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Insilico Analysis of Mosquito (Family: *Culicidae*) Microbiome Diversity and its Role in Insecticide Resistance

by

Ahmad Zafar Baig

A thesis submitted in partial fulfillment for the degree of Master of Science

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I dedicate this thesis to all the great people came in my life specially my beloved Mother and my Supervisor who taught me to stand out in this world with dignity and fearlessly, after that I dedicate it to my lovely sisters that supported me like a solid rock after that to all haters in my life that encouraged me to step ahead without any fear.

CERTIFICATE OF APPROVAL

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Abstract

The gut microbiota of insects is one of the unexplored area. The roles associated with these microbiomes plays a key vital role in supporting their survival and combat with ecological challenges. Mosquito is one of focal attention insects among the Arthopods, being the vector of many pathogenic diseases including dengue and malaria. A variety of strategies have been design and implemented to fight against this vectors including abnoxious use of insecticides. Indiscriminate use of insecticides has lead to development of resistance against broad range of insecticides. Crucial role of bacterial for resistance emergence microbiome has been also under discussion. In this study we focused on the literature mining of the mosquito microbiota. After that those microbiota were fallen into the OTU and the similarity analysis was conducted for those bacteria. The most diversitified group of microbes was found in *Anopheles gambiae* and *Anopheles funestus*. The 47 genera were found to be involved directly or indirectly in the aromatic compound degradation but 7 were identified in the biphenyl pathway which is key pathway to initiate Benzoate degradation. After that the insecticidal resistant pathway was investigated. The boolean network modeling was done to find the significant attractors. Our findings was suggestive that there are many microbes present in the mosquito that are involved in the Biphenyl pathway that degrade aromatic compounds that is the core component of insecticides. This pathway was then model at boolean networks which suggests that the core molecule responsible to trigger the pathway is Biphenyl.

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Abbreviations

Ae.	*Aedes*
An.	*Anopheles*
AMP	Antimicrobial Peptides
DFH	Dengue Fever Hemmoraghe
EST	Expression Sequence Tags
GEM	Genome Metabolic Models
HTS	High Throughput Sequencing
IMD	Imune Defficiency
IRS	Insecticidal Repellent Sprays
MAMBO	Metabolomic Analysis of Metagenomes using FBA and Optimisation
MSA	Multiple Sequence Alignment
NGS	Next Generation Sequencing
OTUs	Operational Taxonomic Units
P.	Plasmodium
PAMPs	Pathogen-associated Molecular Patterna
PRRS	Pattern Recognition Receptors
RBM	Roll Back Malaria
Spp.	Species
St.	Stegomyia
SRS	Substrate Recognition Site
WGS	Whole Genome Sequencing

Symbols

&&	AND Operation
==	Is Equals to
!	NOT Operation
||	OR Operation

Chapter 1

Introduction

The mosquito is a general term used in English to describe biting flies which belongs to the family of *Culicidae*. These biting flies follows the taxonomy as sub-order *Nematocera*, order *Diptera*. This family comprises of approximately 3500 species. The major genera which are of medical importance are *Anopheles* in case of Malaria, *Aedes* for yellow fever, dengue and chikungunya and *Culex* for Japenes encephalitis, West Nile virus and filarisis [1, 2].

They are dual winged biting flies. This family is abundant as it is found throughout the tropical and temperate regions of the world as well as well beyond the Arctic Circle. But only a small proportion of this family act as vector for transmission of disease to human [1].

The mosquito family includes 3,570 extant species which can be classified in two sub families and 113 genera. The two sub families are as follow *Anopheline* which includes three genera and *Culicinae* which include 110 genera theses 110 genera can be segregate into 11 tribes [3]. Mosquitoes are considered one of the most primitive organism whose history trace backs to the ancient history of mankind as they were one of the utmost vital vectors for disease of human. They vectored a large variety of disease from very past to human. From historical literature it has been found that Julius Cesar a Roman Emperor had to drained a swamp in an attempt to control Roman Fever (malaria) [1].

