*, *Klebsiella pneumoniae*, *Rahnella aquatilis*, *Enterobacter hormaechei*, *Brevibacillus brevis,* and *Proteus mirabilis* during the pre-

monsoon. *Priestia aryabhattai*, *Escherichia coli*, *Serratia marcescens*, *Pseudomonas aeroginosa*, *Priestia megaterium,* and *Enterococcus gallinarum* were isolated during monsoon season, and *Bacillus thuringiensis, Enterobacter cloacae, Bacillus cereus*, *Enterobacter bugandensis*, *Brevibacillus parabrevis,* and *Enterobacter ludwigii* were reported during post-monsoon. *Priestia megaterium* was reported for the first time from mosquito larval gut; earlier, it was reported in *Limonius canus* (Pacific Coast wireworm) (Lacey et al., 2007). In agreement with other studies, it is important to highlight that the majority of these bacteria belonged to the class Bacilli and the remaining belonged to the class Gammaproteobacteria (Pidiyar et al., 2004; Mwadondo et al., 2017; Pereira et al., 2021).

Studies on fungal flora in the midgut of the larvae from different populations of the malaria vector *Anopheles stephensi* pointed out the presence of *Aspergillus* sp. this was in agreement with the present study, where the presence of different species of *Aspergillus* like *Aspergillus welwitschiae and Aspergillus aculeatus* was reported during pre-monsoon and post-monsoon (Tajedin et al., 2009 ; Kianifard et al., 2023). It was observed that irrespective of seasonal variation genus *Candida* marked its significance in *Anopheles stephensi* (Jayakrishnan et al., 2018). Most important opportunistic fungi like *Candida metapsilosis*, *Candida albicans*, *Candida tropicalis*, and different strains of *Candida metapsilosis* (CP59 and CBS10907) were found in the guts of mosquito larvae, as pointed out in the recent study of (Djihinto et al., 2022). The highest CFU/ml was observed for *Dipodascus albidus* during the pre-monsoon. The presence of diverse filamentous fungi, *Penicillum* sp., like *Penicillum citrinum*, *Penicillum hispanicum, and Penicillum oxalicum,* was observed in the larval gut of *Anopheles stephensi*. In recent years, this has been supported by high-throughput

sequencing approaches, where the presence of a *Penicillum* sp. named *Penicillum chrysogenum* was isolated from *Anopheles gambiae* (Godfrey, 2021).

Over all, the study showed that bacterial communities within each mosquito species was distinctive irrespective of site and season. This uniqueness holds that vector mosquitoes, eventhough share same habitat are morphologically distinct (Brooks et al., 2016; Novakova et al., 2017). It's interesting to note that the correlation between the microbial population and season shows that post-monsoon diversity is higher. The available data comparing *Aedes aegypti*, *Culex quinquefasiatus*, and *Anopheles stephensi* in terms of bacteria.

In this study, most of the bacteria identified from *Aedes aegypti*, *Culex quinquefasciatus, and Anopheles stephensi* larvae belonged to the phyla Pseudomonadota and Bacillota. Recently phylum Proteobacteria and Firmicutes were renamed as phylum Pseudomonadota and Bacillota (Panda et al., 2022). Earlier analysis, surveyed on both the microbiota of larval gut and aquatic habitat, revealed the presence of the phylum Proteobacteria and Firmicutes, the class Gammaproteobacteria, the genus *Actinobacteria*, and *Bacteroidetes* suggesting that the aquatic habitat strongly influences and plays a pivotal role in shaping the microbial community in each larval species (Favia et al., 2007; Cirimotich et al., 2011; Hegde et al., 2015). Normally, there is microbial reorganisation in mosquitoes while they metamorphose into the next stage as they filter during the course of development. These findings, along with recent observations by (Zouache et al., 2022) justify the influence of the microbial composition of breeding water on structuring gut flora, as larvae were non-selective filter feeders during pupation or as immatures. Another possible cue for the acquisition of larval gut microbiota was the transstadial

transmission of microbes, as demonstrated by the study of (Coon et al., 2014) on *Aedes aegypti*.

Even though the larval gut microbiota serves as a subset of the breeding water microbial consortium, literature on the mosquito larval gut has reviewed a lower abundance of bacteria taxa in comparison with its breeding sites. One reason may be the establishment of bacterial symbiosis within the gut, which further inhibits additional acquisition of bacterial colonies (Dada et al., 2014). Another reason for the lower OTU abundance of gut bacterial taxa could be due to the significant contribution of algae and fungi as dietary constituents, or it may be due to a lack of sufficient screening techniques to get an overall picture of the bacterial consortium (Kaufman et al., 2006).

To date, there has been a lack of research examining the influence of the seasons and habitats on shaping the gut diversity of bacteria and fungi. As well as the impact of these gut entities on larval survival and vectorial competence. While there is a possibility that microbial composition changes in accordance with different habitats and seasonal fluctuations, more studies are required for proper assessment of whether the microbial communities present in larvae are influenced by habitat and seasonal changes and also to track the role. This study was a preliminary attempt to screen the diversity of co-existing bacterial and fungal content in *Aedes aegypti*, *Culex quinquefasciatus,* and *Anopheles stephensi*. These findings may open new windows for understanding bacterial-fungal interactions within mosquito larvae.

The statistical quantification of the bacterial and fungal microbiomes would indicate their significance in the host. The diversity indices quantify microbial diversity within mosquito species during different seasons and describe it numerically.

In the present study, the Simpson's diversity index was found to be moderately high for bacterial and fungal microbiota in all mosquito species on a seasonal basis. This suggests an even and moderate degree of distribution and diversity within mosquito species. In contrast, Shannon diversity was found to be moderate during monsoon and post-monsoon in all mosquito species during all seasons' larvae, pupae, and female midguts, except in *Aedes aegypti* during pre-monsoon. This was due to the presence of a larger number of genera in the male midgut. Shannon evenness is used to estimate how the species are distributed among the individuals (Mohlmann et al., 2017).

Interestingly, the Shannon evenness was found to be uniform for bacterial and fungal gut entities in all mosquito species during all seasons, with an expectation in *Aedes aegypti* in the case of bacteria, which further suggests that the transstadial transmission of some of the genera does occur. On the contrary, Shannon evenness fluctuated in the bacterial diversity in *Aedes aegypti* during the post-monsoon, suggesting the uneven distribution of bacterial genera in the gut of mosquitoes. The analysis of the Bray Curtis index quantified the dissimilarity between gut microbiota on a seasonal basis. In the present study, high and maximum values of index indicate maximum dissimilarity between seasons in mosquito species, except a low value for bacteria was observed in *Aedes aegypti* during monsoon and post-monsoon, suggesting similarity of species during these two seasons.

CONCLUSION

Metazoa harbours a wide array of microorganisms, predominantly composed of bacteria. Other members of the microbiota comprise fungi, protozoa, viruses, etc. When it comes to the case of mosquitoes, they host an exceptionally wide range of microbiota from the larval stage onward. This signifies the substantial influence of the

microenvironment in shaping microbial colonization. Recent years have witnessed a surge in research more focused on the interaction between the host and its microbiota. The focus was exclusively on the role of bacteria as insect symbionts. These studies have highlighted the importance of these bacteria in various mechanisms, like nutrient utilization, the production of metabolites with physiological or immunological functions, which in turn results in greater adaptability to different niches, their defence against stressors, and vector competence. However, our existing knowledge about mycobiome as a part of the gut microbiota of medically important vectors remains relatively restricted.

Aedes aegypti, Culex quinquefasciatus, and *Anopheles stephensi* are important mosquito species capable of vectorizing pathogens that are of public health concern. Furthermore, their ability for cosmotropical distribution also necessitates their importance. Keeping this in view, the present study has focused on characterising the gut content, giving priority to bacteria and fungi present in three mosquito species, namely *Aedes aegypti, Culex quinquefasciatus, and Anopheles stephensi,* from three geographical sites in Thrissur district across three seasons: pre-monsoon, monsoon, and post-monsoon, for a period of two years. The study provides an insight into the co-existence of bacteria and fungi and their comparison within these mosquito species. While considering bacteria at the phylum level, Pseudomonadota and Bacillota marked their presence in all three mosquito larval species. In this work, fungal communities of mosquito larval species are mainly composed of the division Ascomycota, even though the presence of the division Mucromycota was identified in larval species. From the literature survey, it was found that habitat, seasonality, and transstadial transmission play a pivotal role in moulding the gut microbiota. Microbial diversity analysis was carried out to quantify the bacterial and fungal flora on a

seasonal basis. In the study, both alpha and beta diversity were assessed for microbial quantification.

Additional research efforts are required to gain comprehensive information about the whole microbial spectrum in mosquito larval guts. However, further research concentrating more on molecular mechanisms dealing with microbial and host interactions would help to better understand the influence of these microbes on vector longevity and fecundity.

CHAPTER 2

Molecular Phylogeny analysis of Gut microbiota in vector mosquitoes

2.1 INTRODUCTION

Phylogenetics, or cladistics, involves the reconstruction of evolutionary relationships among a set of closely related species or taxa in systematics and taxonomy. The term ‘phylogenetics’ is derived from the Greek word ‘kaldos’ meaning 'clade' (branch) which signifies a group of descendants originating from a common ancestor. The other terms commonly used for phylogenetic interpretation include node, which is a branch point within a tree. Root: It denotes the ancestral population from which subsequent species have evolved. Monophyletic: it refers to a group where all species that share a common ancestor are clustered together. Sister taxa: two taxa or species that are closest relatives in a phylogenetic tree. Paraphyletic group: it encompasses species that share a common ancestor but may not include all of the descendants of that common ancestor. Polytomy: it represents a branch point where two or more species emerge from a common ancestor simultaneously. Polyphyletic: It represents species that do not share an immediate common ancestor (Posada & Crandall, 2021).

The results of phylogenetic analysis are generally depicted in the form of phylogenetic trees. A phylogenetic tree, or phylogeny, is a tree holding nodes that are linked by branches. Each branch symbolises the continuity of a genetic lineage through time, while each node indicates the emergence of a new lineage. If the tree signifies the relationship among a set of species, then the nodes characterise speciation events. (De Queiroz & Gauthier, 1990; Yang & Rannala, 2012).

Nowadays, molecular data, such as nucleotide or amino acid sequences, serves as the primary input for constructing phylogenetic trees. Initially, these trees were exclusively intended to estimate the relationships among the species represented by sequences. However, their applications have evolved to include broader objectives. These include exploring the relationships among the paralogues in the gene family. Deducing the functions of genes that have not been experimentally studied and unravelling mechanisms that lead to microbial outbreaks and genome comparison (Hall & Barlow, 2006; Hall, 2013).

During phylogenetic tree construction, the principle of minimum evolution or maximum parsimony is often employed. The standard algorithm of the tree-making methods depends on this principle to analyse the topology patterns or a subset of likely ones that closely resemble a true tree. The goal is to select a tree that requires the least evolutional change as the final tree. This process is time-consuming, and with a large number of operational taxonomic units, only a small fraction of all conceivable topology patterns can be explored (Saitou & Nei, 1987).

Typically phylogenetic trees are built on the basis of sequence similarities in highly conserved genes like 16S rRNA or a set of housekeeping genes from different organisms. However, this approach has many constraints because the phylogeny of single genes does not necessarily reflect the evolutionary relationship of the entire organism due to the presence of a small set of input sequences. To enhance reliability, it is preferable to use all genes from the core genome when constructing the tree. This was resolved by software tools like EDGAR, MUSCLE and PHYLIP. PHYLIP is a comprehensive software package that offers various algorithms for phylogenetic tree construction. Four prominent algorithms in the PHYLIP are neighbor-joining (NJ),

maximum parsimony (MP), maximum likelihood (ML), and the unweighted pair group method with arithmetic mean (UPGMA) (Takahashi & Nei, 2000).

The phylogenetic methods can be categorised into two groups: character-based methods and distance-based methods. Character-based methods analyse discrete characters from molecular sequences from individual taxa. The theory assumes that characters at corresponding positions in a multiple sequence alignment are homologous among the sequences being studied. These methods evaluate all sequences in the alignment concurrently, examining each character (a site in the alignment) at a time to compute score for each tree. MP, ML and Bayesian inference are types of character-based phylogenetic methods. MP aims to create a phylogeny that requires the least evolutionary change, The 'tree score' represents minimum number of changes for maximum parsimony and ML is suitable for the analysis of distantly related sequences but can be computationally expensive, making it less suitable for larger input data. The tree score indicates log-likelihood value for maximum likelihood. For Bayesian inference, works on the assumption of molecular clock concepts. The tree score is the posterior probability for inference. Whereas distance-based methods rely on the degrees of differences between pairs of sequences and calculate distance as a measure of dissimilarity. This distance will be used to create the distance matrix between individual pairs of taxa. The theory assumes that all sequences involved are homologous, and the resulting weighted tree should satisfy the additive properties. UPGMA and NJ are examples of distance-based phylogenetic methods (Huson & Steel, 2004 ; Desper & Gascuel, 2007).

The distance-based method in phylogenetic analysis can be grouped into cluster-based and optimality-based algorithms. The cluster-based method algorithms construct a phylogenetic tree using a distance matrix, starting from the most similar

sequence pairs. Examples of cluster-based algorithms include UPGMA and NJ. The optimality-based method algorithms, on the other hand, compare several different tree topologies and select the one that best aligns with the computed distances in the trees and the desired evolutionary distances, often referred to as actual evolutionary distances. Examples of optimality-based algorithms include Fitch Margoliash and minimum evolution (Peng, 2007).

UPGMA is a simple clustering method that works under the assumption of a constant rate of evolution following the molecular clock hypothesis. UPGMA requires a distance matrix of the analysed taxa that is derived from multiple alignments for further analysis. The process involves identifying and grouping two taxa with the smallest distances. A new node is introduced at the midpoint of these two taxa, and the two original taxa are positioned on the tree. The distance from the new node to other nodes is determined by the arithmetic mean. Subsequently, the distance matrix is simplified by substituting two taxa with a new node, streamlining the process (Lewis, 1998).

The NJ method is a phylogenetic approach that follows the principle of minimum evolution. While it may not always produce the minimum-evolution tree, computer simulations have demonstrated its effectiveness in determining the tree topology. It is appropriate for any kind of evolutionary distance data (Trees, 1987). NJ constructs phylogenies based on a distance matrix, where the distance between two species is determined by the average number of internodes between them and along the gene tree. The distance-based method can be referred to as NJST. NJ can be used to analyse both rooted and unrooted gene trees (without using an outgroup) to infer species trees (Liu & Yu, 2011).

The Molecular Evolutionary Genetics Analysis (MEGA) software has continually evolved to meet the growing demand for advanced tools for studying evolutionary patterns and processes in organisms and genomes. Its initial release in 1993 offered statistical methods for molecular evolution with an interactive interface on the Microsoft Disc Operating System (MS-DOS). Over a span of more than 25 years, MEGA has expanded its scope and usefulness through the implementation of new methods, tools, and interfaces for comparative sequence analysis, resulting in the formation of modern integrated software (Caspermeyer, 2018). In its early stages, MEGA included distance-based and maximum parsimony methods for molecular phylogenetic interpretation (Kumar et al.,1994). Its functionality was expanded with the addition of data acquisition and the integration of the core sequence alignment technique (Kumar et al., 2004). Subsequently, MEGA incorporates the ML methods and Bayesian methods for molecular evolutionary analyses (Kumar et al., 2001). Today, MEGA offers a wide array of tools for selecting the best-fit substitution model(s), estimating evolutionary distances and divergence times, reconstructing phylogenies, predicting ancestral sequences, testing for selection, and diagnosing disease mutations(Caspermeyer, 2018). In this Chapter, MEGA 11 software was used for phylogenetic reconstruction of bacteria and fungi, which were identified by 16S rRNA and ITS sequencing based on NJ method.

2.2 MATERIALS AND METHODS

The bacteria and fungi isolated from the respective mosquito larvae were identified through DNA isolation and sequencing. The Sanger DNA sequencing technique was employed, utilising the ABI 3500 DNA analyzer and the Big Dye Terminator v3.1 cycle sequencing kit at the Rajiv Gandhi Centre for Biotechnology, Trivandrum. Phylogenetic analyses were conducted to elucidate the relationships among isolated bacteria and fungi isolated from mosquito larval gut. The analyses relied on using partial 16S rRNA and ITS as molecular markers for molecular phylogenetic studies. The gene sequences obtained after the sequencing of these markers were used for the analyses. Additional sequences for constructing the phylogenetic tree were retrieved from the NCBI -Gen Bank (Peng, 2007).

Building a phylogenetic tree requires four distinct steps:

- Identification and acquisition of homologous DNA
- Aligning the sequences
- Estimation of tree using aligned sequences
- Phylogenetic tree interpretation

2.2.1 Identification of homologous DNA

The most dependable method to identify sequences that share homology with sequences of interest involves conducting a Basic Local Alignment Search Tool (BLAST) search by using sequences of interest as the query. BLAST is a freely available software programme provided by the National Centre for Biotechnology Information (NCBI) that is capable of performing various types of analysis. In this particular instance, BLAST programmes are used to compare DNA queries of fungi and bacteria with databases in any combination, often involving the conceptional

translation of DNA sequence before any comparison undergoing conceptual translation before any comparisons are conducted (Altschul et al., 1997).

2.2.2 Aligning the Sequences

Sequence alignment is a process that establishes correspondence between positions in a sequence, indicating their evolutionary relationships. While there are several advanced alignment programmes available, manual editing remains crucial for ensuring alignment quality. However, there is no universally defined set of rules for modifying sequence alignment. The alignment within MEGA 11 was carried out using Clustal W tool integrated with in MEGA 11 software for multiple alignment (Peng, 2007)

2.2.3 Estimation of tree using aligned sequences

MEGA 11 offers several commonly used methods phylogenetic tree estimation including NJ, UPGMA, MP, Bayesian Inference, and ML. In the context of distance based phylogenetic methods, these methods evaluate the dissimilarity between pairs of sequences, which was computed from sequence alignment. The prime assumption of distance based methods that the sequences are homologous, the branches of tree can summarised. In the present study neighbor joining method based on the clustering algorithm was used for tree construction (Sohpal et al., 2013).

2.2.4 Phylogenetic tree interpretation

Interpretation of a phylogenetic tree means understanding the relationships between different taxa based on their evolutionary history. Interpretation encompasses assessing elements such as branch length, topology, clades, whether the tree is rooted or unrooted, inclusion of outgroup, monophyletic, paraphyletic and polyphyletic grouping.

2.3 RESULTS

In this study, phylogenetic analysis were carried on bacterial and fungal isolates retrieved from the gut of three mosquito larval species, *Aedes aegypti*,*Culex quinquefasciatus* and *Anopheles stephensi*. The phylogenetic analysis at family level between bacterial and fungal isolates were carried out. To investigate bacterial phylogenetic relationship partial coding of 16S rRNA sequences were used. For fungal analysis ITS sequencing was employed. To find the closest matches, a NCBI-BLAST search was conducted within the database. 124 sequences generated during the present work were used along with sequences retrieved from the Gen Bank database for the tree construction. These sequences were meticulously aligned using Clustal-W tool within MEGA11 (Tamura et al., 2021). Bootstrapping was performed 1000 times to assess the trees stability.

Further Neighbor-Joining (NJ) method was used to construct the both unrooted and rooted phylogenetic tree (Saitou & Nei, 1987). In phylogeny, the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches(Felsenstein, 1985).The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method.

Table 18: Bacterial and fungal sequences

>*Acinetobacter baumannii* F46| 715 bp|16S ribosomal RNA gene| partial sequence

AGCGGCGGACGGGTGAGTAATGCTTAGGAATCTGCCTATTAGTGGGGGACAACATCTCGAAAGGGATGCT
AATACCGCATACGTCCTACGGGAGAAAGCAGGGGATCTTCGGACCTTGCGCTAATAGATGAGCCTAAGTC
GGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCGGGTCTGAGAGGATGATCCG
CCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGG
GAACCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTATGGTTGTAAAGCACTTTAAGCGAGGAGG
AGGCTACTTTAGTTAATACCTAGAGATAGTGGACGTTACTCGCAGAATAAGCACCGGCTAACTCTGTGCC
AGCAGCCGCGGTAATACAGAGGGTGCGAGCGTTAATCGGATTTACTGGGCGTAAAGCGTGCGTAGGCGGC
TTATTAAGTCGGATGTGAAATCCCCGAGCTTAACTTGGGAATTGCATTCGATACTGGTGAGCTAGAGTAT
GGGAGAGGATGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGATGGCGAA
GGCAGCCATCTGGCCTAATACTGACGCTGAGGTACGAAAGCATGGGGAGCAAACAGGATTAGATACCCTG
GTAGTCCATGCCGTA

>*Acinetobacter baumannii* SL103| 717 bp|16S ribosomal RNA gene| partial sequence

TGCATTCGATACTGGTGAGCTAGAGTATGGGAGAGGATGGGTAGAACTCCAGGTGTAGCGGTGAAATGTG
TAGAGATCTGGAGGAATACCGATGGCGAAGGCAGCCATCTGGCATAATACTGACGCTGAGGTACGAAAGC
ATGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGTCTACTAGCCGTTGGGGCC
TTTGAGGCTTTAGTGGCGCAGCTAACGCGATAAGTAGACCGCCTGGGGAGTACGGTCGCAAGACTAAAAC
TCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACC
TTACCTGGCCTTGACATACTAGAAACTTTCCAGAGATGGATTGGTGCCTTCGGGAATCTAGATACAGGTG
CTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTTTCC
TTACTTGCCAGCATTTCGGATGGGAACTTTAAGGATACTGCCAGTGACAAACTGGAGGAAGGCGGGGACG
ACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCT
ACACAGCGATGTGATGCTAATCTCAAAAAGCCGATCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCAT
GAAGTCGGAATCGCTAG

>*Acinetobacter* sp. HSTU-ASn72| 619 bp|16S ribosomal RNA gene| partial sequence

ATACCGATGGGCGAAGGCAGCCATCTGGCCTAATACTGACGCTGAGGTACGAAAGCATGGGGAGCAAACA
GGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGTCTACTAGCCGTTGGGGCCTTTGAGGCTTTAGT
GGCGCAGCTAACGCGATAAGTAGACCGCCTGGGGAGTACGGTCGCAAGACTAAAACTCAAATGAATTGAC
GGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGCCTTGA
CATACTAGAAACTTTCCAGAGATGGATTGGTGCCTTCGGGAATCTAGATACAGGTGCTGCATGGCTGTCG
TCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTTTCCTTACTTGCCAGCAT
TTCGGATGGGAACTTTAAGGATACTGCCAGTGACAAACTGGAGGAAGGCGGGGACGACGTCAAGTCATCA
TGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCTACACAGCGATGTGA
TGCTAATCTCAAAAAGCCGATCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAA

>*Bacillus cereus* AQ-105| 327 bp|16S ribosomal RNA gene| partial sequence

GGGTTTTTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTTGTCAGCTCGTGTCGTGAGATGTTGG
GTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTCAGTTGGGCACTCTAAGGTGACT
GCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACA
CGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATCTCATAAAACCGTTCTCA
GTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCGCTA

> *Bacillus cereus* AH-43| 781 bp|16S ribosomal RNA gene| partial sequence

AATTTATTTGGGCGTAAAGCGCGCGCAGGTAGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCG
TGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAA
ATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCG
AAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTA
GAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGC
TGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGA
AGAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTCTCCTTCGGGAGCAGAGTGA
CAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC
TTGATCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGG
GGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAAAGAG
CTGCAAGACCGCGAGGTGGAGCTAATCTCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCC
TACATGAAGCT

>*Bacillus cereus* 17|676 bp|16S ribosomal RNA gene| partial sequence

GGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACATCCGGGAAACCGGGGCTAATACCGG
ATAACATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGT
CGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCG
GCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGAC
GAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAA
GAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGC
CAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGG
TTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTG
CAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGA
AAGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGC

> *Bacillus cereus* EB61| 748 bp|16S ribosomal RNA gene| partial sequence

ATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGA
GGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGC
GTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAG
CGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGG
GTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAA
ACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAA
CCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTCTCCTTCGGGAGCAGAGTGACAGG
TGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGA
TCTTAGTTGCCATCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGAT
GACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAAAGAGCTGC
AAGACCGCGAGGTGGAGCTAATCTCATAAAACCGTTCTCAGTTCGGCT

> *Bacillus thuringiensis* NSK-KAU |709 bp|16S ribosomal RNA gene| partial sequence

TAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATT
TTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAG
CTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACT
GGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCT
GACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGT
GCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCC
GCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAA
GTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAG
GAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACT
TTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGGAGCAAACAGGATTAGATACCCTGGTAGTC
CACGCCGTA

> *Bacillus toyonensis* VITSN1505| 688 bp|16S ribosomal RNA gene| partial sequence

AAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACC
GGGGCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTAT
GGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACC
TGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAAT
CTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACT
CTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCAC
GGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAA
GCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACT
GGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGG
AACACCGGTGGCGAAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCG

>*Bacillus cereus* NA5| 690 bp|16S ribosomal RNA gene| partial sequence

AGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCG
GGGCTAATACCGGATAACATTTTGAACTGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATG
GATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCT
GAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATC
TTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTC
TGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACG
GCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAG
CGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTG
GGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGG
GAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACCTGACACTGAGGCGCGAAAGCGT

> *Enterobacter quasihormaechei* WCHEs| 717 bp|16S ribosomal RNA gene| partial sequence

GAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGC
TAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGAT
GGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCA
GCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGC
GCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAG
GAAGGCGATAAGGTTAATAACCTTGTCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCC
AGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTATAGCGCACGCAGGCGGT
CTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCT
TGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAA
GGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTG
GTAGTCCACGCCGTAAA

>*Enterobacter hormaechei* E26| 776 bp|16S ribosomal RNA gene| partial sequence

CGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCCGGGCTCAACTGGGAACTGCATTCGAAACTG
GCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTTGTGTCGGTGAAATGCGTAGAGATCTGGAGG
AATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACA
GGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCT
TCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGAC
GGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGA
CATCCAGAGAACTTAGCAGAGATGCTTTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCG
TCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGG
TTAGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCA
TGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCA
AGCGGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGC
TAGTAA

>*Enterobacter hormaechei* SH19PTE2| 748 bp|16S ribosomal RNA gene| partial sequence

GAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGC
TAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGAT
GGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCA
GCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGC
GCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAG
GAAGGTGTTGAGGTTAATAACCTCAGCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCC
AGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGT
CTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCT
TGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAA
GGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGGAGCAAACAGGATTAGATACCCT
GGTAGTCCACGCCCGTAACGATGTCGACTTGGAGGTTGTGGCCCTTTG

>*Enterobacter hormaechei* EHo5 |729 bp|16S ribosomal RNA gene| partial sequence

TGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCCAGGTGTAGCGGTG
AAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTG
CGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGG
TTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGG
TTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCG
AAGAACCTTACCTACTCTTGACATCCAGAGAACTTAGCAGAGATGGATTGGTGCCTTCGGGAACTCTGAG
ACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACC
CTTATCCTTTGTTGCCAGCGGTCCGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGT
GGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGCATACAAAG
AGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCG
ACTCCATGAAGTTCGGAATCGCTAGTAAT

>*Enterobacter hormaechei* 9 |308 bp|16S ribosomal RNA gene| partial sequence

GCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCAT
GTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGGGAACTTAGCAGAGATGGT
TTGGTGCTTTCGGGAACTTTGAGACAGGTGCTGCATGGCTGTTGTCAGCTCGTGTTGTGAAATGTTGGGT
TAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTCCCAGCGTTTCGGCTGGGAATTCAAAGGAGACTG
CCGGTGATAAACCGGAGGAAGGTGGGGA

>*Enterobacter* sp.LF3 |784 bp|16S ribosomal RNA gene| partial sequence

GAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGC
TAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGAT
GGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCA
GCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGC
GCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAG
GAAGGCGATAAGGTTAATAACCTTGTCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCC
AGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGT
CTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCT
TGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAA
GGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTG
GTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGATGCGTGGCTTCCCGGAGCTAACGC
GTTAAGTCGACCGG

> *Enterobacter* sp. UASB_FL5 | 594 bp|16S ribosomal RNA gene| partial sequence

AGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACG
CCGTAAACGATGTCGACTAGGAGGTTGTGCCCATGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGAC
CGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCA
TGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTTCCAGAGATGG
ATTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGG
TTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTCCGGCCGGGAACTCAAAGGAGACT
GCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACA
CGTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTA
GTCCGGATTGGAGTCTGCAACTCGACTCCATGAA

>*Enterobacter* sp. X114| 791 bp|16S ribosomal RNA gene| partial sequence

CCGAAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACG
TGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGA
AATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGC
GAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGT
TGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGT
TAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGA
AGAACCTTACCTACTCTTGACATCCAGAGAACTTAGCAGAGATGGTTTGGTGCCTTCGGGAACTCTGAGA
CAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC
TTATCCTTTGTTGCCAGCGGTTCGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTG
GGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGCATACAAAGA
GAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGA
CTCCATGAAGTCGGAATCGCT

Enterobacter sp. KR2| 681bp|16S ribosomal RNA gene| partial sequence

CTTTGCCGGCGAGCGGCGAGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTG
GAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGA
TGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGA
GAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATT
GCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTT
TCAGCGGGGAGGAAGGTGCTGAGGTTAATAACCTCAGCAATTGACGTTACCCGCAGAAGAAGCACCGGCT
AACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGC
ACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCA
GGCTAGAGTCTTGTAGAGGGGGGTAGAATTCGAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATA
CCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAAGTGCGAAAG

>*Enterobacter asburiae* CK2_1_40| 727 bp|16S ribosomal RNA gene| partial sequence

TACTTTGCCGGCGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACT
GGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAG
ATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTG
AGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATAT
TGCACAATGGGCGCAAGCCTGATGCACCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACT
TTCAGCGGGGAGGAAGGTGCTGAGGTTAATAACCTCAGCAATTGACGTTACCCGCAAAAGAAGCACCGGC
TAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCG
CACGCAGGCGGTCTGTCAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGC
AGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAAT
ACCGGTGGCGAAAGCGGCCCCCTGGACAAAGACTGACGCTCATGTGCGAAAGCGTGGGGAGCAAACAAGA
TTATATACCCTGGTAGTCCACGCCGTA

>*Enterobacter asburiae* HAOB_L21 |794 bp|16S ribosomal RNA gene| partial sequence