The medical importance of mosquito can be estimated from this fact almost 300 to

500 million people are effected from malaria annually. From which 1 million people lost their lives while the maximum numbers of mortalities are noted in infants and young children. The region which is mostly effected by malaria is Sub-Saharan African region. In the recent years dengue virus has expanded its range to 50 to 100 million population annually with thousands of mortalities due to severe form i.e. dengue hemorrhage fever. In the past few decades a new endemic emerged in East Africa and America by West Nile virus named chikungunya which also caused many deaths in the region [1].

Mosquitoes (family *Culicidae*) are of great medical importance that they have always been in the focus of worlds research due to their property as vector for medically important diseases of human. The wide range of disease spread is due to the dual property of mosquito as the can be biological vector as well as they can be the mechanical vector. The estimation of medical importance can be guessed that almost more than half population of world is at risk of getting mosquito vectored disease like Malaria, Dengue, Chikungunya, West Nile Virus and Japanese encephalitis [4].

According a World Health Organization (WHO) report published in 2010 about 247 million worlds population became ill due to mosquito and around 1 million people get the disease in 2008. The distribution of mosquitoes throughout the world is somehow miss interpreted that they occur only in tropical and subtropical environments but to some extent it is not true as mosquito can cause nuisance/annoyance or can also spread pathogens or viruses in temperate latitudes as well [4, 5].

With the recent out breaks of dengue fever in Pakistan the studies on mosquito distribution and role as vector bloomed in Pakistan. Almost 104 species of mosquitoes have been reported in literature present in Pakistan. The correct vector identification for the controlled strategies is very important in case of vector borne diseases. The DNA based approaches are used to study the mosquitos presence in a specific ecology. In Pakistan many species of *Culex* and *Anopheles* have been identified. Malaria and Dengue is still a threat to this region [6].

Current studies suggested the progress in the overall global malaria control, and it is estimated that 2 million more cases of malaria have appeared in 2017 as

compared to 2016. The number is increasing in the incidence of malaria inside Americas region [7].

This stall is overlapping with and the insecticide resistance reports are increasing with the passage of time [7, 8]. It stances a big challenge for the programs for the control of the vector for the malaria [9]. Fundamental mechanism of the resistance of the insecticide in the vectors of malaria are not clearly identified and recognized. Yet the following four basic mechanisms were described which underlie insecticide resistance in mosquitoes. [10]:

1. The modification of the cuticle.
2. Amplified detoxification of the insecticides.
3. Insensitivity of the sites of target of the insecticides.
4. Behavioral avoidance of insecticides.

Substantial gaps are still available for the young researchers, particularly the high dose insecticides resistance in the population of the mosquito. Increasing exposure to sophisticated genomic methods has now encouraged the study of many facets of the biology of the mosquito, such as the microbiota of the mosquito populations, which might be linked with the resistance of the insecticide [10].

Just like other organisms mosquito is also hosting a vast types of microbes and these microbes are basically acquired during their immature developmental stages, such as from the habitat in which mosquitoes are breeding, and also from the food source of mosquitoes from where these mosquitoes take their food [11]. Furthermore, the microbes obtained from the environment and/or food supply, the transmission of the bacteria from the female mother at the time of laying eggs via transovarial mechanism [12] and in the mosquitoes the transmission from young stage to the adult stage is also being reported in the mosquitoes which is done transstadially [13]. These microbes among which some are also known to the metabolizing nature against the insecticides [14–16], vigorously change as per the physiology of the host [11, 17]. Hence, the microbiota of the mosquitoes have the capabilities to contribute towards the detoxification of the insecticides and also for

the increased resistance present in the host, it is the same phenomenon which was also previously being reported in the case of the pests present in the agricultural domain of biology [18–21] yet, its discussion is purely based on the vectors associated with the diseases [22, 23]. In Pakistan following species of mosquito have been identified which spreads evenly in all regions of the country [6]. According to the research in the Pakistan's mosquito population the *Anopheles* are more prevalent.

TABLE 1.1: Table Showing The Species Present In The Pakistan [6].