AATTACTGGGCGTAAAGCGCACGCAGGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACGTGG
GAACTGCATTCGAAACTGGCAGGCTAGAGTCTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATG
CGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAA
GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTG
CCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAA
ACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAA
CCTTACCTACTCTTGACATCCAGAGAACTTAGCAGAGATGGATTGGTGCCTTCGGGAACTCTGAGACAGG
TGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAT
CCTTTGTTGCCAGCGGTCCGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGA
TGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAG
CGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCC
ATGAAGTCGGAATCGCTAGTAATC

>*Enterobacter asburiae* CK2_1_41|700bp|16S ribosomal RNA gene| partial sequence

ACTTTGCCGGCGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTG
GAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGA
TGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGA
GAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATT
GCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTT
TCAGCGGGGAGGAAGGTGCTGAGGTTAATAACCTCAGCAATTGACGTTACCCGCAGAAGAAGCACCGGCT
AACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGC
ACGCAGGCGGTCTGTCAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCA
GGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATA
CCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCATGTGCGAAAGCGTGGGGAGCAAACAGGA
AGGCTCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCCGTAACGATGAGTGCTA

>*Enterobacter asburiae* E19| 734 bp|16S ribosomal RNA gene| partial sequence

TGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAA
GACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCATATGTGCCCAGATGGGATTATCTAGTAGGTGGGG
TAACGGCTCACCTAGGCGACGATCCCTATCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACAC
GGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAGTGGGCGCAAGCCTGATGCACCCGTGC
CGCGTGTATGAAAAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGCGTTGAGGTTAATAAC
CTCAGCGATTGACGTTACCCGCAAAAAAAGCACCGGCTAACTCCGTGCCAGCACCCGCGGTAATACGGAG
GGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCAGATGTGAAAT
CCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCC
GGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGAC
TGACGCTCATGTGCGAAAGCGTGGGGAGCAAACGAGATTATATACCCTGGTAGTCCACGCCGTAACGATG
TCACTTGGAGGTTGTGCCCTTGAGCGTGGCTTCC

> *Enterobacter ludwigii* VVS01-S1 | 581 bp|16S ribosomal RNA gene| partial sequence

GACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCG
CATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAG
CTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACT
GGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCT
GATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGCGT
TGAGGTTAATAACCTCAGCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCG
CGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAG
TCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGG
GGGGTAGAATTCCTGGTGTAG

Enterococcus gallinarum ClaCZ15| 746 bp|16S ribosomal RNA gene| partial sequence

AGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATT
CCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTTTTGGTCTGT
AACTGACGCTGAGGCTCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC
GATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCAAACGCATTAAGCACTCCGCCTGG
GGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTT
TAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTTTGACCACTCTAGAGATAGAGCTTCC
CCTTCGGGGGCAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTC
CCGCAACGAGCGCAACCCTTATTGTTAGTTGCCATCATTTAGTTGGGCACTCTAGCGAGACTGCCGGTGA
CAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTAC
AATGGGAAGTACAACGAGTTGCGAAGTCGCGAGGCTAAGCTAATCTCTTAAAGCTTCTCTCAGTTCGGAT
TGTAGGCTGCAACTCGCCTACATGAAGCCGGAATCGCTAGTAATCG

>*Enterococcus gallinarum* 1880| 782 bp|16S ribosomal RNA gene| partial sequence

AGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAACACTTGGAAACAGGT
GCTAATACCGTATAACACTATTTTCCGCATGGAAGAAAGTTGAAAGGCGCTTTTGCGTCACTGATGGATG
GACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCATAGCCGACCTGAGA
GGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCG
GCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTT
GTTAGAGAAGAACAAGGATGAGAGTAAAATGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTA
ACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAG
CGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAG
ACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACAC
CAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCGAACAGGATT
AGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTGGAGGGTTTCCCGCCCTTCAGTGCT

>*Enterococcus casseliflavus* D1| 630 bp|16S ribosomal RNA gene| partial sequence

TTGAAAGGCGCTTTTGCGTCACTGATGGATGGACCCACGGTGCATTAGCTAGTTGGTGAGGTAACGGCTC
ACCAAAGCAACAATGCATACCCAACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGAC
TCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGT
GAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGATGAGAGTAAAATGTTCATCCC
TTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCATCAGCCGCGGTAATACGTACGTGGCAAG
CGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGC
TCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAG
CGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTG
AGGCTCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCCGTAACGATGAGTGCTA

> *Enterococcus casseliflavus* 902| 696 bp|16S ribosomal RNA gene| partial sequence

CATTTTCCGCATGGAAGAAAGTTGAAAGGCGCTTTTGCGTCACTGATGGATGGACCCGCGGTGCATTAGC
TAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTG
GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTG
ACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGA
TGAGAGTAAAATGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCG
CGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAG
TCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGG
AGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTC
TCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCA
CGCCGTAAACGATGAGTGCTAAGTGTTGGAGGGTTTCCCGCCCTTCAGTGCTGCAGCAACGCATTA

>*Enterobacter kobei* NPKC3| 729 bp|16S ribosomal RNA gene| partial sequence

ACTTTGCCGGCGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTG
GAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGA
TGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGA
GAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATT
GCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTT
TCAGCGGGGAGGAAGGTGTTGAGGTTAATAACCTCAGCGATTGACGTTACCCGCAGAAGAAGCACCGGCT
AACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGC
ACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCA
GGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATA
CCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAAGAT
TATATACCCTGGTAGTCCACGCCGTAAAC

> *Enterobacter cloacae* 07| 792 bp|16S ribosomal RNA gene| partial sequence

AAGCGTTAATCGGAAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCCGGATGTGAAATCCC
CGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGG
TGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGA
CGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTC
GACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTAC
GGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCG
ATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTAGCAGAGATGGTTTGGTGCCTTCGG
GAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAAC
GAGCGCAACCCTTATCCTTTGTTGCCAGCGGTTAGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACT
GGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGC
GCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGT
CTGCAACTCGACTCCATGAAGT

>*Enterobacter cloacae* CSBB-493| 631 bp|16S ribosomal RNA gene| partial sequence

TGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGA
TACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGC
TAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCC
GCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGA
GAACTTTGCAGAGATGGTTTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCG
TGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTCCGGCCG
GGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTT
ACGAGTAGGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACC
TCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATC
G

>*Enterobacter cloacae* HNXY160623|703 bp|16S ribosomal RNA gene| partial sequence

TGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAA
TACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGATGGG
ATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCC
ACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCA
AGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAA
GGCGATGAGGTTAATAACCTTGTCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGC
AGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTG
TCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGT
AGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGG
CGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGG
GTA

>*Enterobacter bugandensis* KPC863|808 bp|16S ribosomal RNA gene| partial sequence

TGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGG
TAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCC
AGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATG
ACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAAT
GGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGG
GGAGGAAGGTGTTGAGGTTAATAACCTTGTCAATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCG
TGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGG
CGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGA
GTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGG
CGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACC
CTGGTAGTCCACGCCGGTAAACGATGTCGACTTGGAGGTTGTGCGCTTGAGGCGTGGCTTCCGGGAGCTA
CGCGTTTAGTCGACCGCCTGGGGGGAGTACGGCCGCAG

>*Enterobacter ludwiggi* GN091 | 949 bp|16S ribosomal RNA gene| partial sequence

GGACACGGGGAAACCTTGGCTTCTCCGTGTGAGCAGTGGCGGACGGGTGAGTAATGTCTGGGAAGCTGC
CTGATGGAGGGGGAGAACTACTGGAAACGGTGGCTAGTACCGCATAACGTCGCAAGACCGAAGAGGGGG
AGCTTCGGGCCTCTTGCCCTCACATGTGCCCACATGGGATTATCTAGTAGGTGGGGTAATGGCTCACCT
ATGCGACAATCCCTAGCTGGTCTGAGAGGATGACCAACCACACTGGAACTGAGACACGGTCCAGACTCC
TACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCATCCATGCCGCGTGTATGA
AAAAAGCCTTCGGGTTGTAAAGTACTTTCTTCGAGGAGGAAGGAGTTAAGGTTAATAACCTTGTCGATT
GACGTTACCCGCACAAAAAACACCGGCTAACTCCCTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGC
GTTAATCGGAATTACTGGGCGTAAAGCGCACGCACGCGGTCTGTCAAGTCAGATGTGAAATCCCCCGGC
TCAACCTGGGAACTGCATTCGAAACTGGCTGGCTAGAGTCTTGTATAGGGGGGTAGAATTCCCTGTGTG
TCGCTGAGATGCGTACAGATCTGGAGGAATACCGGTGGCGAAAGCGGCCCCCTGGACAAGACTGACACT
CACGTGCGAAAGCGTGTGGAGCAAACAGGATTATATACCACTGGTGAGTCCACGCTCTATACGATGTCT
ACTATGTATGGTTGTGTCCTTGAGGAGTGGCTTCCTGGAGCTAACGCGTTAATTCTACCCGCCCTGGGG
GGAGTACAGGCGGCACGGTGATACTCATCATGAAATGTATCGGGAGGGGCGCGCACAAGCAGGTTGGGA
GGCATGGCTGAGTTGATCGATTGCATCGACGGAACGCAACCATTACCCTTAC

>*Escherichia coli* XJ141J-125-NF1|323 bp|16S ribosomal RNA gene| partial sequence

TCAACTTGTAGAAGGCTTGCTGCTTCGCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCT
GATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACC
TTCGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGC
GACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGG
GAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGAT

> *Escherichia coli* TUM13773| 301 bp |16S ribosomal RNA gene| partial sequence

GAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGT
CGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGT
GGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGA
CACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCAT
GCCGCGTGTATGAAGAA

>*Klebsiella pneumonia*| 510 bp|16S ribosomal RNA gene| partial sequence

GCTCACCTAAGCAACTATCCCTAGCTGGTCTGAAAGGATGACCACCCACACTGGAACTGACACACGGTCC
ACACTCCTACGGGAGGCATCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCATCCATGCCGCGT
GTGTGAAGAATGCCTTCTGGTTGTAAATCACTTTCAGCGGGGAGGAAGGCGATAAGGTTAATAACCTTGT
CGATTGACGTTACCCGCAAAAGAACCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGC
AAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCG
GGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTATAATTCCAGGTG
TAGCGGTGAAATGCGTAAGATCTGGAAGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGC
TCAGTGCGAAAGCGTGGGGA

> *Pseudomonas aeruginosa* KOKG | 403 bp|16S ribosomal RNA gene| partial sequence

CCAATTGGGAACTTGCTCCTGGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTG
GGGGATAACGTCCGGAAACGGGCGCTAATACCGCATACGTCCTGAGGGAGAAAGTGGGGGATCTTCGGAC
CTCACGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATC
CGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAG
CAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGG
ATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGT

>*Proteus mirabilis*| 803 bp|16S ribosomal RNA gene| partial sequence

TTTGGAAAAGCCCCCCGAGGCTTAAACCTTGGGGAAATTGCATTTTGAAATTGGTTGGGTTAGAGTTTTT
GTAGAGGGGGGGGTAGAATTTTCCCTGTGTAGGCGGTGAAAATGCGTAGAGATGTGGAGGAAATACCGGG
TGGGGAAGGCGGCCCCCCTGGACAAAGACTTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTA
GATACCCTGGTAGTTCACGCTGTAAACGATGTTGATTTTGAGGTTGTGGTCTTGAACCGTGGCTTTTGGA
GTAACGCGTTAAATTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCC
CGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTTTTGACATCCAG
AGAATCCTTTAGAGATAGAGGAGTGCCTTTGGGAACTTTGAGACAGGTGCTGCATGGCTGTCGTCAGCTC
GTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCACGTGATGG
CGGGAACTCAAAGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCC
TTACGAGTAGGGCTACACACGTGCTACAATGGCAGATACAAAGAGAAGGGACCTCGCGAGAGCAAGCGGA
ACTCATAAAGTCTTTTTGTAATCCGGATTGGAGTCTGCAACTCGACTTCCATGAAGTCGGAATCGCTAGT
AATTGGAGATCCAAAAATTTTATACGTGTTCGG

> *Priestia aryabhattai*|811 bp|16S ribosomal RNA gene| partial sequence

CGTTAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTGTAAGACTGGGATAACTTCGGGAAACCG
AAGCTAATACCGGATAGGATCTTCTCCTTCATGGGAGATGATTGAAAGATGGTTTCGGCTATCACTTACA
GATGGGCCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCATAGCCGACCT
GAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATC
TTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTC
TGTTGTTAGGGAAGAACAAGTACGAGAGTAACTGCTCGTACCTTGACGGTACCTAACCAGAAAGCCACGG
CTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGC
GCGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGG
GGAACTTGAGTGCAGAAGAGAAAAGCGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAA
CACCAGTGGCGAAGGCGGCTTTTTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGG
ATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTG
CTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGT

> *Priestia megaterium*| 811 bp|16S ribosomal RNA gene| partial sequence

CGTTAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTGTAAGACTGGGATAACTTCGGGAAACCG
AAGCTAATACCGGATAGGATCTTCTCCTTCATGGGAGATGATTGAAAGATGGTTTCGGCTATCACTTACA
GATGGGCCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCATAGCCGACCT
GAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATC
TTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTC
TGTTGTTAGGGAAGAACAAGTACGAGAGTAACTGCTCGTACCTTGACGGTACCTAACCAGAAAGCCACGG
CTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGC
GCGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGG
GGAACTTGAGTGCAGAAGAGAAAAGCGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAA
CACCAGTGGCGAAGGCGGCTTTTTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGG
ATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTG
CTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGT

>*Rahnella aquatilis* MSSRFQS77| 803 bp|16S ribosomal RNA gene| partial sequence

GGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGCGCTTAACGT
GGGAACTGCATTTGAAACTGGCAAGCTAGAGTCTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAA
TGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGA
AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCGACTTGGAGGTTG
TGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTA
AAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAG
AACCTTACCTACTCTTGACATCCACGGAACTTGCCAGAGATGGCTTGGTGCCTTCGGGAACCGTGAGACA
GGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTT
ATCCTTTGTTGCCAGCACTTCGGGTGGGAACTCAAAGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGG
GATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGTATACAAAGAGA

>*Rahnella aquatilis* TW4| 472 bp|16S ribosomal RNA gene| partial sequence

TGGACAAAGATGACGCTCAGGTGTGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGC
TGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACC
GCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCAT
GTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCACGGAACTTGCCAGAGATGGC
TTGGTGCCTTCGGGAACCGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGT
TAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCACTTCGGGTGGGAACTCAAAGGAGACTG
CCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTT

> *Rahnella aquatilis* _H_6_2_191014057| 791 bp|16S ribosomal RNA gene| partial sequence

CTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGCGCTTAACGTGGGAACTG
CATTTGAAACTGGCAAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAG
AGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTG
GGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCGACTTGGAGGTTGTGCCCTT
GAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCA
AATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTA
CCTACTCTTGACATCCACGGAACTTGCCAGAGATGGCTTGGTGCCTTCGGGAACCGTGAGACAGGTGCTG
CATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTT
GTTGCCAGCACTTCGGGTGGGAACTCAAAGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACG
TCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGTATACAAAGAGAAGCAAAC
TCGCGAGAGCCAGCGGACCTCATAAAGTACGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAA
GTCGGAATCGCTAGTAATCGT

>*Rahnella aquatilis* TW5| 506 bp|16S ribosomal RNA gene| partial sequence

CCCCCTGGACAAAGATTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGGATGAGATACCCTGGTAG
TCCACGCTGTAAATGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAA
GTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGT
GGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCACGGAACTTGCCAG
AGATGGCTTGGTGCCTTCGGGAACCGTGAGACAGGTGCTGCATGGCCGTCGTCAGCTCGTGTTGTGAAAT
GTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCACTTCGGGTGGGAACTCAAAG
GAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGC
TACACACGTGCTACAA

>*Serratia marcescens* MICUL| 789 bp|16S ribosomal RNA gene| partial sequence

GGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCA
TTTGAAACTGGCAAGCTAGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAG
ATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGG
GAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTGGAGGTTGTGCCCTTGA
GGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAA
TGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACC
TACTCTTGACATCCAGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCA
TGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGT
TGCCAGCGGTTCGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTC
AAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGTATACAAAGAGAAGCGACCTC
GCGAGAGCAAGCGGACCTCATAAAGTACGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGT
CGGAATCGCTAGTAATCGT

>*Serratia oryzae* J046| 547 bp| 16S ribosomal RNA gene| partial sequence

CGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCCGATGGAGGGGGATAACTACTGGAAACGGTGGCTAA
TACCGCATAACGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCACGCCATCGGATGTGCCCAGATGGG
ATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCC
ACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCA
AGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAA
GGCATCATACTTAATACGTGTGGTGATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGC
AGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTG
TTAAGTCAGATGTGAAATCCCCGCGCTTAACGTGGGAACTGCATTTGAAACTGGCAA

> *Stenotrophomonas maltophilia* PEG-305| 344 bp|16S ribosomal RNA gene| partial sequence

CCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGTATG
TGGTTTAATTTGATGCAACGCGAAGAACCTTACCTGGCCTTGACATGTCGAGAACTTTCCAGAGATGGAT
CGGTGCCTTCGGGAACTCGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTT
AAGTCCCGCAACGAGCGCAACCCTTGTCCTTAGTTGCCAGCACGTAATGGTGGGAACTCTAAGGAGACCG
CCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCT

>*Aspergillus tamarii* | 177 bp|internal transcribed spacer sequence

TGATCTAGTGAAGTCTGAGTTGATTGTATCGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCC
GGCATCGATGAAGAACGCAGCGAAATGCGATAACTAGTGTGAATTGCAGAATTCCGTGAATCATCGAGTC
TTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGG

>*Aspergillus niger* KL3 | 316 bp|internal transcribed spacer sequence

CCCGCCGCTTGTCAGCCGCCGGGGGGGCGCCTCTGCCCCCCGGGCCCGTGCCCGCCGGAGACCCCAACAC
GAACACTGTCTGAAAGCGTGCAGTCTGAGTTGATTGAATGCAATCAGTTAAAACTTTCAACAATGGATCT
CTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAATGTGAATTGCAGAATTCAGTGAAT
CATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCT
GCCCTCAAGCCCGGCTTGTGTGTTGGGTCGCCGTCC

>*Aspergillus chevalieri* CBS | 535 bp|internal transcribed spacer sequence

ACTTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGCGGGCCCTCTGGGTCCAACCTCCCATCCGT
GTCTATCTGTACCCTGTTGCTTCGGCGTGGCCACGGCCCGCCGGAGACTAACATTTGAACGCTGTTTGAA
GTTTGCAGTCTGAGTTTTTAGTTAAACAATCGTTAAAACTTTCAACAACGGATCTCTTGGTTCCGGCATC
GATGAAGAACGCAGTGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAA
CGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCACGGC
TTGTGTGTTGGGCTTCCGTCCCTGGCAACGGGGACGGGCCCAAAAGGCAGTGGCGGCACCATGTCTGGTC
CTCGAGCGTATGGGGCTTTGTCACCCGCTCCCGTAGGTCCAGCTGGCAGCTAGCCTCGCAACCAATTTTT
TTAACCAGTTTGACTTTCGGATTCAAGGGAGGTAAATTACATCGG

>*Aspergillus foetidus* N1| 453 bp|internal transcribed spacer sequence

GTTGCTTCTGCGGGCCCGCCGCTTGTCGGCCGCCGGGGGGGCGCCTCTGCCCCCCGGGCCCGTGCCCGCC
GGAGACCCCAACACGAACACTGTCTGAAAGCGTGCAGTCTGAGTTGATTGAATGCAATCAGTTAAAACTT
TCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAATGTGAATTGC
AGAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTC
CGAGCGTCATTGCTGCCCTCAAGCCCGGCTTGTGTGTTGGGTCGCCGTCCCCCTCTCCGGGGGGACGGGC
CCGAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACATGCTCTGTAGGATT
GGCCGGCACCTGCCGACGTTTTCCAACCATTCT

>*Aspergillus tubingensis* SACCR110743| 421 bp|internal transcribed spacer sequence

CCCGCCGGAGACCCCAACACGAACACTGTCTGAAAGCGTGCAGTCTGAGTTGATTGAATGCAATCAGTTA
AAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAATGTG
AATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATG
CCTGTCCGAGCGTCATTGCTGCCCTCAAGCCCGGCTTGTGTGTTGGGTCGCCGTCCCCCTCTCCGGGGGG
ACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACATGCTCTGT
AGGATCGGCCCACTCCGCCGAGGAGCTTCCCAATCATCGGAGCAGCAGTCAATAAGTCGAGAGCATCTCG
C

>*Aspergillus aculeatus* YLA2| 370 bp|internal transcribed spacer sequence

TAGGAAAAGGCATGGGGTCTTCGGGGCCCAACCTCCCACCCGTGCTTACCGATACCCTGTTGCTTCGGCG
GGCCCGCCTTCGGGCGGCCCGAGGGCCTGCCCCCGGGACCGCAGCCCGCCGGAGACCCCAATGGAACACT
GTCTGAAAGCGTGCAGTCTGAGTCGATTGATACCAATCAGTCAAAACTTTCAACAATGGATCTCTTGGTT
CCGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAATGTGAATTGCAGAATTCAGTGAATCATCGAG
TCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTTCTCCCCTCC
AGCCCCGCTGGTTGTTGGAC

> *Aspergillus nomiae* strain OKra-A |924bp|internal transcribed spacer sequence

CAAAGCGGGAAGACCGTATGTGACCTGCGGCATATCAATAAGCGGAGGATCCGTAGGTGAACCTGCGGCA
TATCAATAAGCGGAGGATCCGTAGGTGAACCTGCGGCATATCAATAAGCGGAGGATCCGTAGGTGAATCT
GTCTCATATCAATAAGCCGAAGATCCTTGTGTCAACCTCCGTTATATCTTTCAACAATGGATCTCTTGGT
TCCGGCATCGATTAACAATGCACCGAAATGCCATAACTAGTGTGAATTGCAGAATTCCGTGAATCATCGA
GTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCAT
CAAGCACGGCTTGTGTGTTGGGTCGTCGTCCCCTCTCCGGGGGGGACGGGCCCCAAAGGCAGCGGCGGCA
CCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGCCGGCGCTTGCCGAACG
CAAATCAATCTTTTTCCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAA
GCGGAGGAAGATCATTTGAAGCTGTGGGTTTCTATTATGCCACCGACCTCCATGTTTAGGTACTCATTTT
TTTCGCGGGCCACCTTTCACGTCACATGCGCTTTATCTTGACCCGCCAGAACTCTAAAATCTGTCTTATC
TGTTGTAGTCGAGTTGATTGTATTGCATCTTTTCAAATATTACAATGGGAGATTGTGTTCGCATCCATCA
AAAAATCACTAATGCGTTTACGTTGGGAATTGCATAAATCCTGTATCACAGTCATTTAAAGATATTCGCC
TATTTTTTCGAGGAGGCAGCTTTCGATCTTCTTTGTTCACACAGCGGACGGTGGTTTTGTGGTTCTTCCT
TCTCTCGGAAGAGA

> *Aspergillus oryzae* DJL-FUNG03B 451 bp|internal transcribed spacer sequence

AGCCCCGGGCCCGCGCCCGCCGGAGACACCACGAACTCTGTCTGATCTAGTGAAGTCTGAGTTGATTGTA
TCGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGC
GATAACTAGTGTGAATTGCAGAATTCCGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTAT
TCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCATCAAGCACGGCTTGTGTGTTGGGTCGTCGTCC
CCTCTCCGGGGGGGACGGGCCCCAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTT
GTCACCCGCTCTGTAGGCCCGGCCGGCGCTTGCCGAACGCAAATCAATCTTTTTCCAGGTTGACCTCGGA
TCAGGTAGGGATACCCGCTGAACTTAAGCAT

>*Aspergillus assiutensis* isolate CBS 132773|702bp|internal transcribed spacer sequence

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CGAGTTGTTTGGGAATGCAGCTCTAATGGGTGGTAAATTTCATCTAAAGCTAAATACTGGCCGGAGACCG
ATAGCGCACAAGTAGAGTGATCGAAAGATGAAAAGCACTTTGAAAAGAGAGTTAAACAGCACGTGAAATT
GTTGAAAGGGAAGCGCTTGCGACCAGACTCGCCCTCGGGGTTCAGCCGGCACTCGTGCCGGTGTACTTCC
CCGGGGGCGGGCCAGCGTCAGCTTGGGCGGCCGGTCAAAGGCCTCCGGAATGTAGTGCCCCCCGGGGCAC
CTTATAGCCGGAGGTGCAATGCGGCCAGCCTGGGCTGAGGAACGCGCTTCGGCACGGACGCTGGCATAAT
GGTCGCAAACGACCCGTCTTGAAACACGGACCAAGGAGTCTAACATCTACGCGAGTGTTCGGGTGTCAAA
CCCGTACGCGCAGTGAAAGCAAACGGAGGTGGGAGCCCCCTCGCGGGGGCGCACCATCGACCGATCCTGA
TGTCTTCGGATGGGATTTGAGTAAGAGCCGTAAATGTGGGGGACCCGAAAGATGGTGAACTATGCCTGGA
AT

>*Aspergillus welwitschiae* strain LayoO1|268bp|internal transcribed spacer sequence

CTGTTTGAAAGCGTGCAGTCTGAGTTGATTGAATGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGG
TTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAATGTGAATTGCAGAATTCAGTGAATCATCG
AGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCT
CAAGCCCGGCTTGTGTGTTGGGTCGCCGTCCCCCTCTCCGGGGGGACGGGCCCGAAAG

>*Candida metapsilosis* CP59| 370 bp|internal transcribed spacer sequence

GTTGCTTACTGCAATCCTTTTCTTTCTACACATGTGTTTTTCTTTTTTTTGAAAACTTTGCTTTGGTGGG
CCCACGGCCTGCCAAAAATTAAACTCAACCAAATTTTTATTTAATTGTCAACTTGATTAACTAATAGTCA
AAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAAAACGCAGCGAAATGCGATAAGTAATATG
AATTGCAAATATTCGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATG
CCTGTTTGAGCGTCATTTCTCCCTCAAACCCTCGGGTTTGGTGTTGAGCGATACGCTGGGTTTGCTTGAA
AAAAAGGCGGAGTATAAACT

> *Candida metapsilosis* CBS 10907| 323 bp|internal transcribed spacer sequence

CGTAGGTGAACCTGCGGAAGGATCATTACAGAAATAGGAGAAGGTTGCTTAACTGCAATCCTTTTCTTTT
TACACATGTGTTTTTTTTTTTTTTGAAAACTTTGCTTTGGTGGGCCCACGGCCTGCCAGAGATTAAACTC
AACCAAATTTTTATTTAATTGTCAACTTGATTAACTTATAGTCAAAACTTTCAACAACGGATTTTTTGGT
TCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATTTGAATTGCAGATTTTCGTGAATCATTGA
ATCTTTGAACGCACCATGCGCCCTTTGGTATTCCAAAGGGCAT

>*Candida tropicalis* Hb39| 371 bp|internal transcribed spacer sequence

TTTTTATTGAACAAATTTCTTTGGTGGCGGGAGCAATCCTACCGCCAGAGGTTATAACTAAACCAAACTT
TTTATTTACAGTCAAACTTGATTTATTATTACAATAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTC
GCATCGATGAAGAACGCAGCGAAATGCGATACGTAATATGAATTGCAGATATTCGTGAATCATCGAATCT
TTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTTGAGCGTCATTTCTCCCTCAAACC
CCCGGGTTTGGTGTTGAGCAATACGCTAGGTTTGTTTGAAAGAATTTAACGTGGAAACTTATTTTAAGCG
ACTTAGGTTTATCCAAAAAGC

> *Candida albicans*| 495 bp| internal transcribed spacer sequence

GATCATTACTGATTTGCTTAATTGCACCACATGTGTTTTTCTTTGAAACAAACTTGCTTTGGCGGTGGGC
CCAGCCTGCCGCCAGAGGTCTAAACTTACAACCAATTTTTTATCAACTTGTCACACCAGATTATTACTTA
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GGGCATGCCTGTTTGAGCGTCGTTTCTCCCTCAAACCGCTGGGTTTGGTGTTGAGCAATACGACTTGGGT
TTGCTTGAAAGACGGTAGTGGTAAGGCGGGATCGCTTTGACAATGGCTTAGGTCTAACCAAAAACATTGC
TTGCGGCGGTAACGTCCACCACGTATATCTTCAAACTTTGACCTCAAATCAGGTAGGACTACCCGCTGAA
CTTAA

>*Candida metapsilosis*19MWFY29.1| 323 bp| internal transcribed sequence

CGTAGGTGAACCTGCGGAAGGATCATTACAGAAATAGGAGAAGGTTGCTTAACTGCAATCCTTTTCTTTT
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AACCAAATTTTTATTTAATTGTCAACTTGATTAACTTATAGTCAAAACTTTCAACAACGGATTTTTTGGT
TCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATTTGAATTGCAGATTTTCGTGAATCATTGA
ATCTTTGAACGCACCATGCGCCCTTTGGTATTCCAAAGGGCAT

>*Candida orthopsilosis* ilia 39| 296 bp|internal transcribed spacer sequence

TAAAAAAATGAAGTGCTTACTGCATTATTTTTTACACATGTGTTTTTCTTTTTTTTTTGAAAACTTTGCTT
TGGTGGGCCCATGGCCTGCCAGAGATTAAACTCAACCAAATTTTTATTTAAGTCAACTGATTAACTAATA
GTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA
TATGAATTGCAGATATTCGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGG
CATGCCTGTTTGAGCG

>*Cladosporium cladosporioides* DBR-4| 523 bp|internal transcribed spacer sequence

TTTCCGTAGGTGAACCTGCGGAGGGATCATTACAAGTGACCCCGGTCTAACCACCGGGATGTTCATAACC
CTTTGTTGTCCGACTCTGTTGCCTCCGGGGCGACCCTGCCTTCGGGCGGGGGCTCCGGGTGGACACTTCA
AACTCTTGCGTAACTTTGCAGTCTGAGTAAACTTAATTAATAAATTAAAACTTTTAACAACGGATCTCTT
GGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCAT
CGAATCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGGCATGCCTGTTCGAGCGTCATTTCACCA
CTCAAGCCTCGCTTGGTATTGGGCAACGCGGTCCGCCGCGTGCCTCAAATCGACCGGCTGGGTCTTCTGT
CCCCTAAGCGTTGTGGAAACTTTTCCCTAAAGGGTGCTCGGGAGGGTACGCCGTAAACTGGCGACCCTGC
GGCATTCAATAAGCGGAGAAATTCCGTAGGGGG

>*Cladosporium tenuissimum* SFC20230103-M83| 261 bp|internal transcribed spacer sequence

GATAAGTAATTTGAATTGCAGAATTCAGTGAATCATTGAATCTTTGAACGCACATTGCGCCCCCTGGTAT
TCCGGGGGGCATGCCTGTTCGAGCGTCATTTCACCACTCAAGCCTCGCTTGGTATTGGGCAACGCGGTCC
GCCGCGTGCCTCAAATCGACCGGCTGGGTCTTCTGTCCCCTAAGCGTTGTGGAAACTATTCGCTAAAGGG
TGCTCGGGAGGCTACGCCGTAAAACAAACCCATTTCTAAGGTTGACCTCGG

>*Cladosporium marinum* strain SFC20230103-M34|230bp| internal transcribed spacer sequence

CAAACTCTTGCGTAACTTTGCAGTCTGAGTAAACTTAATTAATAAATTAAAACTTTTAACAACGGATCTC
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ATCGAATCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTTCAC
CACTCAAGCCTCGCTTGGTA

>*Clavispora lusitaniae* IFM56973| 294 bp|internal transcribed spacer sequence

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TTTTTTATTCTAACAAACTAAATCAAAACTTTCAACAACGGATCTCCTGGTTCTCGCATCGATGAAGAAC
GCAGCGAATTGCGATACGTAGTATGACTTGCAGACGTGAATCATCGAATCTTTGAACGCACATTGCGCCT
CGAGGCATTCCTCGAGGCATGCCTGTTTGAGCGTCGCATCCCCTCTAACCCCCGGTTAGGCGTTGCTCCG
AAATATCAACCGCG

>*Curvularia affinis*| 554 bp|internal transcribed spacer sequence

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GTTGCAATCAGCGTCAGTACAACAAATGTAAATCATTTACAACTTTCAACAACGGATCTCTTGGTTCTGG
CATCGATGAAGAACGCAGCGAAATGCGATACGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTT
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TTGCTTGGTGTTGGGCGTTTTGTCTTTGGTTGCCAAAGACTCGCCTTAAAACGATTGGCAGCCGGCCTAC
TGGTTTCGCAGCGCAGCACATTTTTGCGCTTGCAATCAGCAAAAGAGGACGGCACTCCATCAAGACTCTT
TATCACTTTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAG

> *Cunninghamella blakesleeana* isolate A-11|335bp| internal transcribed spacer sequence

CTCTCTCTCTCTCAATTAAAAATCATCCACAGTGTGGGAAATGTCTTTCATTTAAACGCTTGTGCCTGGT
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AGATAGCACCTCTTGTAAAAGGGGATAAGATTTCATTTTATTATACTTTTTTTTTTTTAACTGAACTGAT
AAACCATAAAATCTATGGTTGTTTTTTATTATAAAAAAATTAAAAACAACTTTCAGCAATGGATCTCTCG
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>*Debaryomyces hansenii* isolate HBLC10|550bp|internal transcribed spacer sequence

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AAAATCTTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGAT
AAGTAATATGAATTGCAGATTTTCGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTCTGGTATTCC
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TCGAACTAGGCGTTTGCTTGAAATGTATTGGCATGAGTGGTACTGGATAGTGCTATATGACTTTCAATGT
ATTAGGTTTATCCAACTCGTTGAATAGTTTAATGGTATATTTCTCGGTATTCTAGGCTCGGCCTTACAAT
ATAACAAACAAGTTTGACCTCAAATCAAGTAGGATTACCCGCTGAACTTAAGCATATCA

>*Dipodascus albidus* strain CBS 766.85|609bp|internal transcribed spacer sequence

GTAAGAGCATAGCTGTTGGGACCCGAAAGATGGTGAACTATGCCTGAATAGGGTGAAGCCAGAGGAAACT
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ATCGAACCATTTAGTAGGTGGTTCCTGCCGAAGTTTCCCTCCGGATAGCAGAAGCTTGTATCAGTTCTAT
GAGGTAAAGCGAATGATTAGAGGTACTGGGGTGTATGTGACCTTAACCTATTCTCAAACTTTAAATAAGT
AAGAAGTCCTTGTTTCTTAATTGAACGTGGACATATGAATGAAGAGCTGTTAGTGGGCCATTTCTGGTAA
GCAGAACAGGCGATGCGGGATGAACCGAACGCGGAGTTAAGGTGCCGGAATACACGCTCATCAGACACCA
CAAAAGGTGTTAGTTCATCTAGACAGCCGGACGGTGGCCATGGAAGTCGGAATCCGATAAGGAGTGGGTA
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>*Geotrichum candidum* AY06| 316 bp| internal transcribed spacer sequence

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TTTTTGAACGCACATTGCACTTTGGGGTATCCCCCAAAGTATACTTGTTTGAGCGTTGTTTCTCTCTTGG
AATTGCATTGCTTTTCTAAAATTTCGAATCAAATTCGTTTGAAAAACAACACTATTCAACCTCAAATCAA
GTAGGATTACCCGCTGAACTTAAGCATATCAATAAG

> *Galactomyces candidus* isolate CCF 5692|744bp| internal transcribed spacer sequence

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TTGAACGCACATTGCACTTTGGGGTATCCCCCAAAGTATACTTGTTTGAGCGTTGTTTCTCTCTTGGAAT
TGCTTTGCTCTTCTAAAATTTCGAATCAAATTCGTTTGAAAAACAACACTATTCAACCTCAGATCAAGTA
GGATTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCAACAGGGATTGCCTTAGTAAC
GGCGAGTGAAGCGGCAAAAGCTCAAATTTGAAATCGGCCCCCAGGTCGAGTTGTAATTTGTAGATTGTAT
CTTGAGAGCGGATTAAAGTCTGTTGGAACACAGCGCCTTAGAGGGTGACAGCCCCGTAAAATCTATTCTC
ATTGTAAGATACTTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGAGGTAAATTCCTTCTA
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GAGAGTGAAAAAGTACGTGAAATTGTTAAAAGGGAAGGGTATTGAATCAGACTTGGTGCTGTTGTTCAAC
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>*Nigrospora oryzae* MPL-S7L2A| 540 bp|internal transcribed spacer sequence

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GGGACCTCGCGCCCCGGGGGGCCCGCCGGCGGACAAACCAAACTCTGTTATCTTCGTTGATTATCTGAGT
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AGTATTCTAGTGGGCATGCCTGTTCGAGCGTCATTTCAACCCCTAAGCACAGCTTATTGTTGGGACTCTA
CGGCTTCGTAGTTCCCCAAAGACATTGGCGGAGTGGCAGCAGTCCTCTGAGCGTAGTAATTCTTTATCTC
GCTTTTGTTAGGCGCTGCCCCCCCGGCCGTAAAACCCCCAATTTGTTCTG

>*Penicillium citrinum*| PechiHA25A01 329 bp|internal transcribed spacer sequence

CCGTTCGGAGTGAGGTTCCTCGGGGCCCAGCTCCCACCCGTGTTGCCCGAACCTATGTTGCCTCGGCGGG
CCCCGCGCCCGCCGACGGCCCCCCTGAACGCTGTCTGAAGTTGCAGTCTGAGACCTATAACGAAATTAGT
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TGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCTCTGGTATTCCGGAGGGCA
TGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCCCGGCTTGTGTGTTGGG

> *Penicillium citrinum* ED2 | 472 bp|internal transcribed spacer sequence

CCGAACCTATGTTGCCTCGGCGGGCCCCGCGCCCGCCGACGGCCCCCCTGAACGCTGTCTGAAGTTGCAG
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CGCAGCGAAATGCGATAACTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATTG
CGCCCTCTGGTATTCCGGAGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCCCGGCTTGTGTGTT
GGGCCCCGTCCCCCCCGCCGGGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCG
TATGGGGCTTCGTCACCCGCTCTAGTAGGCCCGGCCGGCGCCAGCCGACCCCCAACCTTTAATTATCTCA
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>*Penicillium hispanicum*D06| 357 bp|internal transcribed spacer sequence

CCACCTCCCACCCGTGTCGACCGTACCGTGTTGCTTCGGCGGGCCCGCCGTCTGGCCGCCGGGGGGCGTT
CGCCCCCGGGCCCGCGCCCGCCGAAGACCCCCCTGTGAACGCTGTCTGAAGCTTGCAGTCTGAGCGAAAA
CGCACAATCAGTCAAAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATG
CGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTA
TTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCCCGGCTTGTGTGTTGGGCCGCCGTCC
CCCGCCT

>*Penicillium oxalicum* Tingo Ho 4| 374 bp|internal transcribed spacer sequence

CGAAGACACACAAACGAACTCTTGTCTGAAGATTGCAGTCTGAGTACTTGACTAAATCAGTTAAAACTTT
CAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCA
GAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCC
GAGCGTCATTGCTGCCCTCAAGCACGGCTTGTGTGTTGGGCTCTCGCCCCCCGCTTCCGGGGGGCGGGCC
CGAAAGGCAGCGGCGGCACCTGTGGCATCCAATAGGGTGAGGAACATCTCTTTGGAACCTGGGGCATATC
AATAAGCGGAGGAACATCCGTAGG

>*Penicillium coffeae* SCAU032| 290 bp|internal transcribed spacer sequence

GCCCGCCGAAGACACCCCAAACGCTGTCTGAAGATTGCAGTCTGAGCGATAAGCACAAATTAGTTAAAAC
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GCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTG
TCCGAGCGTCATTGCTGCCCTCAAGCCCGGCTTGGTCTTGGGCGCCGTCCCCCCGGGGACGTGCCCGAAA
GGCAGCGGCG

>*Penicillium oxalicum* | 460 bp|internal transcribed spacer sequence

TATGCCGCAGGTTCACCTACGGATGTTCCTCCGCTTATTGATATGCCGCAGGTTCACCTACGGATGTTCC
TCCGCTTATTGATATGCCGCAGGTTCACCTACGGATGTTCCTCCGCTTATTGATATGCCGCAGGTTCACC
TACGGATGTTCCTCCGCTTATTGATATGCCGCAGGTTCACCTACGGATGTTCCTCCGCTTATTGATATGC
CGCAGGTTCACCTACGGATGTTCCTCCGCTTATTGATATGCCACAAGTTCACCTACGGATGTTCCTCCGC
TTATTGATATGCCGCACGTTCACATACGGATGTTCCTCCGCTTATTGATATGCCGCAGGTTCACCTACAG
ATGTTCCTCCGCTTATTGAGATGCCGCAAGTTCACCTAAAGATGTTCCTCCGCTTATTGATATGCCGCAT
GTTCAACAAAAGATGTTCCTCCGCTTATTGATATGCCTCA

>*Penicillium rubens* 2010F5| 274 bp|internal transcribed spacer sequence

CCCGCGCCCGCCGAAGACACCCTCGAACTCTGTCTGAAGATTGTAGTCTGAGTGAAAATATAAATTATTT
AAAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATACGTAATGT
GAATTGCAAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATG
CCTGTCCGAGCGTCATTTCTGCCCTCAAGCACGGCTTGTGTGTTGGGCCCCGTCCTCCGATC

> *Syncephalastrum* sp.KBR1-1| 244 bp| internal transcribed spacer sequence

TAAAAAAGAAAAAAGCTCTCAATTGAGACTTTGGACTTTTTAAACAACTTTAAGCAATGGATCTCTTGGC
TCTCGCATCGATGAAGAGCGTAGCAAATTGCGATAATTAGTGCGATCTGCATTCTGCGAATCATCGAGTT
CTTGAACGCACCTTGCACCCTTTGGCTTGTCCTTGGGGTATGCTTGTTTCAGTACAACTATAAACCCACA
AATGACATTTTTTTTTTGAAATGTCCATTGTGAT

> *Simplicillium subtropicum* strain SC55A01|176bp|internal transcribed spacer sequence

GGAGCATATCAATAAAGCGGAGGAGCATATCAATAAGCGGAGGAGCATATCAATAAGCGGAGGAGCATAT
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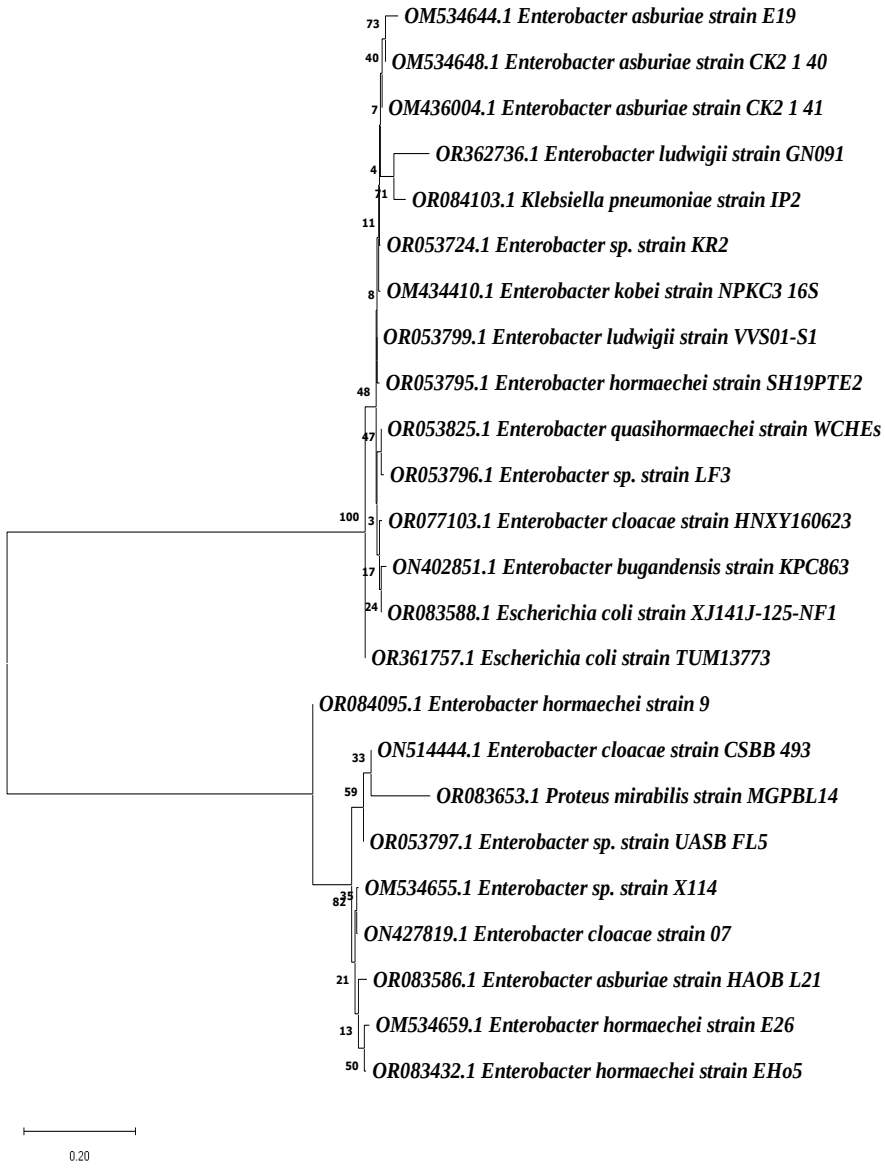

Figure 37. Phylogenetic tree based on partial 16S rRNA gene sequences of family Enterobacteriaceae using Neighbor Joining method and Kimura 2 distance parameter model

Phylogeny of the bacterial species belonging to family Enterobacteriacea, based on partial 16S rRNA gene sequence were resolved. A total of 24 bacterial sequences were involved in the analysis. The tree with a sum of branch length 1.66176010 has obtained. Here the tree has been scaled down to 0.20 meaning each unit length in phylogeny tree corresponds to an evolutionary distance of 0.2 units. The

evolutionary distance were computed using Maximum Composite Likelihood and are in the units of the number of base substitutions per site. Codon positions included in the analysis were $1^{st}+2^{nd}+ 3^{rd}$ +noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 1133 positions in the final data set.

All the species were grouped into distinct clusters according to the genus they belonging to (Figure 37). Among these, *Proteus mirabilis* of the family Enterobacteriaceae showed a high level of divergence from their common ancestor followed by *Enterobacter ludwiggi* and *Klebsiella pneumonia*. The monophyletic clade evolved from common ancestor includes *Enterobacter asburiae* strains, *Enterobacter ludwiggi* and *Klebsiella pneumonia*, *Enterobacter quasihormaechei* and *Enterobacter* species, *Enterobacter cloacae* and *proteus mirabilis* and between *Enterobacter hormaechei*. The other members within the Family Enterobacteriaceae were found to be paraphyletic in nature.

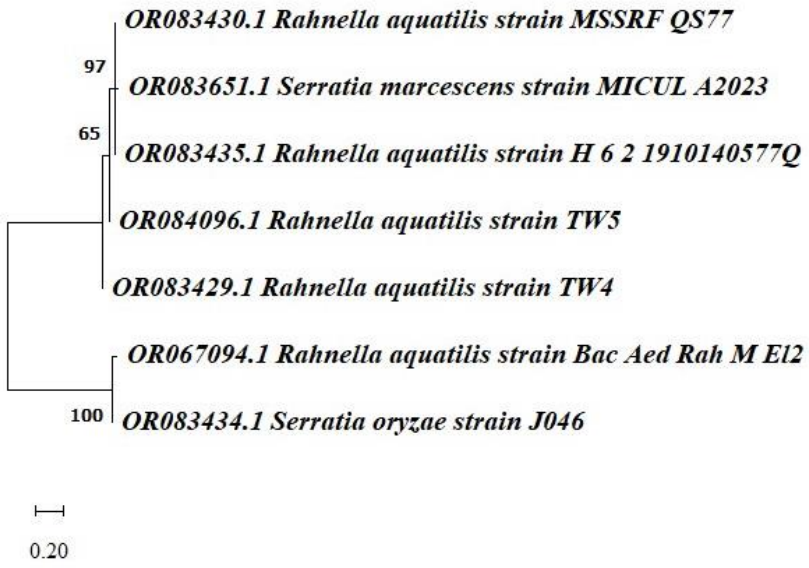

Figure 38. Phylogenetic tree based on partial 16S rRNA gene sequences of family Yersiniaceae using Neighbor Joining method and Kimura 2 distance parameter model

Phylogenetic relationship among the members of family Yersiniaceae based on partial 16S rRNA gene sequences were resolved. Seven bacterial species that were isolated from mosquito larval gut were sequenced during the present work were used for the analysis. The well resolved seven sequence phylogenies were presented in (Figure 38). The tree with a sum of branch length 1.62180851 has obtained. The tree is drawn with a scale of 0.20 was used to indicate branch length within the tree. So, here the branch length are proportional to a genetic or evolutionary distance distance of 0.2 units. The evolutionary distance were computed using Maximum Composite Likelihood and are in the units of the number of base substitutions per site. Codon positions included in the analysis were 1^{st}+2^{nd}+ 3^{rd} +noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 815 positions in the final data set.

The result indicated monophyletic relationship between the genera *Serratia* and *Rahnella* specifically between *Rahnella aquatilis* strain MSSRQS77, *Serratia marcens* strain MICULA2023 and *Rahnella aquatilis* strain H621910140577Q as well as between *Rahnella aquatilis* strain Bac Aed Rah ME12 and Serratia *oryzae* strain J046. The other members in the family were paraphyletic. Among the seven species *Rahnella aquatilis* strain Bac Aed Rah ME12 showed more divergence when compared to other members with in the family.

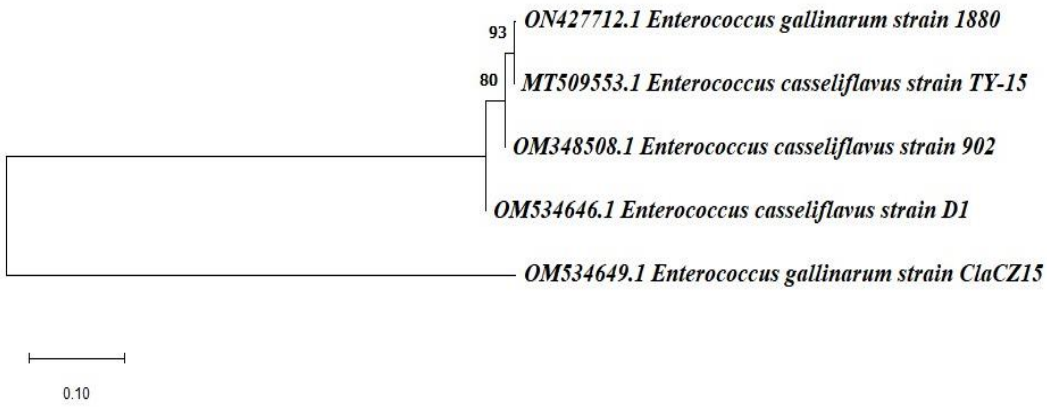

Figure 39 : Phylogenetic tree based on partial 16S rRNA gene sequences of family Enterococcaceae using Neighbor Joining method and Kimura 2 distance parameter model

A total of five bacterial nucleotide sequences exclusively belonged to the family Enterococcaceae were selected for retrieving the phylogenetic tree. The phylogram has been scaled down to 0.10, which means each unit of length in the tree corresponds to represents an evolutionary distance of 0.10 units. The phylogram has a sum of branch length 1.06393076. The evolutionary distance were computed using Maximum Composite Likelihood and are in the units of the number of base substitutions per site. Codon positions included in the analysis were $1^{st}+2^{nd}+ 3^{rd}$ +noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 825 positions in the final data set.

The 16S analysis suggested that, *Enterococcus gallinarum* strain 1880 and *Enterococcus casseliflavus* strain TY-15 exhibited monophyletic relationship, while the other members of the genus *Enterococcus* were paraphyletic. *Enterococcus gallinarum* strain ClaZ15 showed higher degree of divergence followed by *Enterococcus gallinarum* strain1880 (Figure 39).

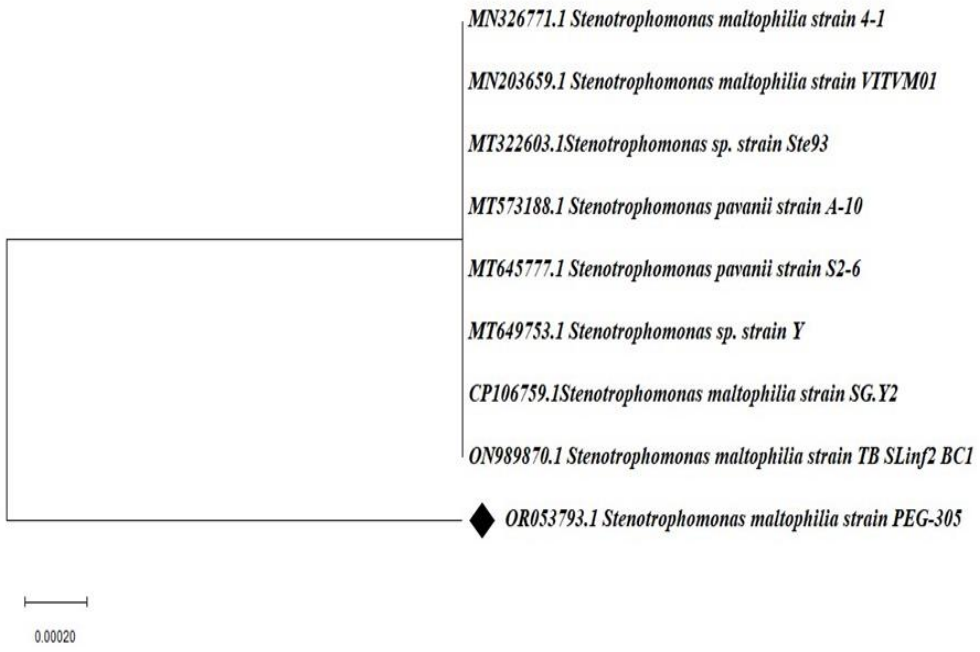

Figure 40. Phylogenetic tree based on partial 16S rRNA gene sequences of family Xanthomonadaceae using Neighbor Joining method and Kimura 2 distance parameter model

The *Stenotrophomonas maltophilia* strain PEG -305, a bacterium isolated from mosquito larval by 16S rRNA sequencing. To construct phylogenetic tree *Stenotrophomonas maltophilia* strain PEG -305 sequence was combined along with sequences retrieved from the Gen Bank database. The phylogram has been adjusted to a scale of 0.00020, where each unit of length in the tree represents an evolutionary distances 0.00020. The evolutionary distance were computed using Maximum Composite Likelihood and are in the units of the number of base substitutions per site. Codon positions included in the analysis were $1^{st}+2^{nd}+3^{rd}$ +noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 344 positions in the final data set. The members of genus *Stenotrophomonas* showed monophyletic origin. *Stenotrophomonas maltophilia* strain PEG -305 showed more divergence when compared with others in phylogeny (Figure 40).

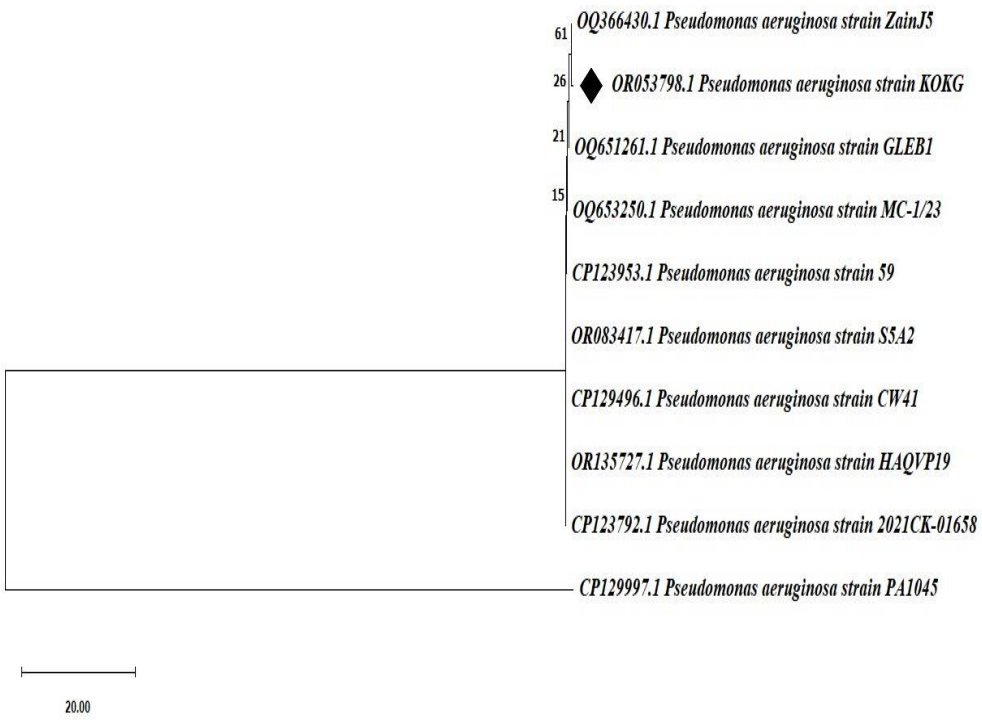

Figure 41. Phylogenetic tree based on partial 16S rRNA gene sequences of family Pseudomonadaceae using Neighbor Joining method and Kimura 2 distance parameter model

The *Pseudomonas aeruginosa*, was identified from mosquito larval gut by 16S rRNA sequencing method. Ten nucleotide sequences were used for phylogenetic construction. Out of this nine nucleotide sequences were retrieved from the Gen Bank database. The phylogenetic tree has been scaled down to represent an evolutionary distance of 20.00 units per unit of length.The resulting phylogenetic tree has a sum of branch length =199.70710549. The evolutionary distance were computed using Maximum Composite Likelihood and are in the units of the number of base substitutions per site. Codon positions included in the analysis were 1^{st}+2^{nd}+ 3^{rd} +noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 424 positions in the final data set.

All the species were grouped into clusters according to the family Pseudomonadaceae they belonging to (Figure 41). Species of the family Pseudomonadaceae was found to possess a monophyletic relationship. The particular clade bears different strains of *Pseudomonas aeuroginoisa*. The highest divergence was observed in *Pseudomonas aeruginosa* strain PA1045.

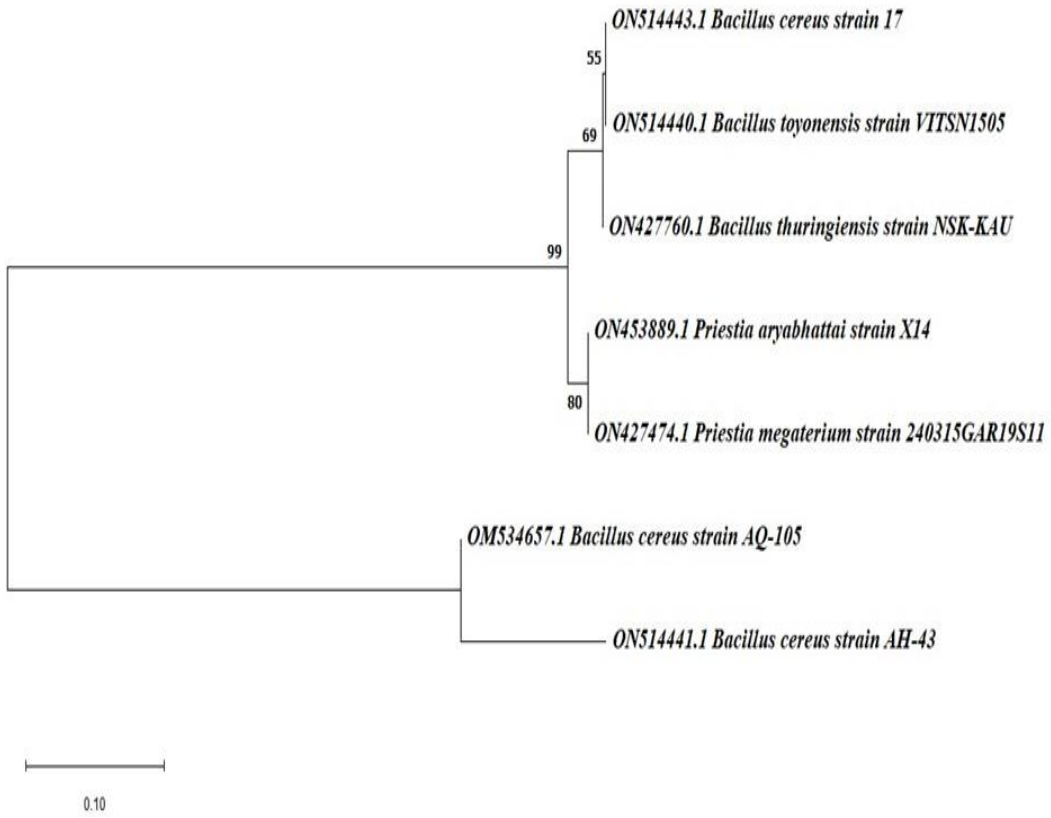

Figure 42. Phylogenetic tree based on partial 16S rRNA gene sequences of family Bacillaceae using Neighbor Joining method and Kimura 2 distance parameter model.