Anopheles	***Culex***	***Aedes***
subpictus	*quinquefasciatus*	*aegypti*
peditaeniatus	*theileri*	*albopictus*
stephensi	*tritaeniorhynchus*	*w-albus*
splendidus	*bitaeniorhynchus*	*unilineatus*
pulcherrimus	*mimeticus*	
annularis	*fuscocephala*	
culicifacies		
gambiae		

1.1 Problem Statement

In human disease the most important vectors are mosquitoes and they are also responsible for the massive pandemics like Dengue, Chikungunya and Zika virus. In current era, many recent studies have focused on the role of insects microbiota in the physiology, development, reproduction, acquisition and transmission of pathogens and in association of resistance development against drugs, antibiotics and insecticides. Keeping in view the significance of microbiome as a supportive instrument in survival of insects particularly as vectors, there is a need to explore role of the microbiota in order to get effective target specific strategies for vector (mosquito) control strategies and to explore the target specific pathways to find out the attractors involved in the resistance of insecticides which can be effective in designing of new insecticides.

1.2 Objectives

The study entails following objectives:

1. To check the prevalence of bacterial microbiota of mosquito.
2. To find the variations in microbiome among different species of mosquitoes at different developemental stages.
3. To explore the role of microbiota in insecticides resistance with respect to aromatic compounds degrading pathways.

1.3 Scope

The study of mosquito microbiome will help us to figure out the role of microbiota in insecticidal resistance. These findings can help the researchers to make more environment friendly and efficient mosquito control strategies and also help to deal with problem of insecticidal resistance. With the recent advancement in the computational studies, researchers are able to explore the association of different organisms. The microbial interactions to their hosts are widely studied in last few decades revealing that microbes are responsible for the various phenotypical+physiological functions in host. *Anopheles* mosquito been the most significant vector specie thus to design a vector controlled strategy we have to study the microbial interactions in the *Anopheles* species. In this research we focused on the identification of microbes reported till date in *Anopheles* species and identify their functional role and to identify bacterial role pathways involved in this. If experimental and computational research is used in a collaborative manner, it will identify not only the representative species of the mosquito microbiota rather it also identify the unknown species which were not reported with which these leading microbial interaction through a wide range of metabolite exchanges [24].

Chapter 2

Literature Review

2.1 Biology of Mosquitoes

To understand the versatile role of mosquitoes as vector it is better to understand the biology of mosquito.

2.1.1 Habitat of Mosquito

The first step in understanding the biology of mosquito is to study its habitat as there is hardly no such places in the world where mosquitoes are not found. Mosquitoes breed almost in every water place this is due to their adaptation mechanism that mosquitoes are able to breed in each type of water places like rivers, swamps, lakes, clean water, large or small water bodies even in permanent and temporary water bodies. This leads to the conclusion that there is hardly any water body that didn't lend itself a breeding site for mosquito. In temporary flooded areas, the areas near rivers and lake with the water flow fluctuations, flood waters mosquitoes like *Aedes vexans* or *Ochlerotatus sticticus* have developed such adaptations that allows them to breed and their ability to fly miles cause nuisance in places even far from their breeding sites [25].

Some species of *Ochlerotatus* have also adapted to breed in the harsh environments like snow-melt, swampy woodlands these mosquitoes include *Ochlerotatus communis*, *Ochlerotatus cataphylla*, *Ochlerotatus cantans*, *Ochlerotatus hexodontus* and *Ochlerotatus punctor*. They encounter conditions and make them ideal. In the flood plains along coastal areas the environment contains large number of salt thus in these area the halophilous species (which prefer salt water or brackish habitats) for example *Ochlerotatus taeniorhynchus*, *Ochlerotatus sollicitans*, *Ochlerotatus vigilax*, *Ochlerotatus caspius*, *Ochlerotatus detritus* are focus in large numbers [25].

The *Anopheles* larvae developed an associative link with mosquito species in every habitat like fresh water, salt water, edges of streams, rice fields, mangrove swamps, grassy ditches or in temporary or permanent water bodies. Some species are known tree living named arboreal species such as *Aedes cretinus*, *Ochlerotatus geniculatus*, *Orthopodomyia pulcripalpis* and *Anopheles plumbeus* these species prefer tree holes as their habitat. Some species can also breed in small water bodies like containers, rain water, water drums, tyre, cemetery pots or small clay pots these species include *Culex pipiens*, *Aedes aegypti* [*Stegomyia aegypti*] *Aedes albopictus* [*Stegomyia albopicta*] or *Ochlerotatus japonicas* [4, 25].