A total of seven bacterial species isolated and identified by 16S rRNA sequences were used for phylogenetic construction. These bacteria belonged to the phylum Firmicutes. The phylogram has been adjusted to a scale of 0.10, where each unit of length in the tree represents an evolutionary distance of 0.10 units. The tree

has a sum of branch length=0.87950977. The evolutionary distance were computed using Maximum Composite Likelihood and are in the units of the number of base substitutions per site. Codon positions included in the analysis were $1^{st}+2^{nd}+ 3^{rd}$ +noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 1006 positions in the final data set. The result indicated the monophyly between *Bacillus cereus* strain 17 and *Bacillus toyonensis* strain VITSN1505, including *Priestia megaterium* strain 240315GAR19S11 and *Priestia aryabhattai* strain X14. Highest evolutionary divergence was exhibited by *Bacillus cereus* strain AH-43 (Figure 42).

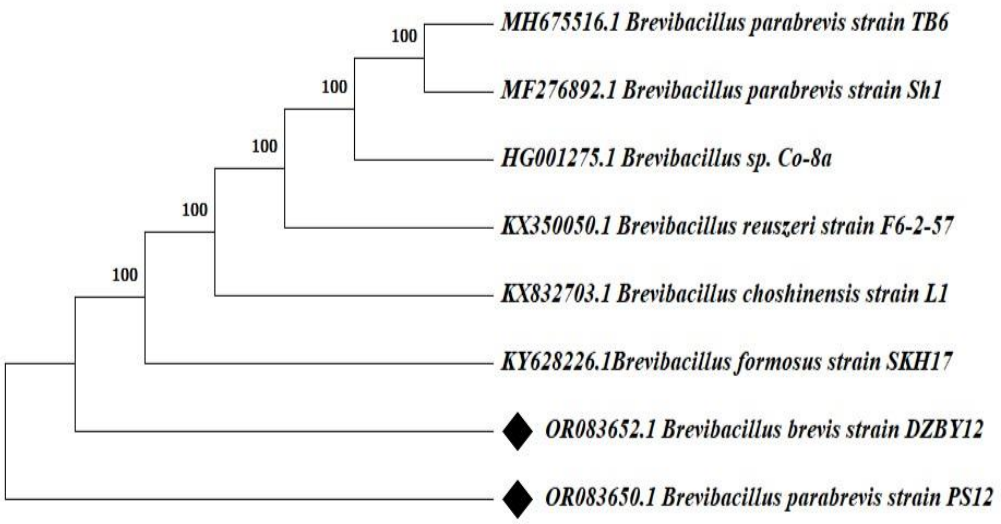

Figure 43. Phylogenetic tree based on partial 16S rRNA gene sequences of family Paenibacillaceae using Neighbor Joining method and Kimura 2 distance parameter model

The 16S rRNA analysis identified the bacterial species as, *Brevibacillus brevis* strain DZBY12 and *Brevibacillus parabrevis* strain PS12 from the gut of mosquito larvae. For constructing phylogenetic tree a total of eight nucleotide sequences were used. Out of this six nucleotide sequences were were taken from the Gen Bank database. Here in the tree the sum of branch length=0.0000. The evolutionary distance

were computed using Maximum Composite Likelihood and are in the units of the number of base substitutions per site. Codon positions included in the analysis were $1^{st}+2^{nd}+ 3^{rd}$ +noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 797 positions in the final dataset. In this phylogeny, all the species belonged to genus *Brevibacillus* exhibited monophyletic origin. Here the scale and branch length is zero indicating no divergence(Figure 43).

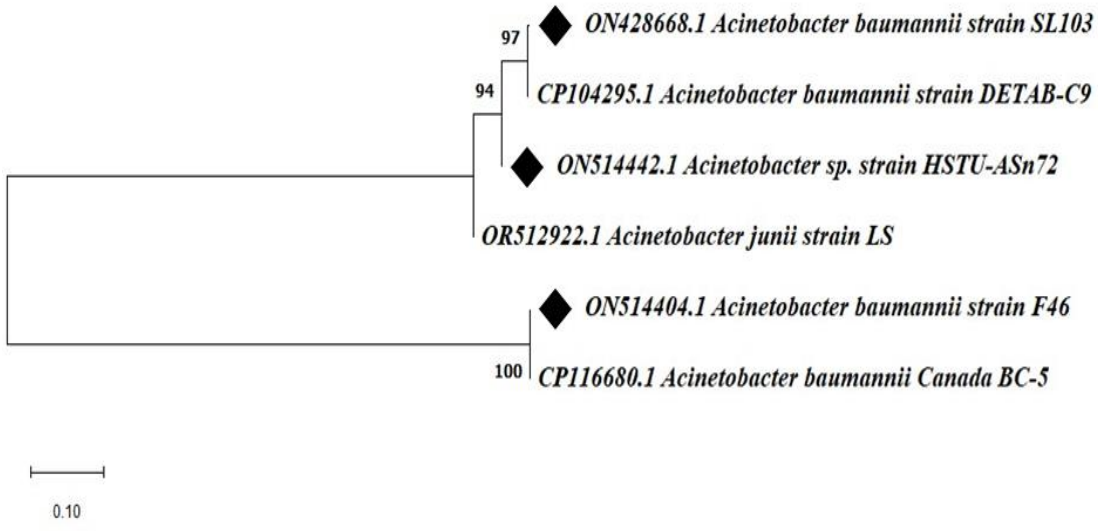

Figure 44. Phylogenetic tree based on partial 16S rRNA gene sequences of family Moraxellaceae using Neighbor Joining method and Kimura 2 distance parameter model

Acinetobacter baumannii strain SL103, *Acinetobacter* sp. strain HSTU-ASn72 and *Acinetobacter baumannii* Canada BC-5 were identified as mosquito gut resident bacteria. The phylogenetic relatedness among the bacteria found in the mosquito larval species was determined by utilising partial coding sequence of 16S rRNA.The phylogenetic tree was constructed by six nucleotide sequences, among these three were retrieved from NCBI-Gen Bank. The resulting phylogenetic tree has a sum of branch length=1.43238833.The tree is drawn with a scale of 0.10 was used

to indicate branch length within the tree. The evolutionary distance were computed using Maximum Composite Likelihood and are in the units of the number of base substitutions per site. Codon positions included in the analysis were $1^{st}+2^{nd}+ 3^{rd}$ +noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 756 positions in the final dataset. The monophyletic group was seen between *Acinetobacter baumannii* strain SL103 and *Acinetobacter baumannii* strain DETAB-C9 as well as between *Acinetobacter baumannii* strain F46 and *Acinetobacter baumannii Canada* BC-5(Figure 44). The highest genetic divergence was shown by *Acinetobacter baumannii Canada* BC-5.

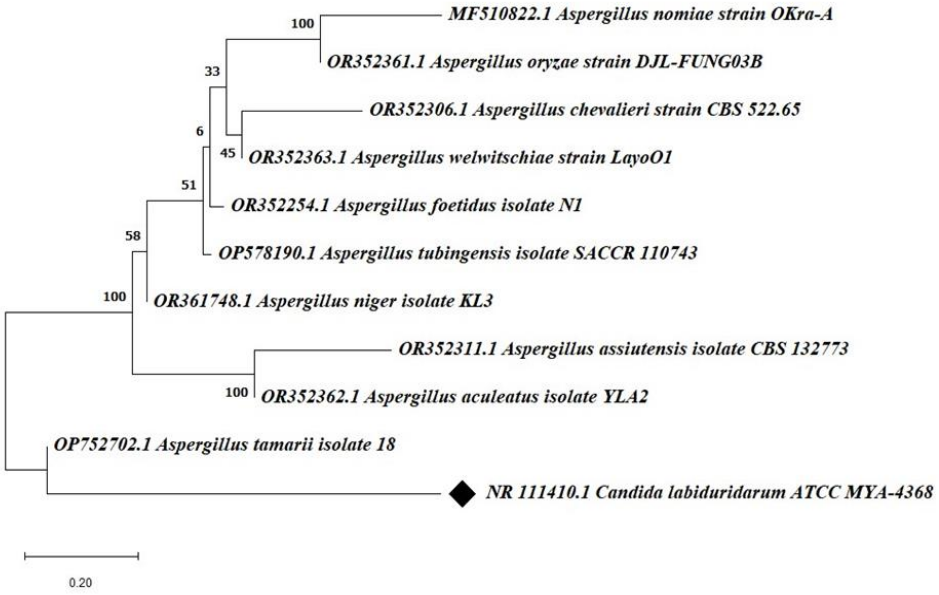

Figure 45. Phylogenetic tree based on ITS sequences of family Aspergillaceae using Neighbor Joining method and Kimura 2 distance parameter model

ITS analysis indicated the presence of ten different species belonged to the genus *Aspergillus*. The sequence of *Candida labiduridarum* was included as out group. The phylogenetic tree was constructed by using these eleven nucleotides. The tree has a sum of branch length =2.27359461. The tree was scaled down to 0.20, indicates branch length within the tree. The evolutionary distance were computed

using Maximum Composite Likelihood and are in the units of the number of base substitutions per site. Codon positions included in the analysis were $1^{st}+2^{nd}+ 3^{rd}$ +noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 944 positions in the final dataset.

The tree showed the presence of monophyletic clade between *Aspergillus nominae* strain Okra-A and *Aspergillus oryzae* strain DJL-FUNG03B, *Aspergillus chevalieri* strain CBS 522.65, and *Aspergillus welwitschaiae* strain LayoO1, *Aspergillus assiutensis* isolate CBS132773 with *Aspergillus aculeatus* isolate YLA2. Other members were paraphyletic to the common ancestor. Here highest evolutionary distance was showed by *Aspergillus nominae* strain Okra-A(Figure 45).

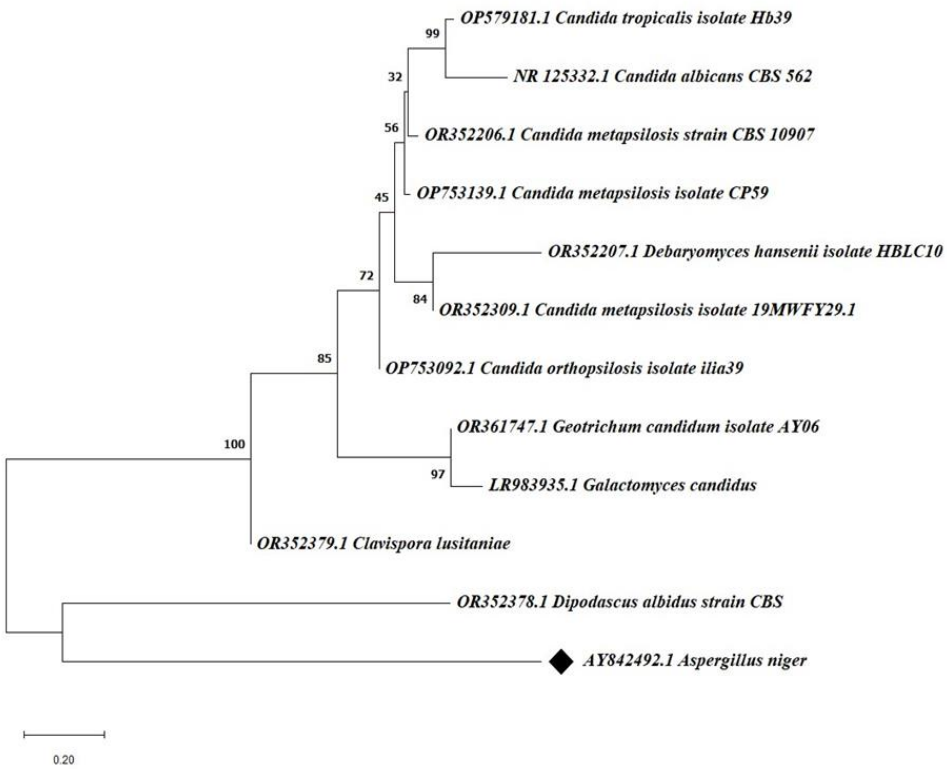

Figure 46. Phylogenetic tree based on ITS sequences of family Debaryomycetaceae using Neighbor Joining method and Kimura 2 distance parameter model

A total of 11 species of fungi belonged to genus *Candida* were identified from the mosquito larval gut across the study by ITS sequencing. The phylogenetic tree

has a sum of branch length= 4.32404850. The analysis involved a total of 12 nucleotide sequences including an outgroup *Aspergillus niger*. The phylogenetic tree has been scaled down to represent an evolutionary distance of 0.20 units per unit of length. The evolutionary distance were computed using Maximum Composite Likelihood and are in the units of the number of base substitutions per site. Codon positions included in the analysis were 1^{st}+2^{nd}+ 3^{rd} +noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 934 positions in the final dataset.

In the phylogram *Candida tropicalis* isolate Hb39 and *Candida albicans* CBS 562, *Debaryomyces hansenii* isolate HBLC10 and *Candida metapsilosis* 19MWFY29.1,*Geotrichium candidum* isolate AY06 and *Galactomyces candidus* remain closely related as sister taxa(monophyly) with same ancestral origin, while other species within the tree remain as paraphyletic (Figure 46). Here a greater divergence was shown by *Debaryomyces hansenii* isolate HBLC10.

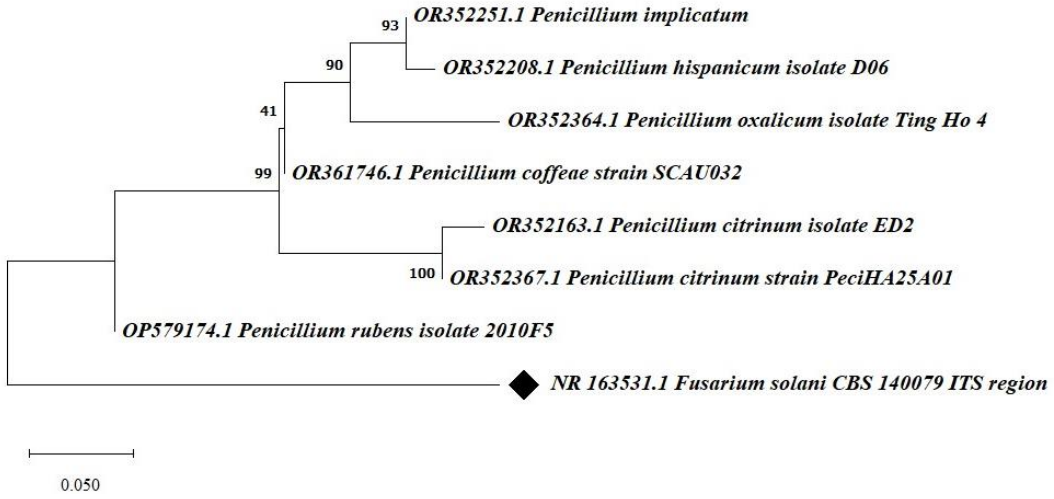

Figure 47. Phylogenetic tree based on ITS sequences of family Trichosphaeriaceae using Neighbor Joining method and Kimura 2 distance parameter model

The phylogenetic relatedness among fungus found in the larval gut of mosquito larvae was investigated by utilising ITS sequencing. The phylogram has

been adjusted to a scale of 0.20, where each unit of length in the tree represents an evolutionary distance of 0.20 units. The tree has a branch length of 0.61306925.The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All ambiguous positions were removed for each sequence pair pairwise deletion option. There were a total of 640 positions in the final dataset.

The phylogenetic tree was reconstructed by using eight nucleotide sequences, out of which one is an outgroup species named *Fusarium solani* CBS 140079. In this tree sister taxa was observed between *Penicillum implicatum* and *Penicillum hispanicum* isolate D06, *Penicillum citrinum* isolate ED2 and *Penicillum citrinum* strain PeciHA25A01. Other members within tree are paraphyletic. Here greatest divergence was exhibited by *Penicillum citrinum* isolate ED2(Figure 47).

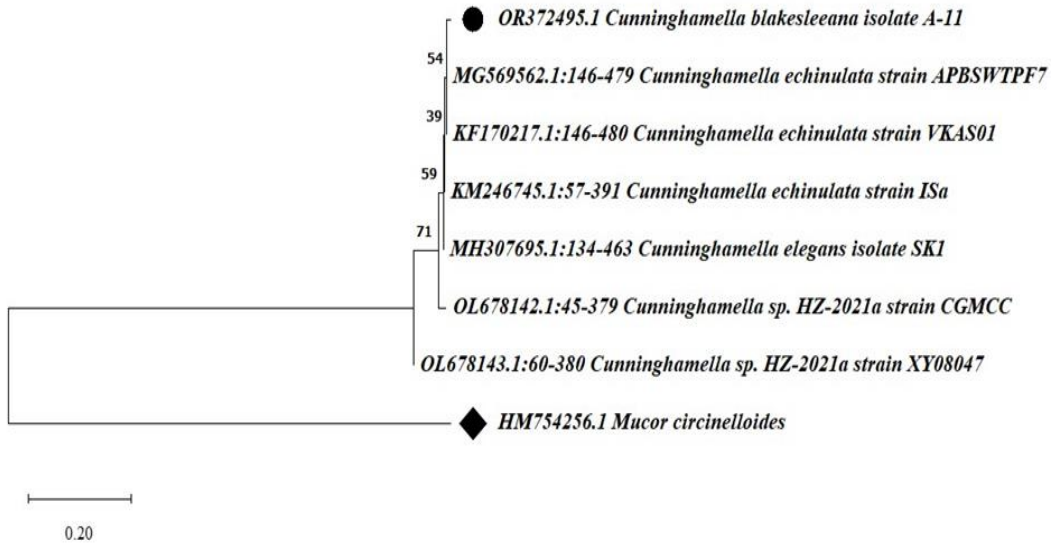

Figure 48. Phylogenetic tree based on ITS sequences of family Cunninghamellaceae using Neighbor Joining method and Kimura 2 distance parameter model

Cunninghamella blakesleena isolate A11 was identified from mosquito larval gut. For constructing phylogenetic tree nucleotide sequences were retrieved from

NCBI Gen Bank. The fungal phylogenetic tree has been scaled down to 0.20 meaning each unit of length in a phylogenetic tree corresponds to an evolutionary distance of 0.2 units. Seven nucleotide sequences were along with *Mucor circinelloides* (as out group) were used for phylogenetic reconstruction. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All ambiguous positions were removed for each sequence pair pairwise deletion option. There were a total of 497 positions in the final dataset. Within the phylogram *Cunninghamella* sp.HZ 2021a strain CGMCC showed higher genetic divergence. Most of them showed paraphyletic relationship with its common ancestor (Figure 48).

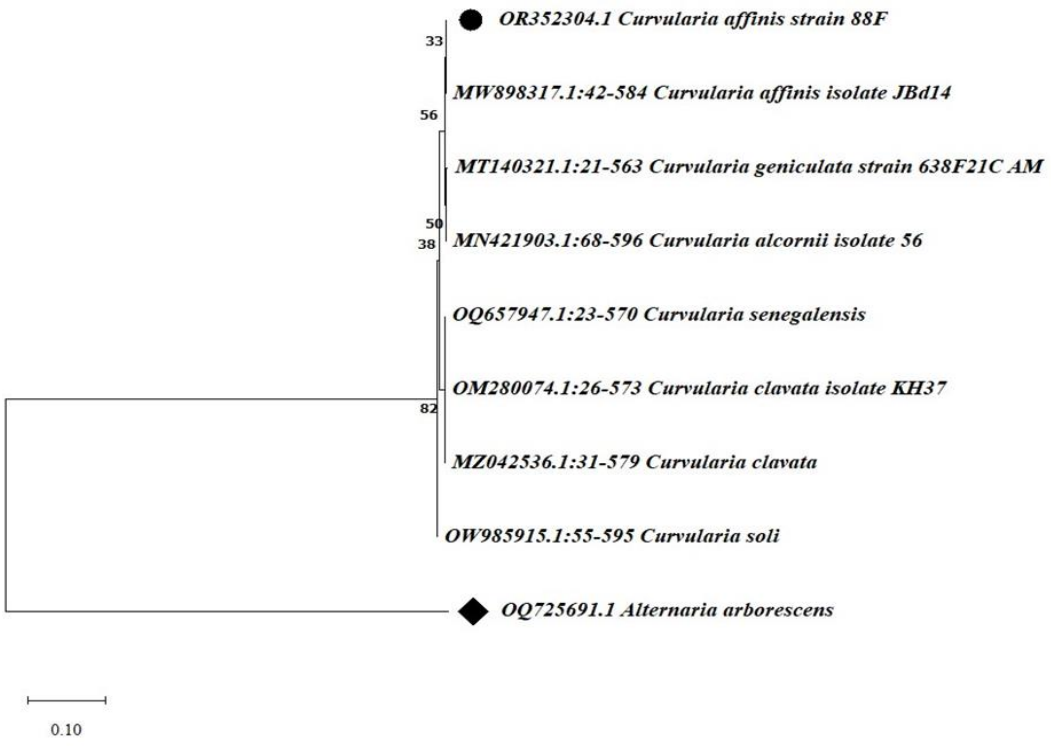

Figure 49. Phylogenetic tree based on ITS sequences of family Pleosporaceae using Neighbor Joining method and Kimura 2 distance parameter model

To analyse the phylogenetic relationship of *Curvularia affinis* strain 88F, nucleotide sequences were retrieved from NCBI gen bank. The tree was analysed by using a total of nine nucleotide sequence including out group *Alternaria arborescens*.

The tree is drawn with a scale of 0.10, indicating each unit of length in phylogenetic tree corresponds to an evolutionary distance of 0.10 units. The tree bears a sum of branch length 1.15424038. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All ambiguous positions were removed for each sequence pair pairwise deletion option. There were a total of 696 positions in the final dataset.

Three species *Curvularia senegalensis*, *Curvularia clavata* isolate KH37 and *Curvularia clavata* with in the phylogram exhibited polytomy (Figure 49). Here greater divergence was represented by *Curvularia clavata* isolate KH37 within the phylogram.

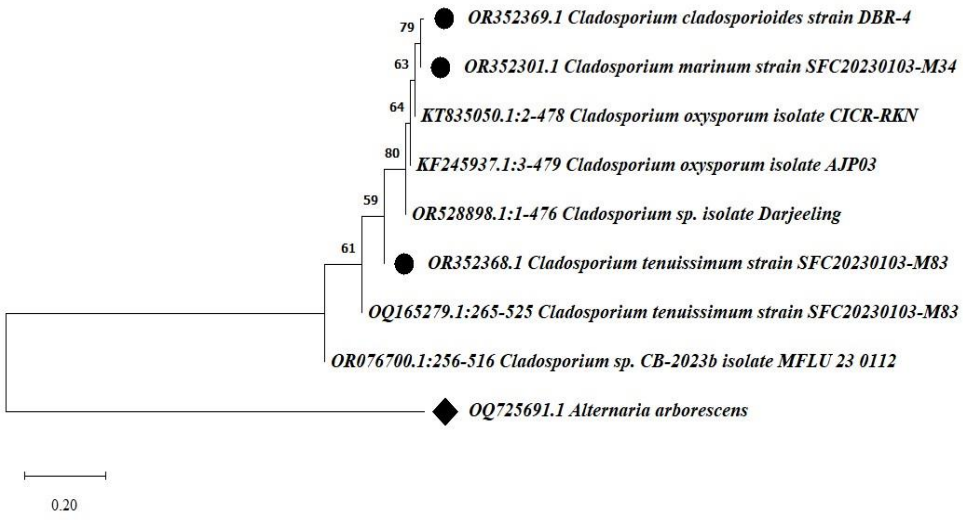

Figure 50. Phylogenetic tree based on ITS sequences of family Davidiellaceae using Neighbor Joining method and Kimura 2 distance parameter model.

Cladosporium tenuissimum strain SFC20230103-M83, *Cladosporium marinum* strain SFC20230103-M34 and *Cladosporium cladosporioides* strain DBR-4 were isolated and identified from mosquito larval gut. The tree was reconstructed by analysing nine nucleotide including *Alternaria arborescens* as out group. The

evolutionary tree has been resized, with each unit of length on the tree representing an evolutionary distance of 0.20 units.

The tree bears a sum of branch length of 2.06457993. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All ambiguous positions were removed for each sequence pair pairwise deletion option. There were a total of 693 positions in the final dataset. While analysis the tree *Cladosporium cladosporioides* strain DBR-4 and *Cladosporium marinum* strain SFC20230103-M34 remained as sister taxa bearing common ancestory. The terminal taxa were paraphyletic in nature (Figure 50). *Cladosporium marinum* strain SFC20230103-M34 showed greater divergence when compared with other species within the tree.

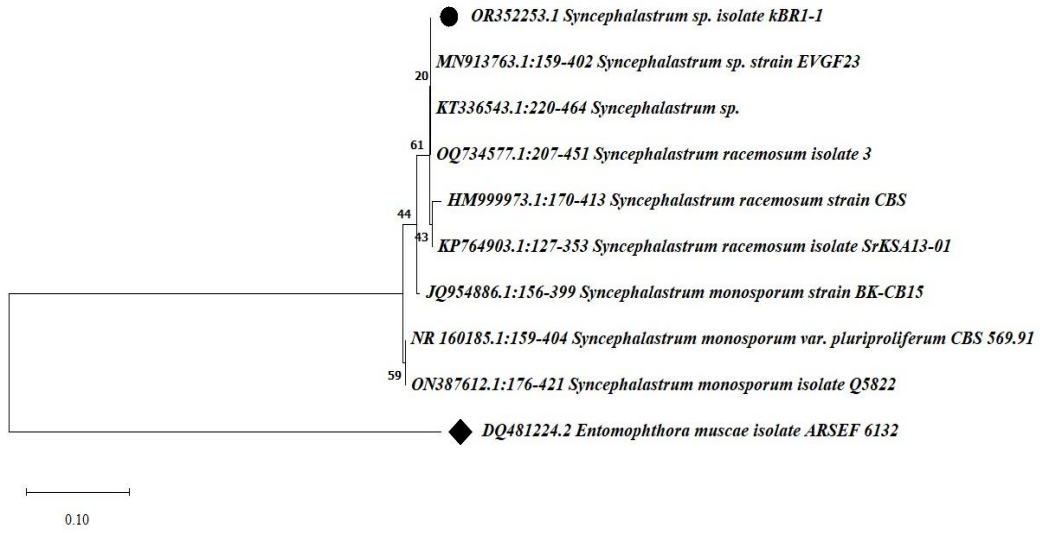

Figure 51. Phylogenetic tree based on ITS sequences of family Syncephalastraceae using Neighbor Joining method and Kimura 2 distance parameter model.

Syncephalastrum sp. isolate kBR1-1 was isolated from mosquito larval gut. Regarding the fungal phylogenetic tree, it was constructed by using ITS sequence. The tree was reconstructed by using sequence of Syncephalastrum sp. isolate kBR1-1 along with sequences retrieved from NCBI- Gen Bank data base. Phylogram

analysis involved 10 nucleotide sequence along with outgroup *Entomophthora muscae* ARSEF 6132. The tree holds a branch length of 2.06457993. The phylogram has been adjusted to a scale of 0.10, where each unit of length in the tree represents an evolutionary distance of 0.10 units. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All ambiguous positions were removed for each sequence pair pairwise deletion option. There were a total of 823 positions in the final dataset. From the phylogenetic tree it was revealed that *Syncephalastrum* sp. isolate kBR1-1, *Syncephalastrum* sp. strain EVGF23, *Syncephalastrum* sp. and *Syncephalastrum racemosum* isolate 3 exhibited polytomy(Figure 51). While *Syncephalastrum racemosum* strain CBS and *Syncephalastrum racemosum* isolate SrKSA13-01 showed monophyletic relationship. *Syncephalastrum racemosum* strain CBS showed greater divergence in the tree.

Figure 52. Phylogenetic tree based on ITS sequences of family Cordycipitacea using Neighbor Joining method and Kimura 2 distance parameter model

The fungus *Simplicillium subtropicum* strain SC55A01 was isolated from mosquito larval gut. The phylogenetic tree was constructed by using ITS sequences. The tree was reconstructed by using sequence of *Simplicillium subtropicum* strain SC55A01 along with sequences retrieved from NCBI- Gen Bank data base. Twelve nucleotide sequences along with out group *Beauveria bassiana* ARSEF 1564 were used for tree reconstruction. The tree is drawn with a scale of 0.10 was used to indicate branch length within the tree. So here the branch length are proportional to a genetic or evolutional distance of 0.10 units. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site Codon positions included in the analysis were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 548 positions in the final dataset. The monophyletic groups having common ancestory was shown by *Fusarium* sp. strain 495F28C-AM and *Lasiodiplodia theobromae* isolate NLBLS and *Cladosporium cladosporioides* strain SC45A01 and *Amycospharella Africana* strain Ct-LP86. In the tree greatest divergence was shown by *Sordariomycetes* sp. HHTG-20151 isolate Y19H3a8epi (Figure 52).

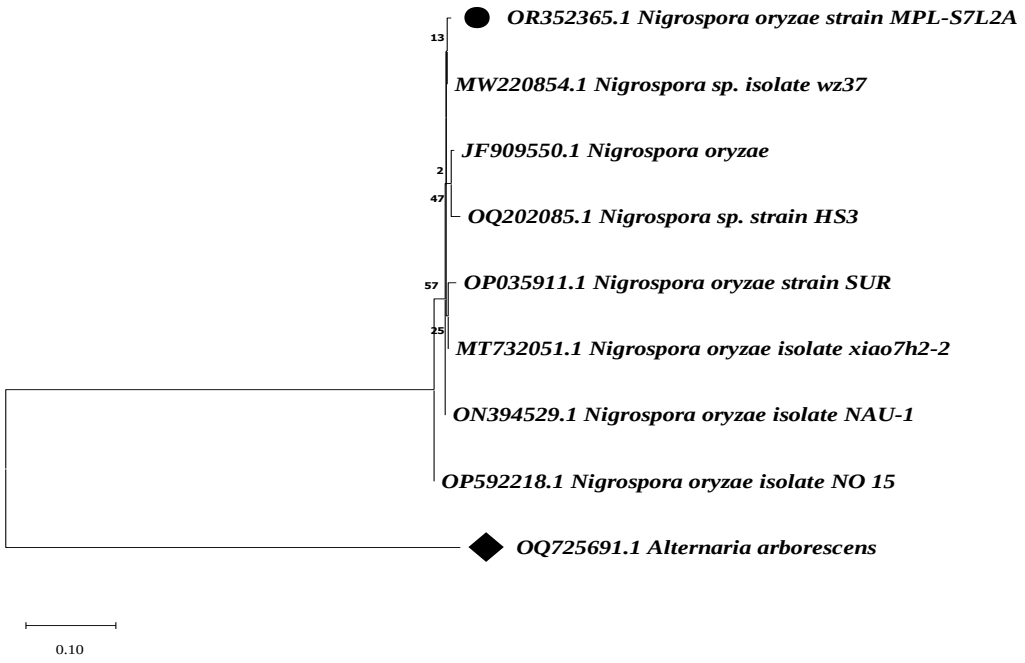

Figure 53. Phylogenetic tree based on ITS sequences of family Trichosphaeriaceae using Neighbor Joining method and Kimura 2 distance parameter model

The fungus *Nigrospora oryzae strain* MPL-S7L2A was obtained from the gut of mosquito larvae. A phylogenetic tree was constructed by using ITS sequence region. The tree was built by incorporating the sequence of *Nigrospora oryzae* strain MPL-S7L2A along with sequences retrieved from NCBI- Gen Bank data base. Nine nucleotide along with *Alternaria arborescens* as out group were used for phylogenetic reconstruction. Here the tree has been scaled down to 0.10 meaning each unit of length in a phylogenetic tree corresponds to an evolutionary distance of 0.10 units. The phylogram has a sum of branch length=1.02784790. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site The codon position included in the analysis were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 691 positions in the final dataset.

Monophyletic groups were seen between taxa *Nigrospora oryzae strain* MPL-S7L2A and *Nigrospora oryzae* sp. isolate wz37, *Nigrospora oryzae* and *Nigrospora sp*. strain HS3, *Nigrospora oryzae* strain SUR and *Nigrospora oryzae* isolate xiao 7h2-2 (Figure 53). Here highest divergence is shown by *Nigrospora oryzae* strain SUR.

2.4 DISCUSSION

Microbial phylogenetics focuses on the evolutionary relatedness among different groups of microorganisms. The molecular approaches to microbial phylogenetic analysis enhance our understanding of their evolutionary history. With the development of molecular taxonomy, more reliable results could be generated. Phylogenetic analysis involving molecular marker genes of diverse origins provides more precise and reliable results. This chapter deals with the study of phylogenetic relationships between bacteria and fungi found in the mosquito larval gut. It employs the 16S rRNA gene for bacteria and ITS region for fungi for getting better resolution (Badotti et al., 2017).

The result of the phylogenetic analysis of the family Enterobacterales demonstrated that *Proteus mirabilis* exhibited a substantially high level of divergence from their common ancestor, followed by *Enterobacter ludwiggi* and *Klebsiella pneumonia*. Abbott's findings mentioned the addition of genera *Proteus, Klebsiella*, *Escherichia*, and *Shigella* to the family Enterobacterales (Abbott, 2011). Within the monophyletic clade evolved from a common ancestor, several species were identified, including *Enterobacter asburiae* strains, *Enterobacter ludwiggi* and *Klebsiella pneumonia*, *Enterobacter quasihormaechei* and *Enterobacter* species, *Enterobacter cloacae* and *proteus mirabilis,* along with the relationship between *Enterobacter hormaechei* strains. Notably, other members of the family Enterobacterales were found to be paraphyletic in nature. Such instances of both monophyletic and paraphyletic relationships are particularly common within the same family.

A number of studies pointed out that the family Enterobacterales has experienced a significant increase in the number of species with the introduction of new molecular techniques for species identification. The inclusion of new members

in this family has been based on characteristics such as phenotypes, habitat, and disease pattern. According to the 2020 List of Prokaryotic Names with Standing Nomenclature, it was discovered that this particular family holds more than 355 species. As of 2020, 14 new genera were listed, along with the transfer of the existing taxa *Klebsiella* sp to new genus *Raultella* (Janda & Abbott, 2021). Moreover, it was observed that members of the family Enterobacterales, especially the genus *Enterobacter*, *Klebsiella*, Proteus, and *Escherichia,* are found as major flora in flying insects of the order Diptera, irrespective of their geographic location (Abbott, 2011).

The result of phylogenetic analysis strongly supported the monophyly of the genera *Serratia* and *Rahnella,* especially evident in *Rahnella aquatilis* strain MSSRQS77 and *Serratia marcens* strain MICULA2023, and *Rahnella aquatilis* strain Bac Aed Rah ME12 and *Serratia oryzae* strain J046. This monophyly was strongly supported by marker genes 16S rRNA with high bootstrap values of 97% and 100%, respectively. This analysis supported the monophyly of the genera *Serratia* and *Rahnella*. In earlier research involving phylogenetic analysis of the endobacterium *Serratia oryzae* strain J11-6T, it was evident that there was a remarkable similarity to *Rahnella aquatilis* JCM 1683^{T} with a bootstrap value of more than 98.3%. The study also highlighted similarity between certain genera like *Yersinia* and *Serratia,* with a 98.0% to 97% bootstrap value (Zhang et al., 2017). Furthermore *Rahnella aquatilis* strain Bac Aed Rah ME12 showed more divergence when compared to other members with in the family.

In the current study, it was reported that *Enterococcus gallinarum* strain 1880 and *Enterococcus casseliflavus* strain TY-15 exhibited a monophyletic relationship with a bootstrap value of 93%, as revealed by phylogenetic analysis of 16S rRNA sequencing. While the other members displayed a paraphyletic relationship,

Enterococcus gallinarum strain ClaZ15 showed a higher degree of divergence, followed by *Enterococcus gallinarum* strain 1880. Prior studies have indicated that the molecular marker *atpA* gene was found to be more effective than 16S rRNA sequencing for distinguishing between species. The *atpA* sequencing revealed the presence of distinct sub-clusters within the genus Enterococcus. Especially two separate subclusters were identified within the *Enterococcus casseliflavus* species group, comprising *Enterococcus gallinarum* and *Enterococcus casseliflavus* and *Enterococcus flavescens.* It was further noted that *Enterococcus casseliflavus* LMG 10745^{T} and *Enterococcus flavescens* LMG 13518T displayed a high degree of relatedness to each other, with a 98.9% *atpA* sequence similarity (Naser et al., 2005). *Stenotrophomonas maltophilia*, previously known as *Xanthomonas maltophilia*, was a common gram-negative bacteria found in soil and water. In the present study, strains from all nine species within the genus *Stenotrophomonas* were examined. The phylogenetic branching pattern is very similar, indicating minimal divergence and a shared ancestral lineage among them. Based on partial *gyrB* gene sequences, it was determined that the stenotrophomonas *maltophilia* complex, which comprises *Stenotrophomonas maltophilia*, *Stenotrophomonas pavanii*, *Stenotrophomonas africana*, *Pseudomonas geniculata*, *Pseudomonas hibisciola*, and *Pseudomonas beteli*, displayed their phylogenetic relatedness (Rhee et al., 2013). Various strains of *Pseudomonas aeruginosa* showed polytomy in their phylogenetic relationships. This observation aligns with several previous studies that involved 112 strains of *Pseudomonas aeruginosa* by whole genome sequencing and tends to cluster into two major groups (Gomila et al., 2015; Lalucat et al., 2020).

The results of the study revealed a monophyletic relationship between *Bacillus cereus* strain 17 and *Bacillus toyonensis* strain VITSN1505, including *Priestia*

megaterium strain 240315GAR19S11 and *Priestia aryabhattai* strain X14. The highest evolutionary divergence was exhibited by *Bacillus cereus* strain AH-43. "*Bacillus cereus* group" comprises Gram positive, spore-forming bacteria that form distinct and homogeneous branches within the *Bacillus genus.* The group includes *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus anthracis*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus weihenstephanensis*, and *Bacillus cytotoxicus*. Earlier studies on phylogenetic reconstruction based on 16S rRNA gene sequences within the *Bacillus cereus* group using the RAxML algorithm pointed out that *Bacillus toyonensis* shared identities greater than 99.5% with the *Bacillus cereus* group and displayed 99.9% similarity to that of *Bacillus thuringiensis*. This is consistent with the present study, which depicts an evolutionary relationship between *Bacillus cereus*, *Bacillus toyonensis*, and *Bacillus thuringiensis with a* slightly lesser bootstrap value (Jiménez et al., 2013). Recently the evolutionary relationship between *Priestia megaterium* strain 240315GAR19S11 and *Priestia aryabhattai* strain X14 was confirmed by examining complete 16S rRNA gene sequences. The analysis revealed the presence of a clade within the cluster containing *Priestia megaterium* *Priestia aryabhattai* and *Priestia flexus* with more than 98% sequence identity, with difference of 3-29 nucleotides(Wagh et al., 2021).

In the present study, two distinct species, *Brevibacillus* strain PS12 and *Brevibacillus brevi*s strain DZBY12, were identified from the mosquito larval gut. About eight nucleotide sequences, including those from the NCBI GenBank, were used to identify near-phylogenetic neighbors. The phylogenomic results showed 100% boostrap value among all the species strains of *Brevibacillus*. In previous studies utilising 16S rRNA sequencing, the presence of the *Bacillus brevis* cluster was identified, which includes ten distinct species: *Bacillus brevis*, *Bacillus agri*, *Bacillus*

centrosporus, *Bacillus choshinensis*, *Bacillus parabrevis*, *Bacillus reuszeri*, *Bacillus fornosus*, *Bacillus borstelensis*, *Bacillus laterosporus*, and *Bacillus thermoruber*. These ten species account for over 93.2% of the sequence similarity between them (Shida et al., 1996 ; Johnson & Dunlap, 2019).

The result of the present study supported genomic distinctiveness and monophyly between *Acinetobacter baumannii* strain SL103 and *Acinetobacter baumannii* strain DETAB-C9, as well as between *Acinetobacter baumannii* strain F46 and *Acinetobacter baumannii Canada* BC-5. The highest genetic divergence was shown by *Acinetobacter baumannii, Canada* BC-5. This intergenomic relationship between the genera *Acinetobacter* was delineated by DNA-DNA hybridization (Nemec et al., 2011).

Phylogenetic studies using ITS region shed light on the relationships between *Aspergillus* species. In this study, the resulting phylogenetic tree showed a monophyletic clade that included *Aspergillus nominae* strain Okra-A, *Aspergillus oryzae* strain DJL-FUNG03B, *Aspergillus chevalieri strain* CBS 522.65, *Aspergillus welwitschaiae* strain LayoO1, *Aspergillus assiutensis* isolate CBS132773, and *Aspergillus aculeatus* isolate YLA2. Other members were paraphyletic to the common ancestor. The highest evolutionary distance was shown by the *Aspergillus nominae* strain Okra-A. The genus *Aspergillus was* vast, and the infrageneric classification split the genus into six subgenus: *Fumigati*, *Nidulantes*, *Polypaecilum Aspergillus, Cremei* and *Circumdati* and 27 sections (Samson et al., 2014; Sun et al., 2022). In this study, *Aspergillus nominae* strain Okra-A and *Aspergillus oryzae* strain DJL-FUNG03B were identified as monophyletic as they belonged to section flavi of the subgenus *Aspergillus*. Other members of this section include *Aspergillus tamari, Aspergillus parasiticus,* and *Aspergillus flavus*. *Aspergillus chevalieri,* strain CBS

522.65, and *Aspergillus welwitschaiae* belonged to the subgenus Circumdati and were found to be evolutionary related. On the contrary, *Aspergillus assiutensis* isolate CBS132773 and *Aspergillus aculeatus* isolate YLA2 belonged to different subgenera, even though they share the same genus. This helps to understand the classification and evolutionary relationship among *Aspergillus* species (Bennett, 2007).

The phylogram obtained with ITS sequencing exhibited the presence of sister taxa between *Penicillum implicatum* and *Penicillum hispanicum* isolate D06 with boots trap value 93%. In addition anamorphic fungus *Penicillum citrinum* isolate ED2 and *Penicillum citrinum* strain PeciHA25A01 were strongly supported as monophyletic with bootstrap value 100% (Houbraken et al., 2010). However the other members within tree are paraphyletic and greatest divergence was exhibited by *Penicillum citrinum* isolate ED2. To date, there have been limited studies investigating the phylogenetic relationship of *Penicillium* at the genus level. Berbee's work in 1995 based on 18S rDNA sequences, showed that *Penicillium* is polyphyletic (Berbee et al., 1995). The genus splits up into four *Biverticillium*, *Penicillium*, *Furcatum* and *Aspergilloides* (Houbraken & Samson, 2011). Earlier literature work on genus *Penicillum* proves the presence of *Penicillum hispanicum* and *Penicillum implicatum* to the subgenus *Aspergilloides* and its phylogenetic significance (Khokhar et al., 2013).

The phylogram in (Figure 46) depicts members of the family Debaryomycetaceae and the order Saccharomycetales. This particular order is presumed to comprise 11 families and 55 genera. In the phylogram, *Candida tropicalis* isolate Hb39 and *Candida albicans* CBS 562 are monophyletic and have strong support with a bootstrap value of 99%. According to the studies by McManus

and Coleman, it is suggested that *Candida albicans* and *Candida dubliniensis* diverged from their common ancestor, *Candida tropicalis,* approximately 20 million years ago due to the separation of the whole genome duplication (WGD) and CTG lineages. This illustrates the relationship between *Candida tropicalis* isolate Hb39 and *Candida albicans* CBS 562 (McManus & Coleman, 2014). *Debaryomyces hansenii* isolate HBLC10 and *Candida metapsilosis* 19MWFY29.1 are closely related and form a monophyletic group with a bootstrap value of 84%, while other species within the tree appear as paraphyletic. Here, a greater divergence was shown by *Debaryomyces hansenii* isolate HBLC10. *Debaryomyces hansenii's* teleomorph is *Candida famata; Candida metapsilosis* is documented as part of the *Candida parapsilosis* species complex (Tavanti et al., 2005 ; Castanheira et al., 2013). This highlights its phylogenetic relationship. *Geotrichium candidum* isolate AY06 and *Galactomyces candidus* are monophyletic and strongly supported, with a bootstrap value of 97%, and they remain teleomorphs (Perkins et al., 2020).

Cunninghamella, like many other genera, belongs to the order Mucorales. In this research, Cunninghamella *blakesleena* isolate A11 was identified from the mosquito larval gut. It belongs to the genus *Cunninghamella* and the order Mucorales. Currently, there are 12 recognised species in the genus *Cunninghamella,* including *Cunninghamella echinulata*, *Cunninghamella bertholletiae*, *Cunninghamella elegans*, *Cunninghamella blakesleeana*, *Cunninghamella binariae*, *Cunninghamella clavata*, *Cunninghamella elegans, Cunninghamella homothallica, and Cunninghamella vesiculosa. Cunninghamella septata* and *Cunninghamella intermedia* (Bragulat et al., 2017).

Curvularia affinis strain 88F was isolated from the gut of mosquito larvae. This genus has a wide spread distribution bearing approximately 164 species.

Through 18S rRNA sequencing, several species of *Curvularia* were identified, including *Curvularia affinis. intermedia*, *Curvularia pallescens*, *Curvularia eragrostidis*, *Curvularia geniculata*, *Curvularia Curvularia verruculosa*, *Curvularia lunata*, and *Curvularia penniseti* notably, these species are known to be phyto pathogens (Alawlaqi & Alharbi, 2020).

In the current study, while analysing the fungal consortium within the mosquito larval gut, three species of *Cladosporium*, such as *Cladosporium tenuissimum* strain SFC20230103-M83, *Cladosporium marinum* strain SFC20230103-M34, and *Cladosporium cladosporioides* strain DBR-4, were identified in the mosquito. *Cladosporium* is a commonly found fungus with a wide ecological niche, primarily functioning as saprophytes. Currently, there are more than 230 species of *Cladosporium* fungus. Recently, phylogenetic analysis based on the LSU rRNA gene has delineated and distinguished the *Cladosporium* genus from other *Cladosporium* like genera *Hyalodendriella*, *Ochrocladosporium*, *Rachicladosporium*. *Verrucocladosporium Rhizocladosporium, Toxicocladosporium* and the newly designated genus *Neocladosporium* (Iturrieta-González et al., 2021).

In the present phylogenetic tree analysis, it was observed that *Cladosporium cladosporioides* strain DBR-4 and *Cladosporium marinum* strain SFC20230103-M34 *are* closely related, forming sister taxa with a bootstrap value of 79% and having a common ancestor. The terminal taxa displayed a paraphyletic nature. *Cladosporium marinum* strain SFC20230103-M34 showed greater divergence when compared with other species within the tree. Recent research involving concatenated phylogenetic analysis based on ITS, *act*, and *tef1* has led to the discovery of five new species: *Cladosporium marinum* sp. nov., *Cladosporium lagenariiforme* sp. nov., *Cladosporium maltirimosum* sp. nov., the *Cladosporium cladosporioides*

species complex, and *Cladosporium snafirmbriatum* sp. nov. in the *Cladosporium herbarum* species complex (Lee et al., 2023).

The genus *Syncephalastrum* belongs to the order Mucorales. In this study, molecular markers like ITS sequencing were employed to identify *Syncephalastrum* sp. isolate kBR1-1 from the mosquito larval gut. There have been a limited number of studies in the past regarding the phylogenetic relationship within this genus. The phylogenetic analysis of the present study showed an evolutionary relationship between *Syncephalastrum* sp. isolate kBR1-1 and various strains of *Syncephalastrum racemosum* and *Syncephalastrum monosporum*. *Syncephalastrum* sp. is a zygomycete species that was originally isolated *from a* pumpkin (Cucurbita pepo) seed sample in Korea.

In the subsequent years, more comprehensive phylogenetic analysis utilising multilocus molecular markers like nuclear 18S ribosomal RNA small subunit (SSU), nuclear large subunit 28S ribosomal RNA (LSU), and translation elongation factor-1α (EF-1α) has been conducted to explore the phylogeny of mucoralean species. These studies have confirmed the placement of *Syncephalastrum monosporum* in the family Syncephalastraceae (Duong et al., 2016).

The genus *Simplicillum* originated from the genus *Verticillium* and is known to inhabit various ecological niches. In this study, the fungus *Simplicillium subtropicum* strain SC55A01 was isolated from the mosquito larval gut. The phylogenetic tree was constructed by using partial coding of 5.8S rRNA ITS-1. For tree reconstruction, sequences of *Simplicillium subtropicum* strain SC55A01 along with sequences retrieved from the NCBI-GenBank data base were used. In the resulting phylogenetic tree, monophyletic groups having a common ancestor were

observed. *Fusarium* sp. strain 495F28C-AM and *Lasiodiplodia theobromae* isolate NLBLS formed one such group with a bootstrap value of 64%. Another monophyletic included *Cladosporium cladosporioides* strain SC45A01 and *Amycospharella Africana* strain Ct-LP86, with a lower bootstrap value of 33%. The tree also revealed the highest divergence exhibited by *Sordariomycetes* sp. HHTG-20151 isolate Y19H3a8epi (Chen et al., 2022).

Nigrospora species exhibits cosmopolitan distribution. The fungus *Nigrospora oryzae strain* MPL-S7L2A was obtained from the gut of mosquito larvae. A phylogenetic tree was constructed by using partial coding of 5.8S rRNA ITS-1 region. By using the sequences retrieved from NCBI- Gen Bank data base along with *Nigrospora oryzae strain* MPL-S7L2A. Within the phylogenetic tree (Figure 53), monophyletic groups were seen between taxa *Nigrospora oryzae* strain MPL-S7L2A and *Nigrospora oryzae* sp. isolate wz37, *Nigrospora oryzae* and *Nigrospora* sp. strain HS3, *Nigrospora oryzae* strain SUR and *Nigrospora oryzae* isolate xiao 7h2-2 , as they differ only in their type strains(Hao et al., 2020). The phylogenetic studies on bacteria and fungi helped to reveal the diversity and evolution of microorganism. This knowledge can be exploited in various disciplines of biological research like bioremediation vector control etc.

2.5 CONCLUSION

The proper grouping of microorganisms and understanding their interrelationships are crucial aspects of microbiological research. Bacteria represent one of the diverse group of organism, having a wide range of distribution. Small subunit ribosomal RNA, due to its combination of highly conserved and variable regions of the molecule, remains as a prime tool for determining the evolutionary relationship between bacteria. This approach of establishing phylogenetic links has served as the primary basis for classifying prokaryotic organisms. Fungi are multicellular, flagellated, eukaryotic organisms (although some unicellular forms are present) exhibits a wide range of habituation. Explicating the phylogenetic interactions among fungi is pivotal for understanding this group in a broader prespective.

Normally, ITS is used as a housekeeping gene in eukaryotes for molecular identification and phylogenetic analysis. It comes in two forms: ITS1 is positioned between 18S and 5.8S rRNA genes, while ITS2 is flanked by 5.8S and 28S rRNA genes. The ITS1 region corresponds to the ITS in bacteria and archaea, while ITS2 originated as an insertion that interrupted the ancestral 23S rRNA gene. The mechanisms governing microbial community dynamics and patterns of co-occurrence during ecological succession have not been investigated. In the present study, due importance is given to co-occurrence patterns, dynamics, and mechanisms of community dynamics and in bacterial and fungal communities in mosquito larval guts on a seasonal basis for a period of two years. For bacterial and fungal phylogenetic analysis, the microbes were clustered into families.

This study provides new insights into taxonomic and evolutionary slits that occurred in bacterial and fungal communities. This knowledge will help develop new bio control strategies as part of the vector control programme. In the coming decades, we can witness a substantial influx of information regarding molecular phylogeny, which will provide new insights to various branches of science.

CHAPTER 3

Comparative analysis of physicochemical parameters in natural habitats across seasons

3.1 INTRODUCTION

The relevance of mosquitoes as vectors of diseases in context of public health, necessitated proper surveillance of their habitat ecology and environmental conditions involved in mosquito proliferation and species assemblage (Chaves & Koenraadt, 2010). Most of the mosquito species utilize a wide range of lentic aquatic environment with vegetation for their breeding purposes. The mosquito breeding habitats can be categorised as either natural (e.g., lakes, ponds, and bromeliads) or artificial (e.g., tires, cemetery urns, and plastic containers). These habitats can further be classified on their duration falling into permanent, semi-permanent, and temporary. The breeding habitat plays an imperative role in the population dynamics of mosquitoes, serving as the site for vital activities like oviposition, larval maturation and emergence.

As immature aquatic forms, the dispersion, abundance, and individual fitness of mosquitoes are predominately shaped by three key factors: abiotic factors, biotic factors and their interaction between each other and with other related species in the breeding area (Blaustein et al., 2004 ; Yee et al., 2004 ; Blaustein & Chase, 2007). These interactions encompassed predation, competition, parasitism, and pathogenism. The co habituation of various mosquito species in their immature stages and their mutualistic behaviour with other living organisms, within the same habitat form a guild (Devi & Jauhari, 2007; Nikookar et al., 2017).

Conversely, larval density with in a breeding habitat, is linked to oviposition of female mosquitoes (gravid female mosquitoes uses olfactory cues to select breeding

grounds), survival of immatures and its spatial distribution. These dynamics are chiefly influenced by distinct abiotic characteristics of specific breeding site such as vegetation, temperature, turbidity, pH, conductivity, total dissolved solids (TDS), the concentration of ammonia, salinity, nitrite and nitrate, sulfate, phosphate, chloride, calcium, water hardness, hydrology, light/shade and nutrient availability (Mutero et al., 2004 ; Emidi et al., 2017).

Temperature of mosquito breeding water is one such environmental factor which has an influences embryonic development of mosquitoes and also governs the pace of metabolic and reproductive functions in aquatic organism. pH is another indicator of acidic or alkaline nature of breeding water and most of the aquatic forms exhibits sensitivity towards pH variations. Mosquitoes are normally tolerant towards extreme variations in pH, which helps them to survive in various environmental conditions. The optimal pH of 6.8-7.2 was found to be effective for cracking the mosquito egg shells, there by facilitating the first instar emergence. The electrical conductivity of water mainly depends on quantity of dissolved solids in water. Total dissolved solids (TDS) offers insight into the quantity of ions within water, thereby providing quality assessments. Elevated level of TDS can impede water clarity and indicates reduced photosynthesis in the aquatic area. Dissolved oxygen signifies aquatic capacity to sustain aquatic organisms, whereas biological oxygen demand quantifies bacterial sustenance in water. Total hardness predominantly relies on calcium and magnesium ions. Elevated levels of nitrate, calcium, magnesium, phosphate contribute to eutrophication of water. These chemical parameters are found to be affect larval survival and proliferation (Oyewole et al., 2009 ; Jasmine & Robin, 2020).

Mosquito breeding grounds also remains as the habitat of a diverse array of microorganism known as micro biota. This comprised of several species of bacteria, zooplankton, virus, filamentous fungi along with unicellular protists, plant debris, entomopathogenic nematodes crustaceans, and insect scales. These micro biota act as biotic factor which influences mosquito survival (Obi et al., 2019). These microorganisms serves as a potential food source, competitors and even predators of mosquito larvae. Consequently specific micro biota present in these habitats perform the role of natural regulators, effectively controlling mosquito larvae populations. The composition of microbial communities linked to mosquito breeding sites frequently fluctuates due to the presence of species highly sensitive to shift in nutrient cycling and fluctuation in environmental conditions, including temperature (Ranasinghe & Amarasinghe, 2020).

The microbiota in mosquito breeding water can influence the female ovipositioning, development of larvae and can eventually leads to modifications in the bacterial composition within the gut of mosquitoes. The source of microbial communities within mosquitoes and the extent to which these microbial communities are influenced by their surroundings are still debated. For example many research indicates that a portion of bacteria associated with mosquito early stages is acquired during their early developmental stages with in breeding sites (Dada et al., 2014; Scolari et al., 2019). Meanwhile numerous research studies have indicated that physicochemical factors like pH, dissolved oxygen (DO), nutrient concentrations significantly influence in shaping the composition and variety of microbial community present in breeding water. This represents a strong relation between the physicochemical parameters, micro biota and larval survival in breeding water.

In addition to physico-chemical factors, bacteriological characteristics of water holds a notable influence on oviposition behaviour of gravid female mosquitoes. The bacterial composition within the breeding water possesses the capacity to influence the physico-chemical parameters of water, there by rendering it either more or less suitable for the survival of diverse mosquito species (Merritt et al., 1992). On one hand, these bacteria within the breeding sites serve as a direct nutritional source for mosquito larvae. While on the other hand, these bacteria emit certain volatile compounds that acts a chemical attractants, luring female mosquitoes to lay their eggs (Rejmanková et al., 2005 ; Nilsson et al., 2019; Seal & Chatterjee, 2023).

There occurs a strong correlation between the physicochemical characteristics of breeding sites and in the number and dispersion of mosquito larval stages. Mosquito larval abundance and survival were dependent upon physico-chemical parameters of breeding water. Especially pH, temperature, TDS, conductivity, salinity, DO were some of the important parameters influencing larval survival. Control of larval mosquito populations is often preferable because the larvae are relatively immobile, and habitually occupied in minimal habitat area when compared with adults which can rapidly disperse over large areas. Being exclusively aquatic the larval distribution is determined by the presence of suitable water bodies. Hence, comprehending the ecological traits of these larval habitats and the environmental aspects along with understanding the impact of the physicochemical parameters of breeding water become essential. This understanding helps in unveiling how these parameters helps in reducing mosquito richness and offer insight on their potential use in controlling mosquito immatures.

3.2 MATERIALS AND METHOD

3.2.1 Sampling sites

The water sampling was carried out simultaneously with larval collection from three mosquito breeding localities of Thrissur(southwest of Kerala) district along with larval collection namely site1: (Thrissur taluk 10° 31' 49.2420" N 76° 12' 53.0244" E), site 2: (Chavakkad taluk 10° 34' 59.91" N 76° 1' 7) and site 3: (Mukundapuram taluk 10° 19' 60.00" N 76° 13'60.00" E) (Figure.4). Assessment of the physicochemical characteristics of the water were carried out exclusively on seasonal basis during pre-monsoon, monsoon and post-monsoon for two years from 2018-2020. For physico chemical analysis, breeding water, sampling were done with the help of 500ml ladle dippers from the three sampling stations. The pH and temperature was measured in the field, along with the fixation of water sample for the estimation of DO and BOD. Conductivity, total dissolved solids, total alkalinity, calcium, magnesium, total hardness, nitrite, phosphate, DO and BOD were measured separately for all the samples in the laboratory by employing standard methods described in the APHA (Gilcreas, 1966; Rice et al., 2012).

3.2.2 Determination of physicochemical parameters of breeding water

3.2.2.1 Physical parameters

3.2.2.1.1 Temperature

Temperature of water samples were measured by using a high quality digital thermometer (HM digital TM1). The procedure involved immersing the probe of the thermometer into the water sample taken in a beaker and then the water temperature was recorded in degree celsius. Temperature (°C) were recorded on-site at the time of collection.

3.2.2.1.2 Conductivity

The conductivity of the water samples were assessed in the laboratory by utilizing microprocessor controlled conductivity meter (Systronic 306). To ensure accuracy the instrument's sensor was prior calibrated with 0.1M KCl solution at 25^{o}C. The recorded values were expressed in (mS).

3.2.2.2 Chemical parameters

3.2.2.2.1 pH

The pH levels of water samples was recorded by using pH meter (Hanna Hi 96107). The equipment was calibrated by using freshly prepared pH 7 buffer solution. After calibration it was rinsed in distilled water before the recording the pH values of water samples. pH was recorded on-site at the time of collection.

3.2.2.2.2 Total Dissolved Solids (TDS)

Water samples for measuring Total Dissolved Solids was taken in clean conical flask. Normally TDS meter (HM digital TDS3) was calibrated by using NaCl at 342ppm. The sensor of TDS meter was immersed into conical flask for about 20 seconds. The TDS meter will display the readings of water sample in ppm.

3.2.2.2.3 Total Alkalinity

The Alkalinity of water samples were determined by titrimetric method. 50ml of water sample was titrated against 0.1N HCl (Himedia TC536M) using phenolphthalein (0.1% w/v) (Hi Media I009) indicator till the sample turns colourless and then methyl orange (Hi Media GRM 958) indicator was added till the endpoint changes from yellow to orange red. The total alkalinity was calculated using the formula

$$\text{Total alkalinity mg/l} = \frac{\text{MBR x N x 1000 x 50}}{\text{50 ml of sample}}$$

Where, MBR = Mean Burette Reading N = Normality of HCL (0.1N)

3.2.2.2.4 Total Hardness

Hardness of water sample was determined by titrimetric method. To 50 ml of water sample added 2 ml of buffer solution (dissolved 1.179 g of disodium salt of EDTA [Ethylene Di amine Tetra Acetic acid, Hi Media PCT0105] and 780 mg of magnesium sulphate heptahydrate (Hi Media GRM683) in 50 ml distilled water. Added to this, 16.9g of ammonium chloride (Hi Media GRM730) and 143ml of concentrated ammonium hydroxide (Sigma Aldrich 338818) with mixing and diluted to 250 ml using distilled water). The water sample was titrated against 0.05M EDTA[0.05M EDTA titrant: 18.612 g EDTA dissolved in distilled water and made upto 1litre]. A few drops of Eriochrome Black T (Hi Media GRM 939) indicator was added until the colour changes from wine red to blue.

$$\text{Hardness mg/l} = \frac{\text{MBR} \times 1000}{\text{50 ml of sample}}$$

Where, MBR = Mean Burette Reading

3.2.2.2.5 Calcium

The calcium content of the water samples were determined by titrimetric method. To 50 ml of the water sample added 2 ml of sodium hydroxide (Hi Media GRM1183) and 100mg of Murexide (ammonium purpurate Hi Media GRM481) indicator were added to this. The water sample was titrated against 0.05M EDTA solution(0.05M EDTA titrant: 18.612 g EDTA dissolved in distilled water and made

up to 1 litre standardized against zinc sulphate (0.05M Hi MediaGRM1180). 0.05M zinc sulphate was prepared from 14.378 g of zinc sulphate, dissolved in distilled water and diluted to 1litre and titration was continued until no further color change occurs. The calcium content of the water samples were calculated by using the following formula,

$$\text{Calcium mg/l} = \frac{\text{MBR} \times 400.8}{\text{50 ml of sample}}$$

Where, MBR = Mean Burette Reading

3.2.2.2.6 Magnesium

The magnesium content of the water sample were determined using the volume of EDTA (titre value) solution used in determination of hardness and calcium.

$$\text{Magnesium mg/l} = \frac{\text{Y- X} \times 400.8}{\text{Volume of sample} \times 1.645}$$

Where, X = titer value of calcium, Y= titer value of hardness

3.2.2.2.7 Nitrate

Nitrate content of water sample is determined by colorimetric method. To 10 ml water sample (1ml diluted to 10ml) added 0.4ml of buffer reagent [phenol solution (Hi Media MB082) and sodium hydroxide (Hi Media GRM1183) in the ratio 1:1 added 0.2 ml of [hydrazine sulfate (Hi MediaGRM2847) and copper sulphate (Hi Media GRM6391)(1:1 ratio)]incubate in dark for 18-24 hours and added 0.4 ml of acetone (Hi Media AS024) and 0.2 ml of sulphanilamide (Hi Media GRM7528) and 0.2 ml of NED (N-1-naphthyl ethylene diamine dihydrochloride Hi Media RM1073). Blank also prepared in similar manner using 10 ml of distilled water. The optical

density of the sample was measured at 540 nm after 10 minutes incubation using photo electric calorimeter. A series of standards (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0mg/l) of standard nitrate solution were also run to prepare calibration curve. The standard curve was plotted using concentration against absorbance. The concentration of the nitrate was determined from the standard graph

$$\text{Concentration of nitrate (mg/l)} = \frac{\text{OD of sample} \times \text{OD of standard}}{\text{Concentration of standard}}$$

Where, OD = Optical density value

3.2.2.2.8 Phosphate

Phosphate content of water sample is determined by colorimetric method. To 10 ml water sample (1ml diluted to 10ml) added 1.6 ml of mixed reagent[a) sulphuric acid (5N) (Hi Media AS016) : 14 ml of sulphuric acid to 100 ml distilled water, b) potassium antimonyl tartrate (Hi Media GRM1779) solution: dissolved 0.686g of potassium antimonyl tartrate in 250 ml distilled water, c) ammonium molybdate (Hi Media PCT0119) solution: dissolved 4 g of ammonium molybdate in 100 ml distilled water d) ascorbic acid (Hi Media PCT027) solution: dissolved 1,76 g of ascorbic acid in 100 ml distilled water. Mixed reagent of 100 ml was prepared by 50 ml of 5N sulphuric acid, 5ml of potassium antimonyl tartrate solution, 15 ml of ammonium molybdate solution and 30 ml of ascorbic acid solution] and incubated for 10 minutes at room temperature. The absorbance was measured at 880 nm using reagent blank as reference solution. A series of standards (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0mg/l) of standard phosphate solution were also run to prepare calibration curve. The standard curve was plotted using concentration against absorbance. The concentration of the phosphate was determined from the standard graph

$$\text{Concentration of phosphate (mg/l)} = \frac{\text{OD of sample} \times \text{OD of standard}}{\text{Concentration of standard}}$$

Where, OD = Optical density value

3.2.2.2.9 Dissolved Oxygen (DO)

The DO of the water samples was determined by Classical Winkler's method. DO was fixed at the time of collection, for this water samples were taken in 250 ml BOD bottles without air bubbles. Around 2 ml of manganese sulphate (MnSO4-Wrinkler A) (Hi Media MB202) followed by 2 ml of alkaline potassium iodide (KI-Wrinkler B) (Hi MediaGRM252) were added below the surface of water sample. The bottles were sealed tightly using glass stopper to exclude air bubbles. When precipitate settles, leaving a clear supernatant above manganese hydroxide floc, added 2 ml of concentrated sulphuric acid (Hi Media AS016) to dissolve precipitation. 20 ml of water sample was titrated against 0.025 N sodium thiosulphate (Himedia GRM1420) until the colour changes to straw yellow. 1% freshly prepared starch (Himedia GRM3029) solution was used as indicator. Titrate until blue colour was completely discharged. The concentration of DO was subsequently calculated by using the formula,

$$\text{DO (mg/l)} = \frac{\text{MBR} \times \text{N} \times 8 \times 1000}{\text{V2 (V1-V/V1)}}$$

Where, MBR=Mean Burette Reading, N=Normality of sodiumthiosulphate (0.025 N), V1=Volume of BOD bottle(250ml), V2= Volume of sample titrated (20 ml),V=Volume of manganese sulphate and potassium iodide(2+2ml)

3.2.2.2.10 Biological Oxygen Demand (BOD)

To determine BOD, water samples were taken in two 250 ml BOD bottles and fixed at the time of collection. One BOD bottle was placed in BOD incubator at a temperature of 20^{o}C for a period of five days. The DO level of other BOD bottle was measured using Classical Winkler's method. This gave DO_0 value. After five days of incubation, DO level of the incubated sample was determined using the same Winkler's method providing DO_5 value.The BOD value was then calculated by finding the difference between the initial DO_0 and final DO_5 concentration.

$$\text{BOD (mg/l)} = DO_0 - DO_5$$

Where, DO_0 = Dissolved oxygen at day 0, DO_5 = Dissolved oxygen at day 5

3.2.2.3 Data Analysis

Data generated were processed into means and standard deviation (mean ± SD) using Microsoft Office Excel 2016. The mean characteristics of mosquito breeding water parameters (physical and chemical) were compared by using One-way analysis of variance (ANOVA) to examine the significance of seasonal variation of physical and chemical parameters. To assess the linear correlation between the physicochemical parameters measured Karl Pearson's correlation coefficient (r) were employed. Kruskal- Wallis test was performed to determine the correlation between the larval abundance and seasons. The following statistical analysis was performed using SPSS 29.0.1.1 version to determine the significance of the seasonal variation and the correlation between larval abundance.

3.3 RESULTS

The physical and chemical characteristics of an aquatic ecosystem play a significant role in determining the existence of aquatic organisms. Being an immature form, mosquito larvae breed in aquatic environments. The present study investigated the variations in physical and chemical profiles of mosquito breeding water during pre-monsoon, monsoon, and post-monsoon for a period of two years, from February 2018 to January 2020, at three sites, namely site 1: Thrissur, site 2: Chavakkad and site 3: Mukundapuram. The study also explored the relationship between larval abundance and seasonal changes. The *Aedes aegypti*, *Culex quinquefasciatus* and *Anopheles stephensi* were found to be co-exist in the breeding sites. The mean larval abundance was calculated by using Kruskal-Wallis test on seasonal basis.

Temperature

The water temperature in mosquito breeding sites varies between 29 and 32 °C (Table 19). In the first year, the mean temperature was 29.60 °C, and in the second year, it was 29.70°C.The peak temperature of 33°C was recorded in April and May 2018 of both 2019. While the lowest temperature of 27°C was noted in August 2018 and July 2019, on a seasonal basis, the highest temperature was recorded during the pre-monsoon at 31.46°C). Conversely, the lowest mean temperature for breeding water, 27.92°C, was documented during monsoon seasons. The mean temperature during the post-monsoon was 29.75°C, as reported from these sites. The graphical representation of the monthly variation in the water temperature of mosquito breeding sites is shown in (Figure 54). The graphical representation of the mean temperature is shown in (Figure 55). It was generally noted that the water temperature is comparatively higher during the pre-monsoon season in all the systems studied. Temperature showed positive correlation with conductivity (r=0.421,p=0.000), TDS

(r=0.691,p=0.000), total alkalinity (r=0.249,p=0.035), calcium (r=0.556,p=0.000), magnesium (r=0.576,p=0.000), total hardness (r=0.384,p=0.001), nitrate (r=0.336,p=0.004) DO (r=0.286,p=0.015) and BOD (r=0.288.p=0.014). Statistical analysis indicated a significant variation in water temperature between seasons (P value<0.001*, Fvalue 52. 854).

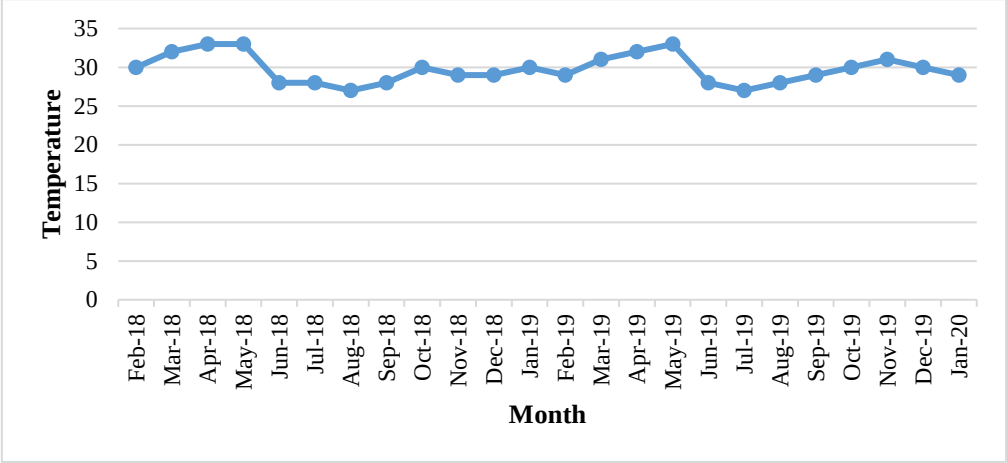

Figure 54: Monthly variations in temperature (°C) of breeding water, Feb2018-Jan2020

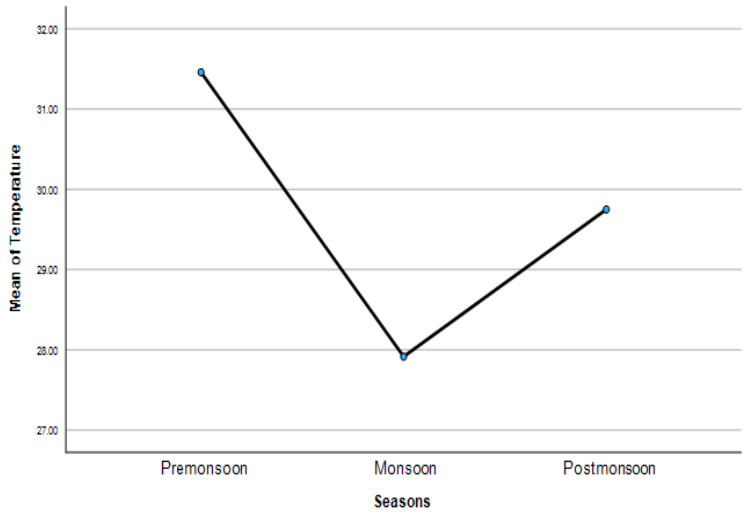

Figure 55 : Mean plot of temperature (°C) of breeding water on seasonal basis, Feb 2018-Jan 2020

Table: 19 Physical and chemical parameters of mosquito breeding water(Feb 2018 to Jan 2020

Values are expressed as Mean ± SD.

Physico-chemical parameters	Pre-monsoon	Monsoon	Post-monsoon
Temperature (^{0}C)	31.46 ±1.47	27.92 ± 1.10	29.75 ± 0.94
pH	6.78 ± 0.20	6.83 ± 0.14	6.92 ± 0.24
Conductivity (mS)	153.32 ± 5.99	135.41 ± 3.65	164.66 ± 3.41
Total Dissolved Solids (ppm)	249.99 ± 6.82	235.69 ± 5.16	238.07 ± 4.03
Total alkalinity (mg/l)	65.99 ± 0.39	66.04 ± 0.43	66.19 ± 0.39
Total hardness (mg/l)	46.00 ± 0.38	46.04 ± 0.45	46.19 ± 0.38
Calcium (mg/l)	16.00 ± 0.37	16.04 ± 0.42	16.19 ± 0.38
Magnesium (mg/l)	8.25 ± 0.36	8.00 ± 0.38	8.12 ± 0.34
Nitrate (mg/l)	6.00 ± 0.38	6.03 ± 0.42	6.18 ± 0.38
Phosphate (mg/l)	0.88 ± 0.08	0.85 ± 0.09	0.86 ± 0.08
Dissolved Oxygen (mg/l)	5.07 ± 0.66	4.75 ± 0.35	5.13 ± 0.48
Biological Oxygen Demand (mg/l)	2.47 ± 0.27	2.43 ± 0.25	2.59 ± 0.21

Table: 20 Seasonal comparison of physical and chemical parameters of mosquito breeding water (Feb2018 to Jan 2020)

Parameter	Pre monsoon	Monsoon	Post monsoo	F-value	P-value
Temperature (^{0}C)	31.46	27.92	29.75	52.854	<.001*
pH	6.78	6.83	6.92	3.303	0.043*
Conductivity (mS)	153.32	135.41	164.66	257.454	<.001*
Total Dissolved Solids (ppm)	249.99	235.69	238.07	47.265	<.001*
Total alkalinity (mg/l)	65.99	66.04	66.19	1.621	0.205
Total hardness (mg/l)	46.00	46.04	46.19	1.492	0.232
Calcium (mg/l)	16.00	16.04	16.19	1.508	0.229
Magnesium (mg/l)	8.25	8.00	8.12	2.889	0.062
Nitrate (mg/l)	6.00	6.03	6.18	1.436	0.245
Phosphate (mg/l)	0.88	0.85	0.86	1.155	0.321
Dissolved Oxygen (mg/l)	5.07	4.75	5.13	3.810	0.027*
Biological Oxygen Demand (mg/l)	2.47	2.43	2.59	2.530	0.087

Each parameter represents average of 8 months.
Significant in relation to seasons at * $P \leq 0.05$.

Table: 21Pearson's Correlation coefficient between physico-chemical parameters

Parameter	pH	Temp	Cond.	TDS	TA	Ca2+	Mg2+	TH	$NO3^-$	PO_4^{3-}	DO	BOD
pH	1											
Temp	0.183 0.124	1										
Cond.	0.136 0.255	.421** 0.000	1									
TDS	0.044 0.717	0.691** 0.000	0.220 0.064	1								
TA	-0.074 0.538	0.249* 0.035	0.242* 0.040	0.332* 0.004	1							
Ca2+	0.239* 0.043	0.556** 0.000	0.406** 0.000	0.473** 0.000	0.127 0.2888	1						
Mg2+	0.060 0.619	0.576** 0.000	0.430** 0.000	0.577** 0.000	0.358** 0.002	0.649** 0.000	1					
TH	0.239* 0.043	0.384** 0.001	0.231 0.051	0.307** 0.009	0.253* 0.032	0.515** 0.000	0.557** 0.000	1				
$NO3^-$	0.178 0.134	0.336** 0.004	0.279* 0.018	0.184 0.122	0.578** 0.000	0.458** 0.000	0.501** 0.000	0.670** 0.000	1			
PO_4^{3-}	0.097 0.419	0.169 0.155	0.145 0.223	0.070 0.559	-0.021 0.862	0.176 0.140	0.031 0.797	0.024 0.844	-0.126 0.292	1		
DO	0.216 0.069	0.286* 0.015	0.088 0.461	0.401** 0.000	0.150 0.207	0.250* 0.034	0.243* 0.040	0.312** 0.008	0.236* 0.046	-0.164 0.170	1	
BOD	0.201 0.091	0.288* 0.014	0.084 0.484	0.400** 0.000	0.118 0.324	0.226 0.056	0.225 0.057	0.240* 0.042	0.161 0.176	-0.089 0.458	0.963** 0.000	1

(Temp-Temperature, Cond.- Conductivity, TDS-Total dissolved solids, TA- Total alkalinity, Ca2+- Calcium, Mg2+- Magnesium, TH-Total Hardness, $NO3^-$Nitrate, PO_4^{3-}-Phosphate, DO-Dissolved Oxygen, BOD-Biological Oxygen Demand)

Conductivity

The average electrical conductivity of breeding water falls within the range of 128 to 171 mS. In the first year, mean electrical conductivity was 152.22 mS and 150.03 mS in the second year. The highest electrical conductivity of 165 mS was observed in November 2018, while the lowest value of 135 mS was recorded in June 2018. Seasonal data revealed a significant difference in conductivity; a higher value was obtained during the post-monsoon season (164.66 mS) and the lowest during the monsoon season (135.41 mS). During the pre-monsoon season, the conductivity ranged between 140 and 163 mS, with a mean value of 153.32 mS. In the monsoon season, it ranged from 128 to 140 mS, with a mean value of 135.41 mS. During the post-monsoon, it ranged between 159 and 170 mS, with a mean value of 164.66 mS. The graphical representation of the monthly variation in the electrical conductivity of mosquito breeding water is shown in (Figure 56). The graphical representation of the mean temperature is shown (Figure 57). Statistical analysis indicated a significant variation in conductivity between seasons (P value<0.001, F value 257.454).

Electrical conductivity shows strong positive correlation with temperature (r= 0.0421, p= 0.000), total alkalinity(r= 0.242, p= 0.000), magnesium (r= 0.430, p= 0.000), calcium (r= 0.406, 0.000) and nitrate (r= 0.279, p= 0.018).

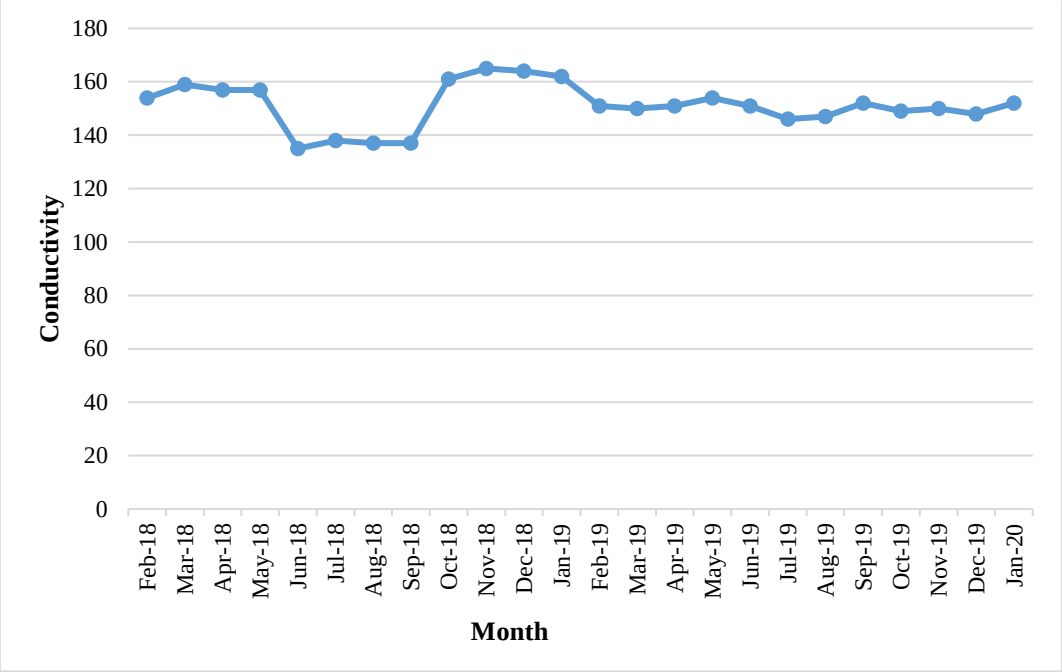

Figure 56: Monthly variations in conductivity (mS) of breeding water, (Feb2018-Jan2020)

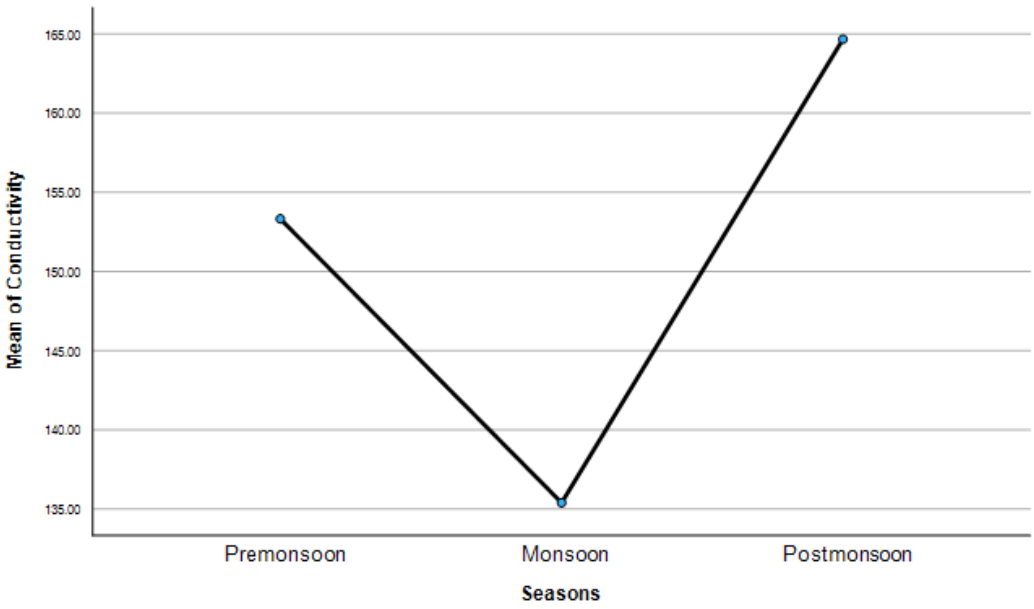

Figure 57. Mean plot of conductivity (mS) of breeding water on seasonal basis, (Feb 2018-Jan 2020)

Chemical parameters

pH

The pH levels of breeding water were recorded at the time of collection. In the first year, the mean pH was 6.82, and in the second year, it was 6.86. The highest pH value of 7.3 was recorded in January 2020, and the lowest value of 6.71 was noted in the month of July 2019. When analysing the data seasonally, it was found that the pH was higher during the post-monsoon season and lower during the monsoon season. During the pre-monsoon season, the pH value ranged between 6.6 and 7.0, with a mean pH of 6.78. During monsoon season, it varied from 6.7 to 6.9 with an average pH of 6.83, and during the post-monsoon period, it ranged from 6.8 to 7.3 with a mean value of 6.92. The graphical representation of the monthly variation in the pH of mosquito breeding water is shown in (Figure 58). The graphical representation of the mean temperature is shown in (Figure 59). Statistical analysis indicated a significant variation in pH between seasons (P value 0.043*, F value 303). pH showed positive correlation with calcium (r=0.239, p= 0.043) and total hardness (r= 0 .239, p= 0.043) and significant negative correlation with total alkalinity (r= -0.074, p = 0.538).

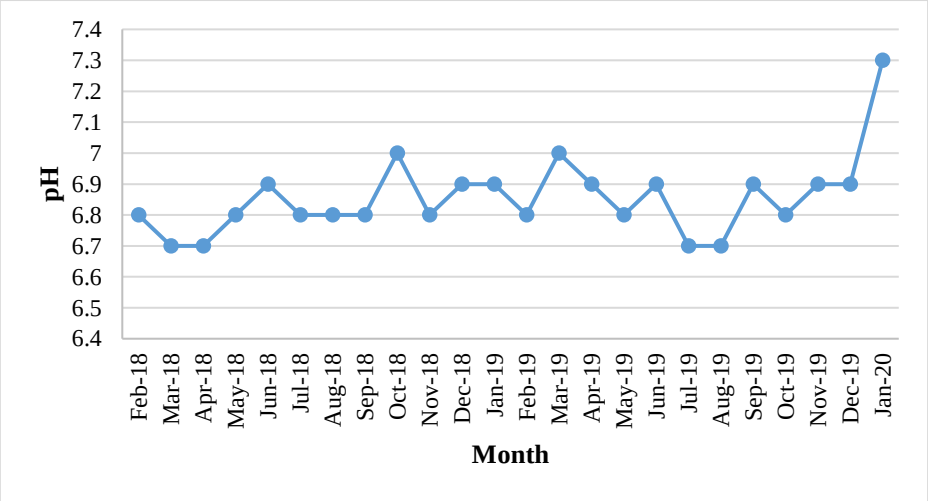

Figure 58. Monthly variations in pH of breeding water, (Feb2018-Jan2020)

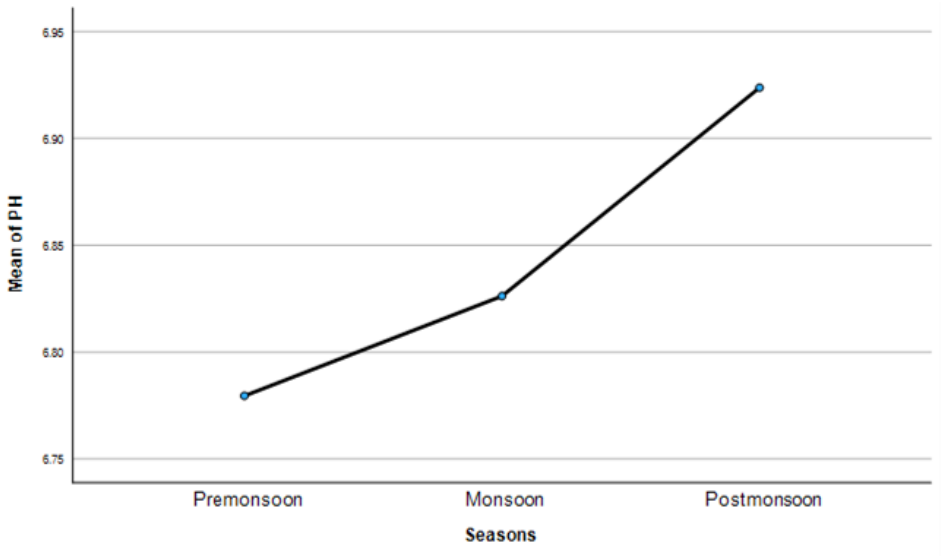

Figure 59. Mean plot of pH of breeding water on seasonal basis,(Feb 2018-Jan 2020)

TDS

The TDS in breeding water had an average value of 239 ppm during the first year and 243 ppm during the second year. The highest value of 255 ppm was recorded in May 2019, while the lowest value of 233 ppm was recorded in June 2018. Based on seasonal aspects, a peak value of 249.99 ppm was recorded during the pre-monsoon season, followed by the post-monsoon (238.07 ppm), and the lowest values were found during the monsoon season (235.69 ppm). Specifically, during the pre-monsoon season, TDS ranged from 244 to 255 ppm, with an average value of 249.99 ppm. During the monsoon season, it was between 233-241 ppm, with a mean value of 235.69 ppm. During the post-monsoon season, it varied from 236-241 ppm to an average of 238.07 ppm. The graphical representation of the monthly variation in the TDS of mosquito breeding water is shown in (Figure 60). The graphical representation of the mean temperature is shown in (Figure 61). Statistical analysis indicated a significant variation in TDS between seasons (P value 0.043*, F value 303).TDS showed positive correlation with temperature (r= 0.691, p= 0.000), total alkalinity

(r=0.332, p=0.004), calcium (r=0.473,p=0.000), magnesium (r=0.577,p=000) total hardness (r=0.307,p=0.009), DO (r=0.401,p=0.000) and BOD (r=0.400,p=0.000).

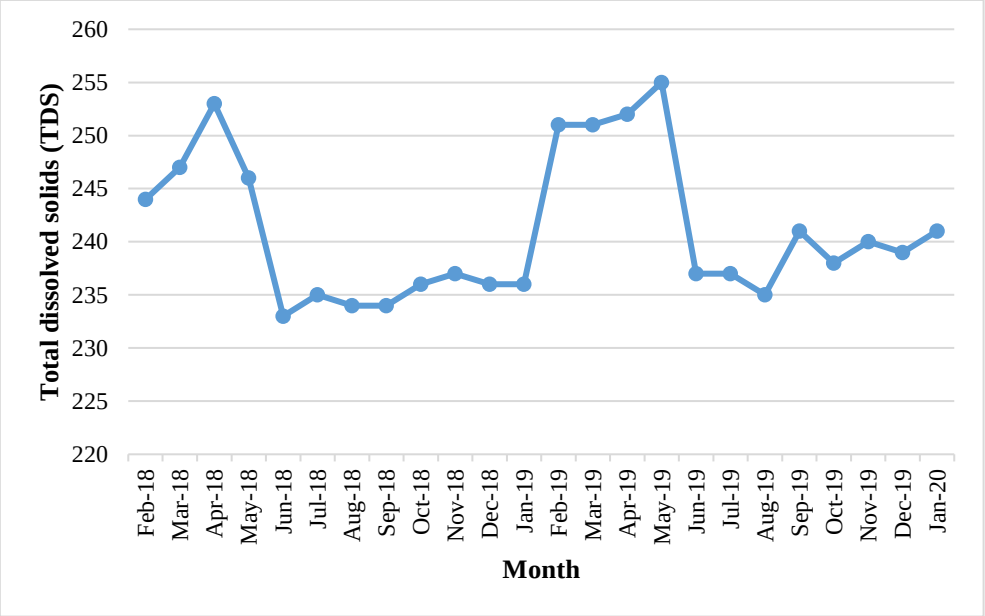

Figure 60: Monthly variations in TDS of breeding water, Feb2018-Jan2020

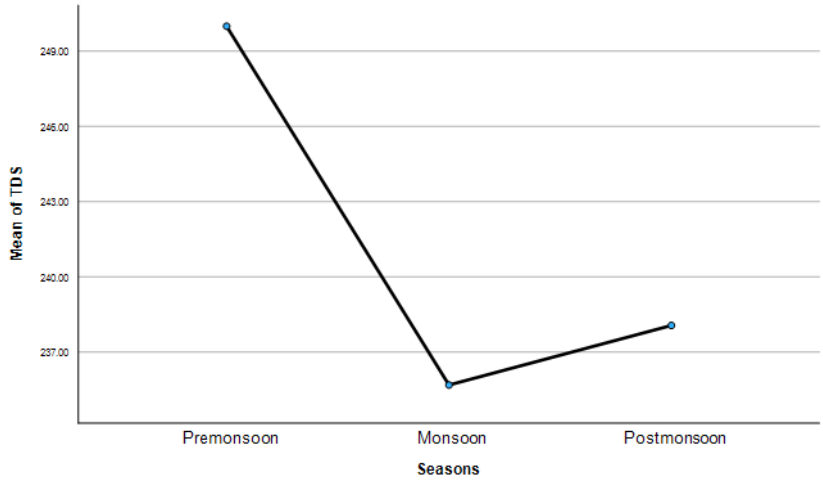

Figure 61: Mean plot of TDS of breeding water on seasonal basis, Feb 2018-Jan 2020

Total Alkalinity

Total alkalinity of breeding water remained relatively stable over the course of the investigation during two years, with a value of 65.7 mg/l in the first year and

66.3 mg/l in the second year exhibiting the minimum increase. The highest value of 66.6 mg/l was recorded in January 2020, and the lowest value of 65.0 mg/l was noted in September 2018. When analysed seasonally, the total alkalinity during pre-monsoon season varied from 65 to 66.3 mg/l with a mean value of 65.99 mg/l, followed by monsoon season within a range of 65 to 66.5 mg/l, having an average value of 66.04 mg/l, while it was within the limit of 65.6 to 66.6 mg/l with a mean value of 66.19 mg/l during post-monsoon. The graphical representation of the monthly variation in the total alkalinity of breeding water is shown in (Figure 62). The graphical representation of the monthly variation in the total alkalinity of mosquito breeding water is shown in (Figure 63), along with the graphical representation of the mean total alkalinity. Statistical analysis indicated a significant variation in total alkalinity between seasons (P value 0.205, F value 1.621). Alkalinity shows significant positive correlation with temperature (r= 0.249 p= 0.035, conductivity (r= 0.242, p=0.040), TDS (r= 0.332, p= 0.004), magnesium (r=0.358, p=0.002), total hardness (r=0.253, p=0.032), nitrate (r=0.578,p=0.000) and DO (r=0.150, p=0.207).

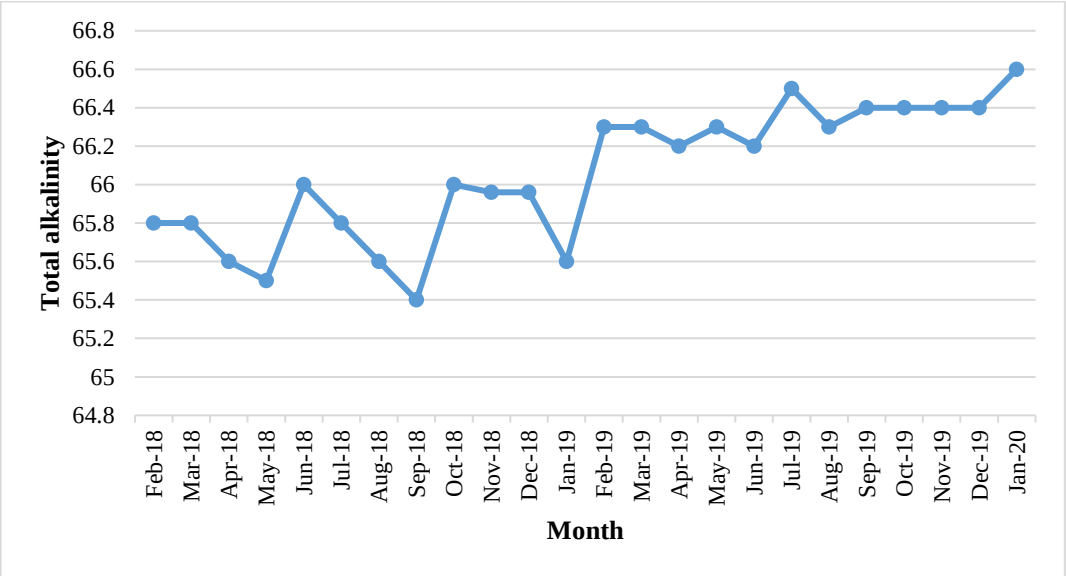

Figure 62 : Monthly variations in total alkalinity of breeding water, Feb2018-Jan2020

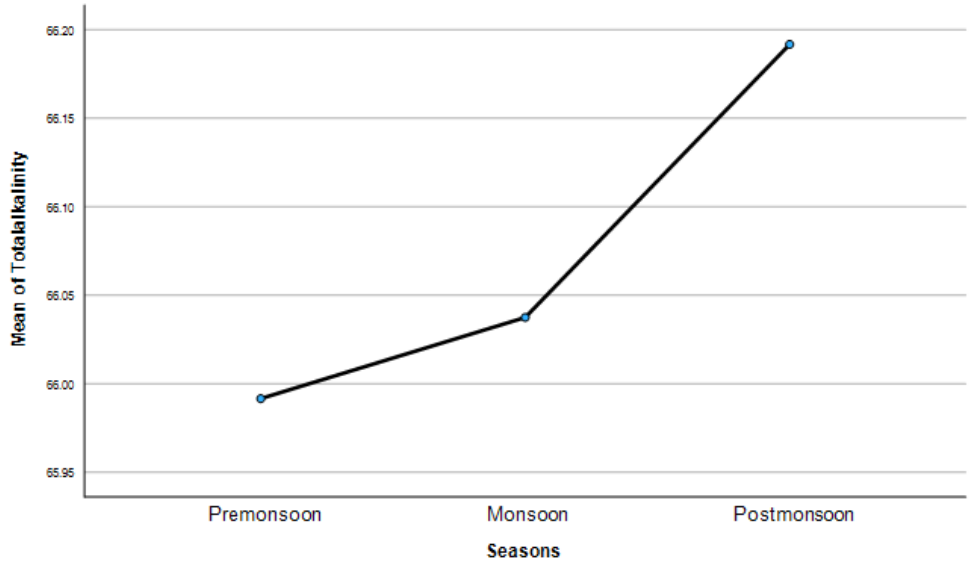

Figure 63. Mean plot of total alkalinity of breeding water on seasonal basis, Feb 2018-Jan 2020

Total Hardness

In the first year, the average total hardness of the breeding water was 45.76 mg/l, while in the second year, it slightly rose to 46.38 mg/l. The highest recorded total hardness value was 46.6 mg/l in January 2020, and the lowest value was 45.46 mg/l in September 2018. Seasonal interpretation revealed consistency in total hardness with minor variations. In the pre-monsoon season, it varied from 6.6 to 7.0 mg/l. In the monsoon season, it was within a range of 6.7–6.9 mg/l, and during the post-monsoon season, it fell between 6.8 and 7.3 mg/l. Notably, the highest concentration of total hardness in breeding water was observed during the post-monsoon period, with an average of 46.19 mg/l. The lowest reading was monitored with a mean value of 46.00 during the pre-monsoon period. The monsoon season exhibited a subtle difference, with a mean value of 46.04 mg/l. Figure 64 illustrates the graphical representation of the monthly variation in the total hardness of mosquito breeding water. The graphical representation of the mean total hardness is shown in

(Figure 65). Statistical analysis indicated a significant variation in total hardness between seasons (P value 0.232, F value 1.492).Total hardness shows significant positive correlation with pH (r= 0.239, p= 0.043), temperature (r= 0.384, p= 0.001), TDS (r= 0.307, p= 0.009), total alkalinity (r=0.253,p=0.032), calcium (r=0.515,p=0.000), magnesium (r=0.557,p=0.000), nitrate (r=0.670, p=0.000), DO (r=0.312, p=0.008) and BOD (r=0.240, p=0.042).

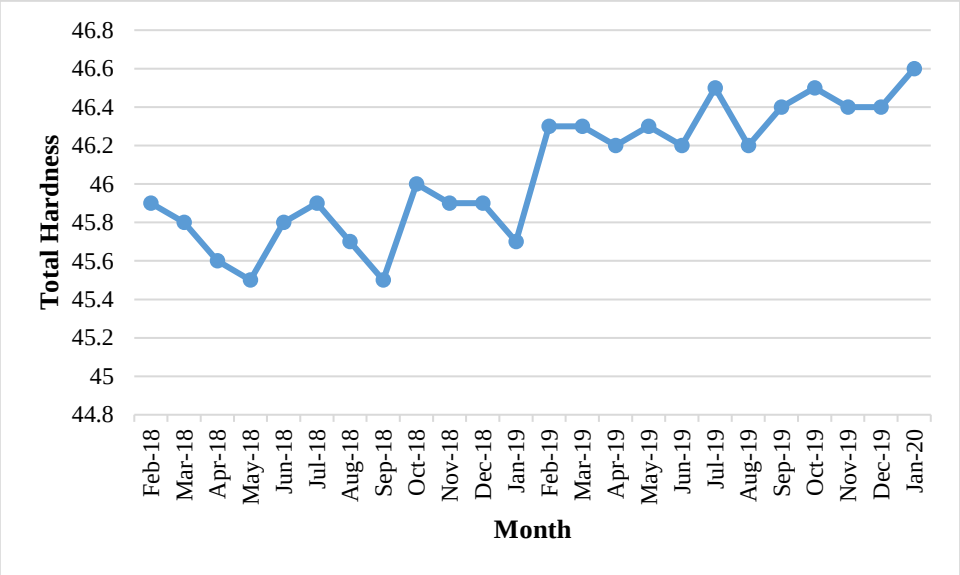

Figure 64 : Monthly variations in total hardness of breeding water, Feb2018-Jan2020

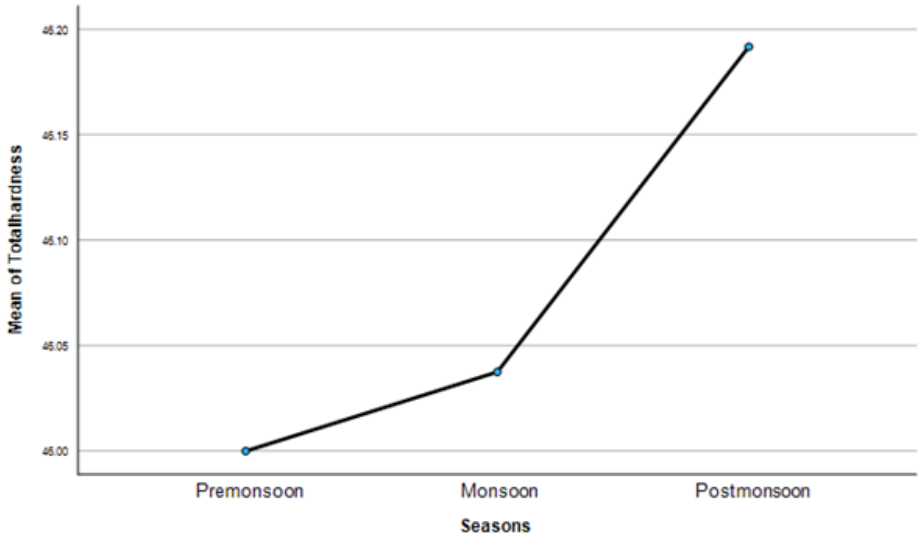

Figure 65: Mean plot of total hardness of breeding water on seasonal basis, Feb 2018-Jan 2020

Calcium

The average calcium concentration in mosquito breeding water was 15.97mg/l in the first year and bears a mean value of 16.38mg/l in the second year. The highest concentration of calcium was observed in January 2020, while the lowest amount was shown in September 2018. On a seasonal basis, the highest value was recorded during the post-monsoon season and the least during the monsoon season. Specifically, during the pre-monsoon season, the calcium levels varied from 15.56 to 16.33 mg/l, with an average of 16.00 mg/l. In the monsoon season, the range was 15.48–16.46 mg/l, with a mean value of 16.04 mg/l. During the post-monsoon season, calcium levels varied from 15.68 to 16.61 mg/l, with an average of 16.19 mg/l. The graphical representation of the monthly variation in the amount of calcium in mosquito breeding water is shown in (Figure 66). The graphical representation of the mean calcium is shown in (Figure 67). Statistical analysis indicated a significant variation in total alkalinity between seasons (P value 0.229, F value 1.508). Calcium shows significant positive correlation with ph (r= 0.239, p= 0.043), temperature (r=0.556,p=0.000) total hardness (r=0.515, p= 0.000), conductivity (r=0.406, p=0.000), TDS (r=0.473,p=0.000), magnesium (r=0.649, p=0.000), nitrate (r=0.458,p=0.000) and DO (r=0.250, p= 0.034).

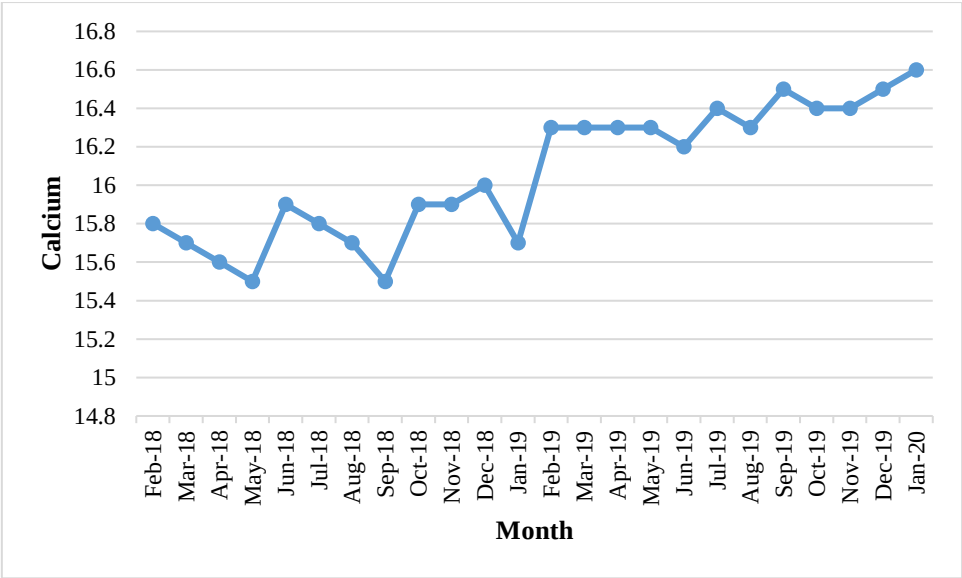

Figure 66. Monthly variations in calcium of breeding water, Feb2018-Jan2020

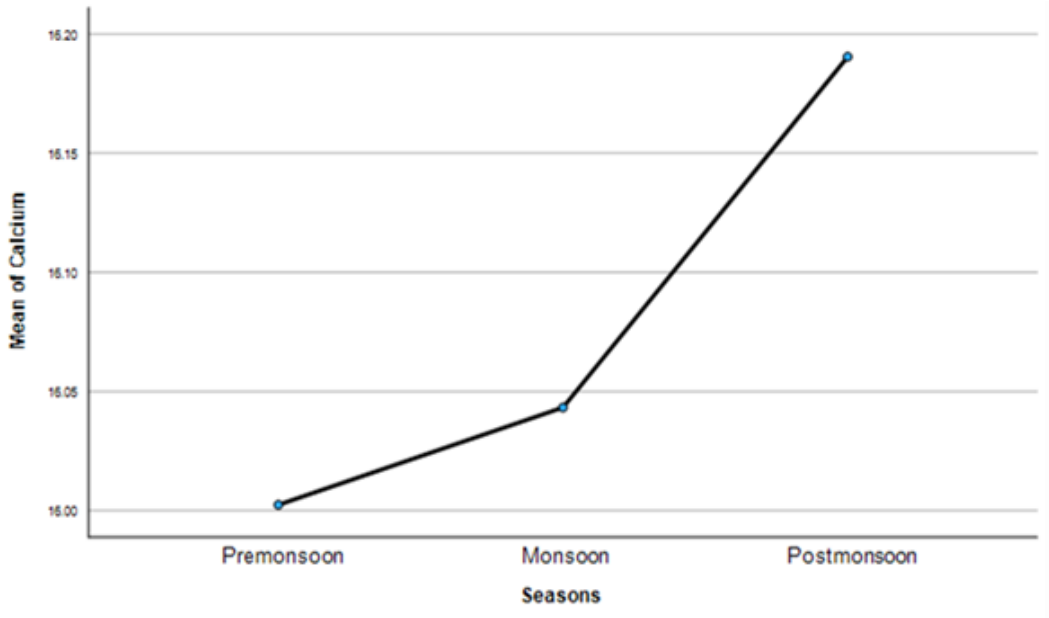

Figure 67. Mean plot of calcium of breeding water on seasonal basis, Feb 2018-Jan 2020

Magnesium

The mean magnesium content in the breeding water of mosquitoes was 7.83 mg/l in the first year and increased to 8.40 mg/l in the second year. A maximum

recorded value occurred in February and March 2018, while the lowest value was noted in May 2019. Seasonal scrutiny of magnesium level: during the pre-monsoon season, the magnesium content ranged from 7.67 to 8.94 mg/l, with a mean value of 8.25 mg/l. In the monsoon season, magnesium levels varied from 7.44 to 8.88 mg/l, with an average of 8.00 mg/l. In the post-monsoon season, the magnesium levels fell between 7.64 and 8.81 mg/l, with a mean value of 8.12 mg/l. The graphical representation of the monthly variation in the amount of magnesium in mosquito breeding water is shown in (Figure 68). The graphical representation of the mean magnesium is shown in (Figure 69). Statistical analysis indicated a significant variation in magnesium between seasons (P value 0.062, F 2.889 value). Magnesium shows significant positive correlation with temperature (r=0.576, p= 0.000), conductivity (r= 0.430, p= 0.000), TDS (r=0.577, p=0.000) alkalinity (r= 0.358, p= 0.002), calcium (r=0.649,p=0.000), total hardness (r= 0.557, p= 0.000), nitrate (r=0.501,p=0.000) and DO (r=0.243,p=0.040).

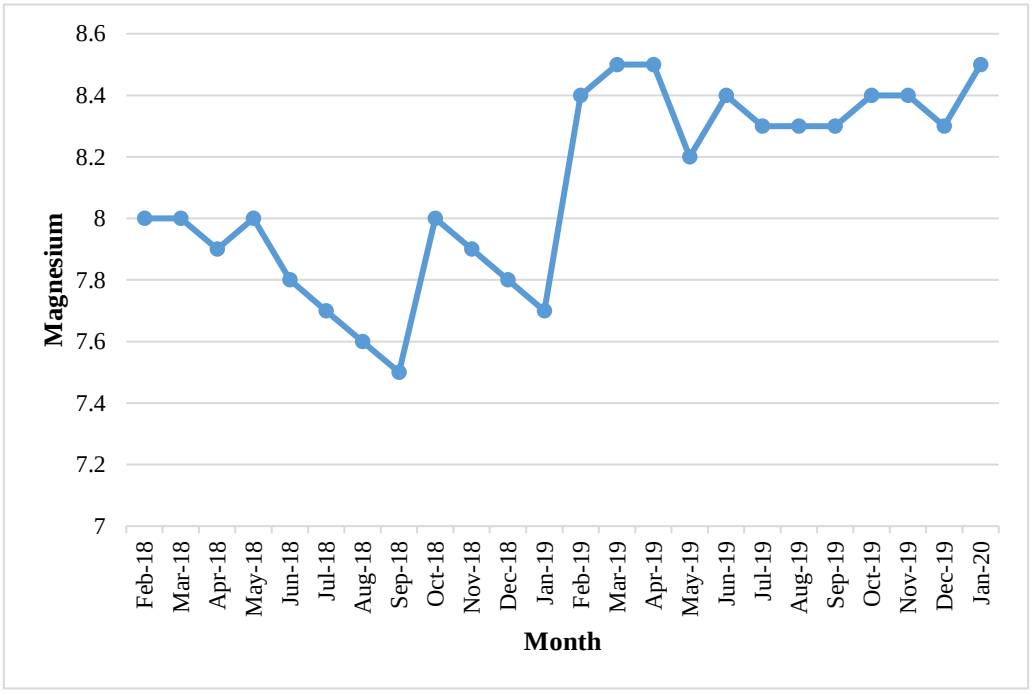

Figure 68: Monthly variations in magnesium of breeding water, Feb2018-Jan2020

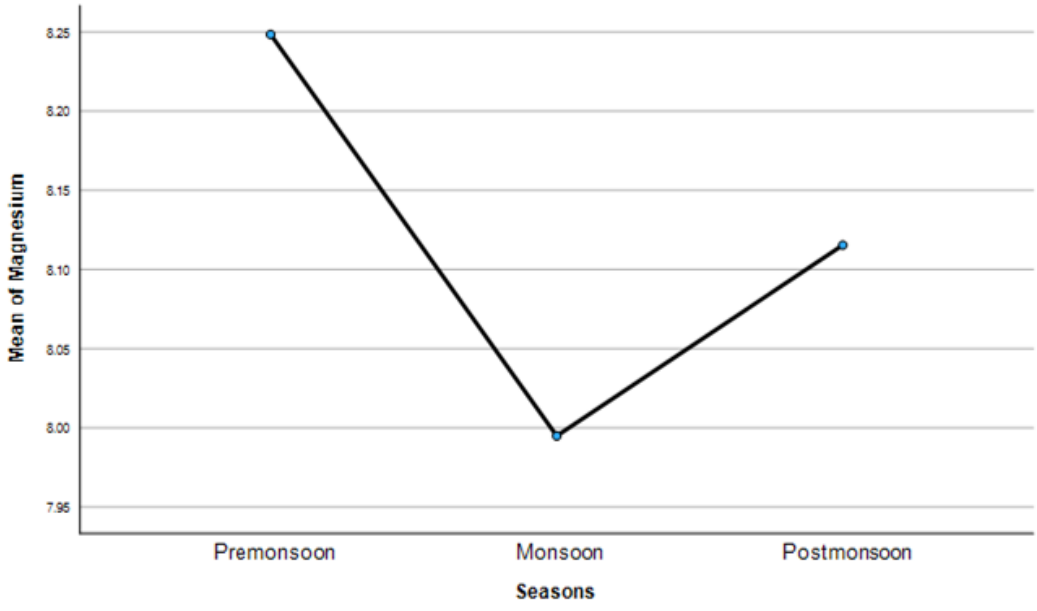

Figure 69 : Mean plot of magnesium of breeding water on seasonal basis, Feb 2018-Jan 2020

Nitrate

The mean amount of nitrate was 5.48 mg/l during the first year and 6.61 during the second year. The highest recorded nitrate value was observed in January 2020, while the lowest was in September 2018. When considering seasonal patterns, the highest nitrate concentration was recorded in the post-monsoon season and the lowest in the monsoon season. Especially during the pre-monsoon season, nitrate levels ranged from 5.34-6.79 mg/l, with a mean of 6.00 mg/l. In monsoon season, it varied from 5.44 to 6.94 mg/l, with an average of 6.03 mg/l. During the post-monsoon season, the concentration of nitrate was in the range of 5.64–7.01 mg/l (Figure 70) depict the graphical representation of the monthly variation in the nitrate of breeding

water. The graphical representation of the mean nitrate is shown in (Figure 71). Statistical analysis indicated a significant variation in the level of nitrate between seasons (P = 0.245, F = 1.436). Nitrate shows significant positive correlation with temperature (r= 0.336, p= 0.004), conductivity (r= 0.279, p= 0.018), alkalinity (r= 0.578, p= 0.000), calcium (r= 0.458, p= 0.000), magnesium (r= 0.501, p= 0.000), total hardness (r= 0.670, p= 0.000), and DO(r= 0.236, p= 0.046) and negative correlation with phosphate(r=-0.126,p=0.292).

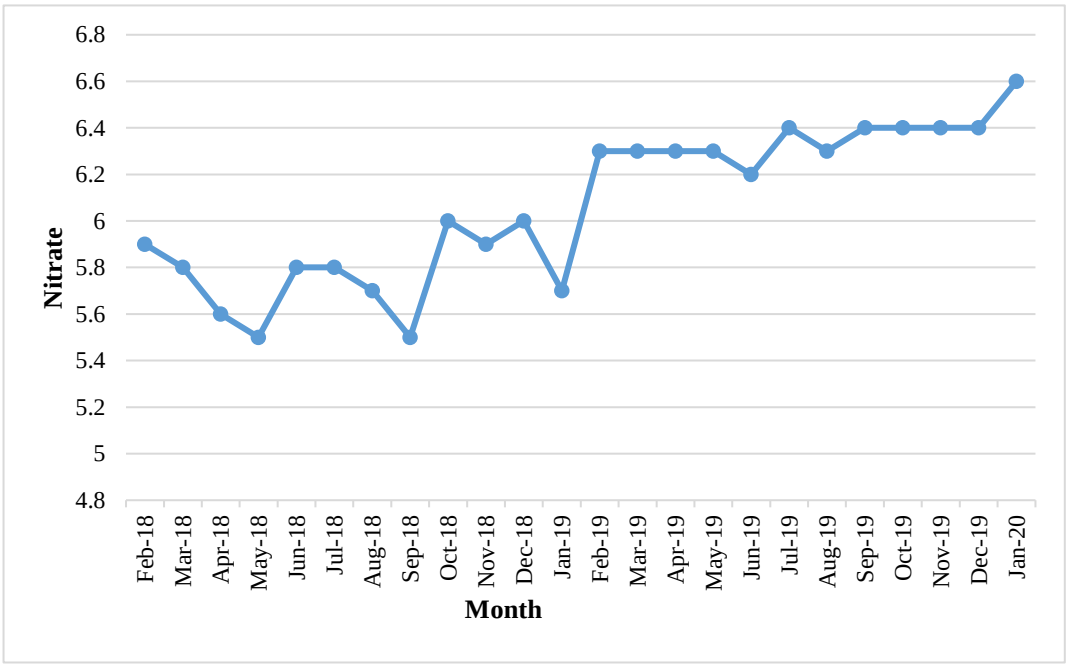

Figure 70. Monthly variations in nitrate of breeding water, Feb2018-Jan2020

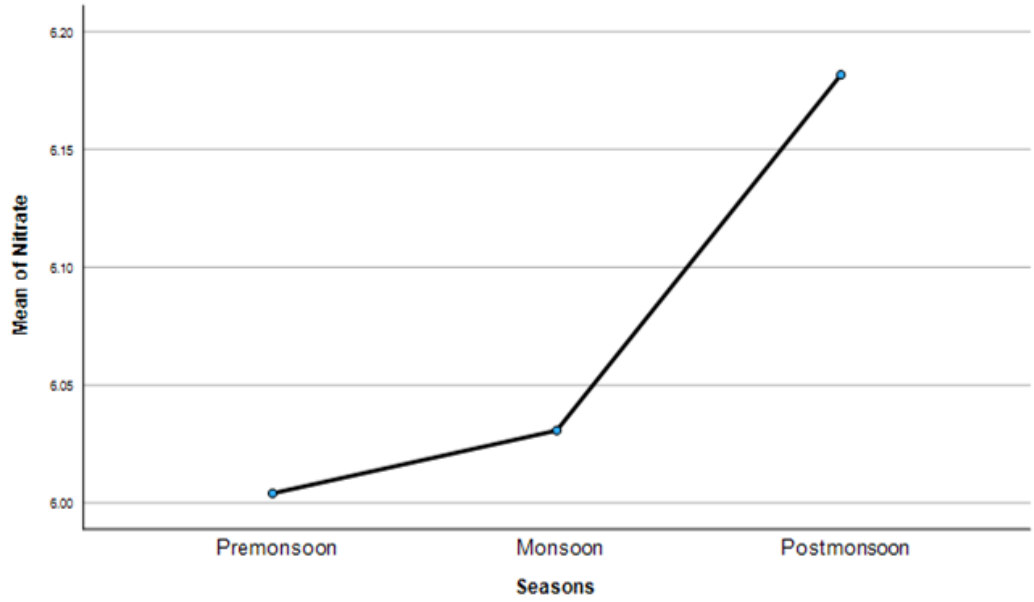

Figure 71. Mean plot of nitrate of breeding water on seasonal basis, Feb 2018-Jan 2020

Phosphate

During the first year, the mean phosphate level was 0.85 mg/l, and in the second year, it was slightly higher at 0.87 mg/l. The highest recorded phosphate value was noted in May 2018, while the lowest was recorded in June 2019. Seasonal interpretation of phosphate levels shows that the highest concentration of phosphate recorded during the pre-monsoon season (0.85 mg/l) and the lowest during the monsoon (0.87 mg/l). The phosphate level ranged from 0.66 to 0.99 mg/l, with a mean of 0.88 mg/l during the pre-monsoon season. It ranged from 0.66-0.96 mg/l, with an average of 0.85 mg/l. During the post-monsoon season, it varied between 0.62-0.97 mg/l, with a mean of 0.86 mg/l. (Figure 72) depicts the graphical representation of the monthly variation in the phosphate of breeding water. The graphical representation of the mean nitrate is shown in (Figure 73). Statistical analysis indicated a significant variation in the level of phosphate between seasons (P = 0.321, F =1.155). Phosphate

showed negative correlation with alkalinity (r=-0.021,p=0.862), nitrate (r=-0.126,p=0.292), DO (r=-0.164,p=0.170) and BOD (r=-0.089, p=0.458).

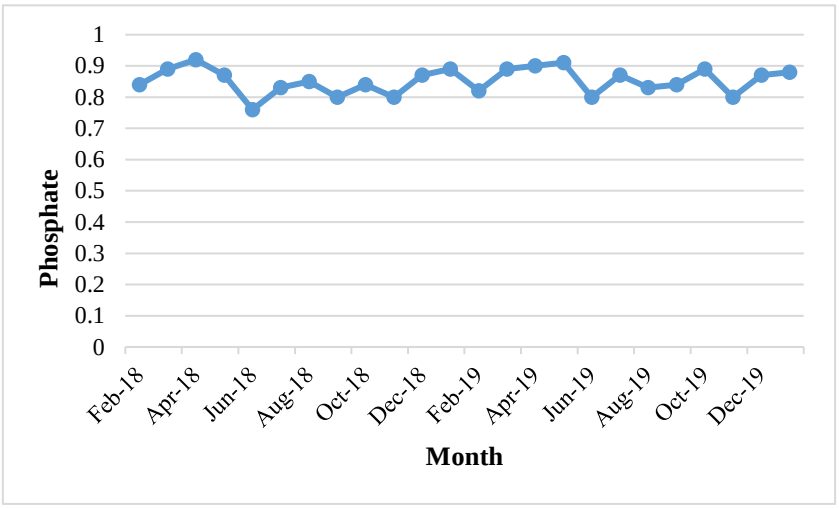

Figure 72. Monthly variations in phosphate of breeding water, Feb2018-Jan2020

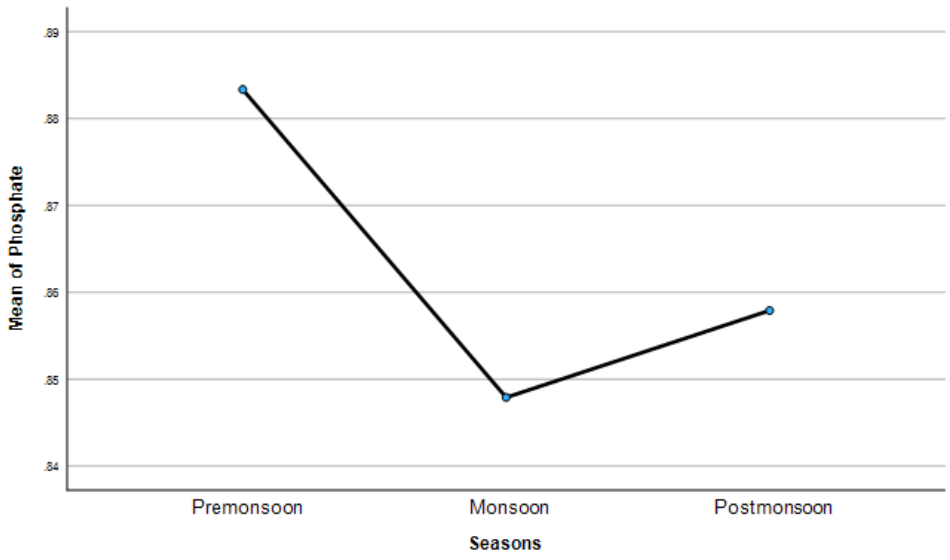

Figure 73. Mean plot of phosphate of breeding water on seasonal basis, Feb 2018-Jan 2020

Dissolved oxygen (DO)

The mean amount of dissolved oxygen in breeding water was found to be 4.89 mg/l, and it was slightly higher (5.07 mg/l) in the succeeding year. The highest recorded value of 5.7 mg/l was noted in May 2018, and the lowest value of 4.43 mg/l was recorded in September 2019. Seasonal elucidation regarding dissolved oxygen explained the highest concentration during the pre-monsoon season and the lowest during the monsoon season. The dissolved oxygen ranged from 3.8 to 6.3 mg/l during the pre-monsoon season with a mean of 5.07 mg/l, while in the monsoon season, it varied from 4.2 to 5.4 mg/l with an average of 4.75 mg/l. During the post-monsoon season, it was between 4.3 and 6.1 mg/l, with a mean of 5.13 mg/l. Figure 74 depicts a graphical representation of the monthly variation in the dissolved oxygen of breeding water. The graphical representation of the mean dissolved oxygen is shown in (Figure 75). Statistical analysis indicated a significant variation in the level of dissolved oxygen between seasons (P =0.027*, F = 3.80). Dissolved oxygen shows significant positive correlation with temperature (r= 0.286, p= 0.015) TDS (r= -0.401, p= 0.000), calcium (r=0.250, p= 0.034), magnesium (r=0.243, p= 0.040), hardness (r=0.312, p= 0.008), nitrate (r=0.236,p=0.046) and BOD (r=0.963, p= 0.022) and negative correlation with phosphate(r=-0,164,p=0.170).

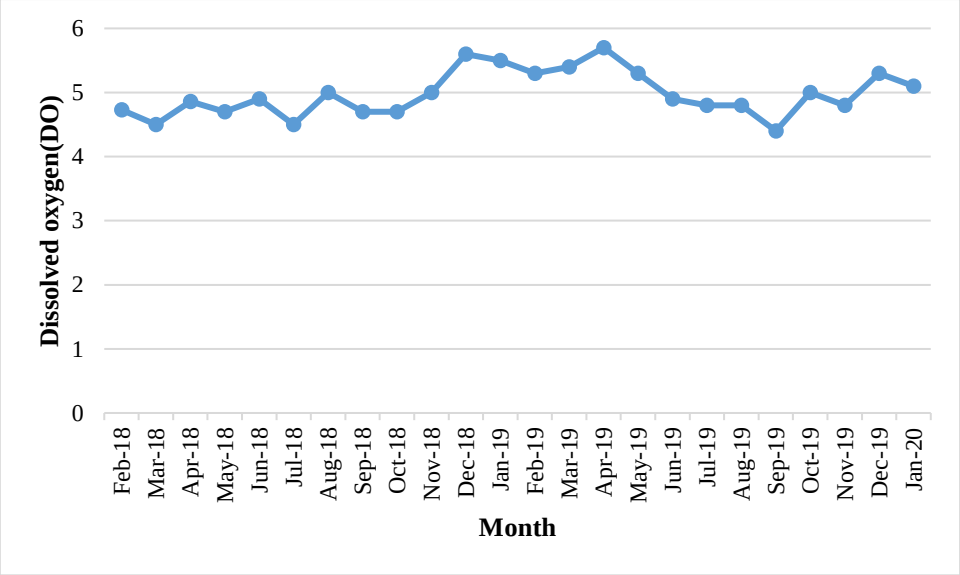

Figure 74. Monthly variations in DO of breeding water, Feb2018-Jan202

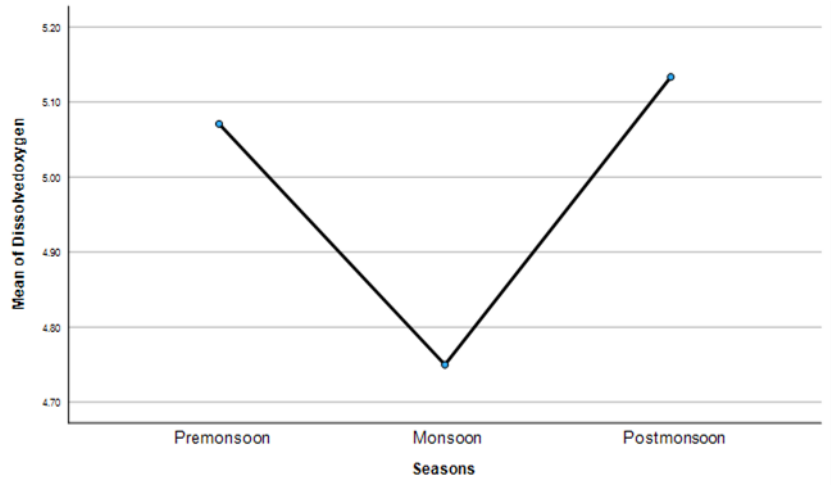

Figure 75. Mean plot of DO of breeding water on seasonal basis, Feb 2018-Jan 2020

Biological Oxygen Demand (BOD)

In the first year of investigation, the average BOD level was 2.66 mg/l, whereas it was 2.33 mg/l in the second year. A maximum mean value of 2.86 mg/l was recorded in October 2018, and the lowest mean of 2.19 mg/l was recorded in May 2019. Regarding the seasonal perspective, the highest BOD concentration was recorded during the post-monsoon and the lowest in the pre-monsoon season. The

dissolved oxygen ranged from 2.16-2.93 mg/l, with a mean of 2.47 mg/l during the pre-monsoon season. It varied from 2.02-2.92 mg/l during the monsoon season, bearing an average of 2.43 mg/l. During the post-monsoon, it varied from 2.22-2.96 mg/l, bearing a mean of 2.59 mg/l. Figure76 depicts a graphical representation of the monthly variation in the BOD in breeding water. The graphical representation of the mean BOD is shown in (Figure 77). Statistical analysis indicated a significant variation in the level of BOD between seasons (P = 0.087F = 2.530). BOD shows significant positive correlation with temperature (r= 0.288, p= 0.014), TDS (r= 0.400, p= 0.000), total hardness (r= 0.240, p= 0.042), DO(r=0.963,p=0.000)and significant negative correlation with total hardness (r= -0.089, p= 0.458).

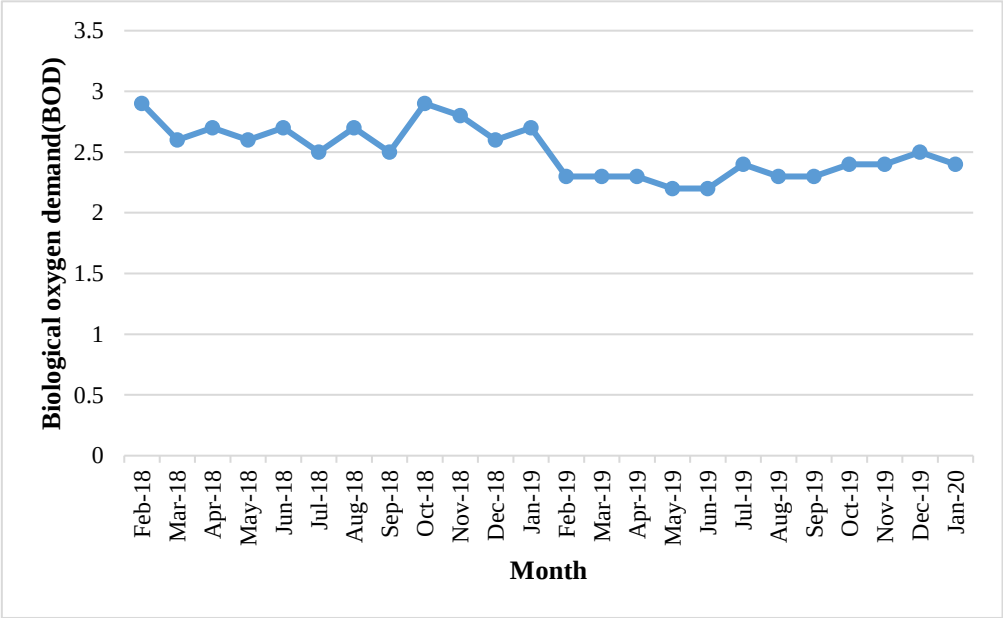

Figure 76. Monthly variations in BOD of breeding water, Feb2018-Jan2020

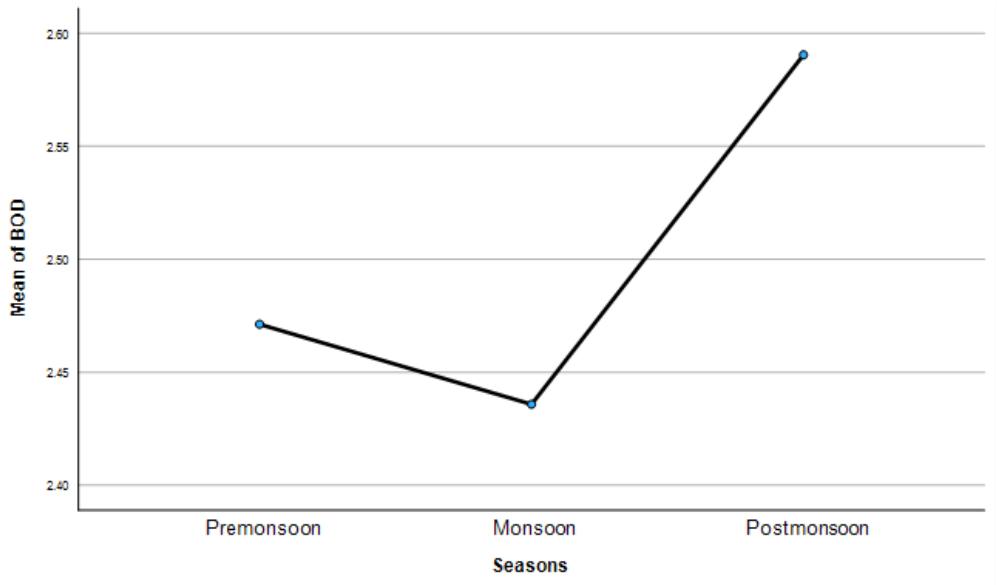

Figure 77. Mean plot of BOD of breeding water on seasonal basis, Feb 2018-Jan 2020

3. Mosquito larval Abundance

Aedes aegypti, *Culex quinquefasciatus*, and *Anopheles stephensi* were found to co-exist in the same breeding sites. The study focused on the larval abundance of these mosquito species during different seasons. A greater larval abundance was observed for *Aedes aegypti* than *Culex quinquefasciatus* and *Anopheles stephensi* during the study. *Aedes aegypti's* larval abundance was found to be 17.62±3.2 during pre-monsoon, 16.58±2.12 during monsoon, and 12.20±1.99. A significant difference in the mean larval density was noted across the seasons (Kruskal-Wallis test, H=35.669[a], *df*=2, P=<.001) (Table: 22-23).

For *Culex quinquefasciatus, mean* larval abundance was 16.75±1.62 during pre-monsoon, 15.70±1.96 during monsoon, and 11.75±1.26 during post-monsoon. The effect of seasonal variations on larval abundance was found to be significant (Kruskal-Wallis test, H=45.537[a], *df*=2, P=<.001)(Table: 22-23).

Anopheles stephensi was found to be less common than *Aedes aegypti* and *Culex quinquefasciatus* during the study. Its mean abundance was found to be 13.87 ±1.80, 14.16 ±0.96, and 11.45 ±1.14 during the pre-monsoon, monsoon, and post-monsoon seasons, respectively. This mosquito species also displayed a significant association between larval density and season (Kruskal-Wallis test, H=33.529[a], *df*=2, P=<.001)(Table: 22-23).

Table 22: Correlation of larval abdundance by Kruskal-Wallis test

Mosquito larvae	Degree of freedom *df*	H statistics	P value
Aedes aegypti	2	35.669[a]	<.001
Culex quinquefasciatus	2	45.537[a]	<.001
Anopheles stephensi	2	33.529[a]	<.001

The significance level is .050. **a** - H statistic is adjusted for ties

Table: 23 Distribution of mean levels of physicochemical parameters and mean mosquito larval abundance based on seasons

Values are expressed as Mean ± SD. DO - Dissolved oxygen, BOD- Biological oxygen demand

Seasons	**Physico-chemical parameters and mean mosquito larval abundance**														
	Temperature(^{0}C) (mean ± SD)	pH (mean ± SD)	Conductivity (mS) (mean ± SD)	Total dissolved Solids (ppm) (mean ± SD)	Total alkalinity (mg/l) (mean ± SD)	Total hardness (mg/l) (mean ± SD)	Calcium (mg/l) (mean ± SD)	Magnesium (mg/l) (mean ± SD)	Nitrate (mg/l) (mean ± SD)	Phosphate (mg/l) (mean ± SD)	DO (mg/l) (mean ± SD)	BOD (mg/l) (mean ± SD)	*Aedes aegypti* (mean ± SD)	*Culex quinquefasciatus* (mean ± SD)	*Anopheles stephensi* (mean ± SD)
Pre-monsoon	31.46 ±1.47	6.78 ± 0.20	153.32 ± 5.99	249.99 ±6.82	65.99 ± 0.39	46.00 ±0.38	16.00 ±0.37	8.25 ± 0.36	6.00 ±0.38	0.88 ±0.08	5.07 ± 0.66	2.47 ±0.27	17.62 ±3.2	16.75 ±1.62	13.87 ±1.80
Monsoon	27.92 ± 1.10	6.83 ± 0.14	135.41 ± 3.65	235.69 ± 5.16	66.04 ± 0.43	46.04 ±0.45	16.00 ±0.42	8.00 ± 0.38	6.03 ±0.42	0.85 ±0.09	4.75 ± 0.35	2.43 ±0.25	16.58 ±2.12	15.70 ±1.96	14.16 ±0.96
Post-monsoon	29.75 ±0 .94	6.92 ± 0.24	164.66 ± 3.41	238.07 ± 4.03	66.19 ± 0.39	46.19 ±0.38	16.19 ±0.38	8.12 ± 0.34	6.18 ±0.38	0.86 ±0.08	5.13 ± 0.48	2.59 ±0.21	12.20 ±1.99	11.75 ±1.26	11.45 ±1.14

3.4 DISCUSSION

The distribution and community composition of mosquito larvae can be influenced by a number of factors, like urbanisation, climate, vegetation, interspecific associations, and the physicochemical parameters of the water in its habitat. This study investigated assessing the physicochemical characteristics of mosquito breeding sites during different seasons and understanding the impact of seasonal variations on influencing the population density of mosquito larvae, including *Aedes aegypti*, *Culex quinquefasciatus,* and *Anopheles stephensi*. The study observed that these three mosquitoes co-existed at the same sites, each with a variable larval abundance. Indicating their adaptability to changes in environmental conditions. This observation concur with the earlier studies where they commented about of adaptability of *Culex* and *Anopheles* mosquito to both polluted and unpolluted environment along with *Aedes* mosquitoes (Yadav et al., 2017).

For aquatic organisms, pH is a vital factor that regulates the abundance and distribution of mosquitoes. This is because pH directly involved in cell functioning and influences the permeability of cell membrane Naturally mosquitoes exhibit a high degree of tolerance to extreme pH values, which mak them competent to survive in various types of environmental conditions. Even some vector mosquito possess inherent mechanisms that enable them to thrive in both acid and alkaline aquatic habitats. This indicated that the ability to tolerate varying pH levels can be associated with the abundance of mosquito species (Multini et al., 2021).

As per the present study, pH was identified as a significant parameter ($p = 0.043$) across seasons influencing the larval survival and abundance of mosquito larvae. The study consistently observed nearly neutral pH throughout the study,

implying the importance of maintaining neutral pH for larval survival. Recent research supports the role of pH in the occurrence of mosquito immatures. The study also pointed out that fluctuations from neutral pH to alkaline or acidic conditions can affect larval diversity and favour the abundance of specific mosquito species. Parameter ($p = 0.043$) influencing the larval survival and abundance of mosquito larvae. The study consistently observed nearly neutral pH throughout the study, implying the importance of maintaining neutral pH for larval survival. Recent research supports the role of pH in the occurrence of mosquito immatures. The study also pointed out that fluctuations from neutral pH to alkaline or acidic conditions can affect larval diversity and favour the abundance of specific mosquito species (Multini et al., 2021).

Another study specified the prominence of *Aedes aegypti* from neutral to slightly alkaline pH (Dalpadado et al., 2022). It was suggested that *Culex quinquefasciatus* larvae reared at neural pH exhibited the fastest development, while larvae reared at extreme pH levels such as highly acidic pH (4) or highly alkaline pH (10) showed increased survival time(Ukubuiwe et al., 2020). Furthermore, in the previous studies, it was quoted that a pH range of 6–8 was ideal for the growth of all species in their natural habitat (Ukubuiwe et al., 2018). On the other hand, studies on the larval abundance of anopheline mentioned that pH, temperature, and turbidity were not significantly related to their abundance (Getachew et al., 2020).

In the current study it was observed that pH showed positive correlation with calcium and total hardness. This observations agrees with the findings of (Saalidong et al., 2022), where they studies various water parameters like turbidity, calcium, total suspended solids, nitrate, magnese and total coliform were linked with optimal water

pH conditions. Conversely sulphate, electrical conductivity, zinc, copper, and faecal coliform were associated with non-optimal water pH levels.

Normally, the temperature of breeding water plays a significant role in influencing mosquito metabolism and development. Temperature also moulds certain characteristics associated with vector-competence and population density of mosquitoes. It has been noted that higher temperature result in faster development and smaller adult mosquitoes (Christiansen-Jucht et al., 2014). The temperature of breeding sites remained as a significant physical parameter ($p=<0.001$) that consistently influenced larval abundance of mosquito larvae throughout the seasonal changes.

The study revealed that a moderately high temperature within the limit of 25^{o}C-32^{o}C remained relatively constant during the study, which was quite favourable for larval growth and survival with minor variations. This was proved in the study where high larval density was observed during pre-monsoon season. As per the literature survey it was found that temperature within the range of 25^{o}C-32^{o}C was conducive for the development of all mosquito species, including *Culex quinquefasciatus* and *Anopheles stephensi*, while temperature exceeding this range was found to negatively affect the larval survival (Oda et al., 1999; Tuno et al., 2023). These findings align with a study by (Tun-Lin et al., 2000) which also emphazised the importance of temperature in influencing the development of *Aedes aegypti* larvae, particularly within a temperature range of 25-30^{o}C.

Benyaha and co-workers reported that, as the temperature raises the solubility of oxygen and other gases tends to decrease. The mobility of ions is affected by the viscosity of the solution, which is in turn depend upon temperature. Consequently,

changes in temperature can lead to shifts the ion concentrations, ultimately influencing pH level, alkalinity, hardness, calcium, magnesium and nitrate levels (Benyahya et al., 2007). This agrees with the current investigation.

The rise in the breeding water conductivity and hardness leads to increase the average larval developmental time in mosquitoes. TDS is the combined concentration of inorganic salt and organic matter that are dissolved in water. The conductivity of water dependent on temperature and TDS which complies with current study. The present study evidently establishes a significant relationship between physico-chemical parameters such as conductivity ($p =<.001$) and total dissolved solids ($p =<0.001$) in different seasons, which in turn influences larval distribution and abundance. In a recent study by Dalpadado and colleagues, it was mentioned that a favourable TDS range for the survival of *Aedes aegypti* larvae falls between 250 and 350 ppm; this aligns with the TDS limits observed in the present study (Dalpadado et al., 2022).

Similar observations were made concerning *Anopheles stephensi* and *Culex quinquefasciatus*, where there was a positive correlation between TDS and larval abundance (Muturi et al., 2008; Thomas et al., 2016). These findings contrast with results of a study conducted by (Alahmed, 2012) which stated that TDS and pH were not significant factors affecting larval abundance during their research on mosquito fauna and its sesonal abundance. Similarly, observations regarding water conductivity, as indicated by (Emidi et al., 2017), clearly suggest its positive impact on *Anopheles* larvae.

In a previous study, there was a positive correlation observed between dissolved oxygen, water transparency, and conductivity with respect to *Culex* larval abundance (Dejenie et al., 2011). The studies by Burke et al., reported that higher TDS levels

above the range of 0–0.188 p.p.t. were negatively associated with *Aedes aegypti* larval abundance (Burke et al., 2010). Similarly, conductivity in the range of 162.9–616.9 µS/cm exhibited a negative correlation with mosquito larval abundance (Gopalakrishnan et al., 2013).

The current study exhibited a significant ($p = 0.027$) relationship between dissolved oxygen (DO) and seasonal variations, which eventually has an impact on the survival of mosquito larvae. The relationship between larval abundance and DO was clearly elucidated by (Silberbush et al., 2015) through their experiments involving *Culex* mosquitoes. Their findings indicated that reduced DO levels in breeding water result in diminished larval survival and prolonged larval developmental time. Such conditions are often associated with extreme water temperatures, nutritional deficiencies, overcrowding, etc. The previous study supported the positive influence of dissolved oxygen on the survival and abundance of *Anopheles* mosquitoes (Thomas et al., 2016). On the contrary, *Aedes aegypti* has been observed to lay eggs and develop normally in raw sewage with low levels of dissolved oxygen, suggesting a wide tolerance range (Overgaard et al., 2017).

This present study indicates that several chemical parameters, including total alkalinity, total hardness, calcium, magnesium, nitrate, phosphate, and BOD, were not significantly affected by seasonal changes. This finding was supported by the studies of (Nikookar et al., 2017) reported a positive correlation between the density of *Culex pipiens* and factors like alkalinity, conductivity and hardness. In which they investigated Culex *quinquefasciatus* larval abundance and its association with various physico-chemical factors of breeding water. In their study, a significant variation ($P < 0.0001$) was observed among physico-chemical parameters like pH, alkalinity, dissolved oxygen, temperature, hardness, phosphates, chloride, and TDS. Conversely,

another study conducted by (Noori et al., 2015) reported a positive correlation between the density of *Culex pipiens* and factors like alkalinity, conductivity, and hardness.

The mesocosm experiments conducted by (Noori et al., 2015) were designed to identify specific water components that are conducive to the development of *Culex* mosquito larvae. The study revealed a strong correlation between the emergence pattern of *Culex* mosquitoes and certain nutrients. Their results indicated that the breeding sites with higher PO_4 or NO_3 concentrations had increased larval survival rates. High concentrations of NO_3 were found to promote the development of male mosquitoes while inhibiting the development of female mosquitoes. However, among the adult females who emerged, those from containers with a high level of NO3 developed faster compared to the reference group. When PO_4 was added to the larval habitat in the absence of nitrogen sources, it slowed down larval development. Conversely, it took a few days for larvae to reach the pupal stage in containers with combinations of NO_3 and PO_4 or NH_4 and PO_4 nutrients.

In a study, ammonium, fluoride, phosphate, and conductivity were found to have positive correlations with each other and with *Aedes* mosquito larvae. On the other hand parameters like nitrates, DO and temperature displayed positively correlated with each other and *anopheles.* Both *Anopheles gambiae* and *Aedes aegypti* showed significant correlations with certain variables. Especially ammonium and phosphate exhibited positive correlations with *Aedes aegypti* larval abundance (ammonium: $P = 0.001$; phosphate: $P = 0.001$). However, the presence of *Anopheles gambiae* larvae was significantly correlated only with rising temperature ($P = 0.033$), not with DO ($P = 0.333$) or nitrates ($P = 0.983$). Importantly, the *Culex* genus did not exhibit a

significant correlation with any of the investigated variables (Onchuru et al., 2016).The precise role of calcium and magnesium in larval abundance requires further research to establish conclusive information.

Ukubuiwe and co workers reported a strong connection between the abundance and diversity of culicine and anopheline larvae and several other water parameters, including pH, temperature, dissolved oxygen, alkalinity, phosphates, and chloride concentrations. It was also found that elevated hardness values of ≥150 mg/L $CaCO_3$ resulted in decreased success in the development of immatures and had a negative impact on various biological fitness indicators in adult mosquitoes (Ukubuiwe et al., 2020).

While analysing the role of BOD, it was observed that effective removal of BOD resulted in a decrease in the development of mosquito larvae (Diemont, 2006). The larval environment of *Culex quinquefasciatus* was characterised by low levels of dissolved oxygen and a high BOD in comparison to other culicine mosquitoes. These types of habitats were often associated with tubificid worms (Annelida) and chironomid larvae (Diptera). Larval survival of *Culex quinquefasciatus* experienced a rapid decline when the pH level reached 9.4 (Amarasing & Dalpadado, 2014)

Seasonal changes plays a significant role that modulate the physico-chemical parameters of breeding water, which can have an impact on larval abundance and vectorial capacity of mosquitoes. In this regard many research have been focused on understanding the role of seasonal changes on mosquito borne diseases. Temperature has been identified as a key factor in predicting the mosquito population dynamics. The changes in temperature patterns during different seasons, especially a warmer temperatures, can lead to expansion of mosquito borne diseases (Franklinos et al., 2019). For example, when the breeding water has a neutral pH, and high dissolved

oxygen creates favourable conditions for the growth of various *Bacillus* species like *Bacillus subtilis* and *Bacillus tequilensis Bacillus cereus, Bacillus megaterium* . These bacteria release volatile substances that attracts the gravid female mosquitoes for oviposition. The other genera of bacteria including *Enterobacter*, *Aeromonas*, *Pseudomonas Acinetobacter*, and *Klebsiella* are involved in attracting mosquitoes for oviposition. However *Stenotrophomonas maltophilia* was found to deter oviposition of gravid *Anopheles gambiae* mosquitoes (Seal & Chatterjee, 2023). The physicochemical characteristics of breeding water provide an ambient environment for the development of fungi, especially the *Candida* species. The physicochemical parameters of mosquito breeding plays a pivotal role in shaping the microbial content of breeding water. This micro biota directly influence the larval abundance as wellas gut micro biota within mosquito species.

3.5 CONCLUSION

Even though many mosquito species can co-exist in the same breeding habitat, mosquitoes exhibit distinctive preferences for aquatic habitat with varying physicochemical characteristics to lay their eggs and ensure the survival and proliferation of mosquito larvae. In a natural environment, certain vector mosquito populations are abundant in specific aquatic habitats, while others remain unoccupied. This indicates that some locations are more appealing to gravid female mosquitoes. The selection of suitable oviposition sites is very important for the vector population dynamics of vector mosquitoes, as these preferred habitats enable immature vectors to complete their life cycle and develop into adults. The physico-chemical properties, microbial composition, and organic content of the breeding habitat water bodies are crucial factors for the survival of mosquito larvae. The physico-chemical parameters of larval habitat have a great effect on the various life stages of mosquito vectors, including larval survival, developmental duration, and fitness of adult mosquitoes. In the present study, about twelve physico-chemical parameters like D.O., BOD, alkalinity, pH, turbidity, TDS, temperature, hardness, conductivity, and the presence of various ions such as nitrate, phosphate, and magnese were studied. Among these parameters, pH, temperature, conductivity, dissolved oxygen, and TDS were identified as significant factors, particularly during seasonal variations, influencing mosquito larval existence.

Specific environmental conditions create unique habitats that attract particular types of microbial species to the inhabiting mosquitoes. Therefore, it is imperative to understand various factors that influence the daily oviposition pattern of gravid female vector mosquitoes in order to effectively control them in natural settings. As the

mosquito larvae are exclusively aquatic, their distribution is intricately linked to the availability of suitable water sources, as well as the microbial content and physiochemical parameters within the breeding water. Therefore, a deep understanding of the ecological traits of these larval habitats and the environmental characters affecting mosquito abundance can help in designing better vector control strategies.

RECOMMENDATIONS

Studies perturbing the mosquito microbiome provide an overview of gut bacterial and fungal consortium. The mosquito gut also harbours viruses, algae and protozoa other than bacteria, and fungi. So, the studies that characterise these gut entities other than bacteria and fungi should be addressed urgently. More research elucidated microbial role in arboviral transmission will be very beneficial in producing new vector control options. Exposing the core microbiome within mosquitoes and determining their influence on the host, as well as whether bacterial and fungal members have functional redundancy, are further challenges. While a systematic understanding of these tripartite interactions is necessary for designing and developing high-throughput in vitro assays capable of evaluating host-microbe-pathogen interactions in this regard. The exploration of the molecular phylogeny of bacteria and fungi helps us establish species delimitations and determine sibling species. From a broader perspective, the phylogenetic analysis of the gut consortium will be helpful to access their taxonomy as well as its evolutionary history. More research in this field may shed light on the role of microbiota in organism's survival. Being an aquatic form, larval growth and survival depend on many parameters, like seasonal fluctuations, nutritional requirements, competition, etc. So, it's better to determine the role of these physicochemical parameters and their relevance to its larval densities, as well as how this relates to the natural variation of the mosquito microbiome. More research is needed to understand the role of environment in shaping the gut profile of mosquito larvae. Anthropogenic alteration of the environment by the use of chemicals like antibiotics and pesticides could influence the microbiota of mosquitoes, thus having wide-reaching implications for mosquito biology and pathogen transmission. This area of research warrants further investigation.

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