The adaptations helped mosquito to change their habitat like Asian Tiger Mosquito *Aedes albopictus* originally found in the tropical regions. But in the course of this climate change they brought evolution in them as they became photoperiodic sensitive. When the days are shorter the photoperiodic sensitive female lays different eggs as it lays egg in longer days. The eggs laid in shorter days are inactive and hatch themselves in suitable seasons which ensures the species survival in the winter [4].

The ability of mosquitoes to adapt the climate and the adaptation of eggs to be resistant to drought, their survival capacity for greater than a year and the ability to breed at almost every site whether it is artificial or natural such as running water, tyres, container helped mosquitoes to ensure their fitness from centuries. This also contributed to the spread of mosquitoes internationally from one border to other. They can be transported from one place to another in hours or days via

aircrafts, cars or containers [26].

2.1.2 Life Cycle

The tremendous ecological tendency and flexibility of mosquito can be understood by successful metamorphosis of mosquitoes. As mosquitoes belong to *Diptera* thus they also exhibit complete metamorphosis like *Diptera* species. All mosquitoe species need aquatic environment for breeding and development, but few *Aedes* and the ability of *Ochlerotatus spp.* to lay eggs in moist soil as well [4].

After hatching process the mosquitoes undergoes larval instars and a pupal stage. In the pupal stage mosquito follows metamorphosis for the successful adult production. Most mosquito species are unautogenous means to follow copulation female mosquito needs blood meal. Only few species which donot require blood meal thus known as autogenous. They first develop egg batches without blood meal e.g. *Culex pipiens* [4].

After the blood meal female mosquitoes lay between 50 and 500 eggs within 2 to 4 days (may be longer in temperate or cold environments). Generally, on the basis of egg laying behavior the mosquitoes are divided into two of the main groups, second group criteria are on the basis of embryo that whether the embryo enters in the dormancy period of the enter the diapause. Some of the parameters which are critical in the determination of the choice for the site of breeding with respect to the egg laying procedure by the females on to the surface of water are not yet known for most of the species. The factors such as the quality of water, the quantity of existing eggs, the incidence of light, quantity of the available food and the vegetation at the local level are quite decisive for the site where the egg could be layed by the mosquito with great favor [4, 14]. The content of the organic material present inside the water also plays a very critical and important role in the attraction of the female mosquitoes to lay their eggs. Visibly, the decomposition of the organic material creates several of the gases inside the water body such as methane, ammonia, and also the carbon dioxide and the presence of these gases in turn attracts the female mosquitoes of Culex pipiens to lay their eggs on that

water surface [4, 14]. The deposition of the eggs is done by the female mosquitoes in two main forms such singly or in the form of batches. When the Culex females lay their eggs they just not lay it randomly they lay in a clustered manner having several of hundreds eggs locked together one another in a shape of a boat. Whereas, Anopheles lay single eggs and this is done when they are standing onto the surface of water or when they are just hovering near the surface of the water. The eggs layed by the Anopheles remain floating onto the surface of water or and they could easily be damaged by the process of desiccation [16].

The embryos of the first group do not enter dormancy or diapause and hatch when the embryonic development is completed. Those species which are producing the non-dominant eggs mostly have multiple generations each year. The developing stages of these species are mostly done in the permanent waters and here one generation follows the others during the breeding seasons. The numbers of the generations basically depend upon the length of the seasons during which the species breeds, as well as the biotic and the abiotic conditions and most critically temperature influences the time period of the development [4].

The eggs laid in the second group do not instantly hatch just after the oviposition. The most interesting fact is that the mosquitos belonging to *Ae. vexans* and the subgenus *Culicella* of the genus *Culiseta* are the floodwater mosquitoes not only lay eggs on to the surface water but also in the moist soil so when the water level rises those eggs also complete their developing stages inside the water. These mosquitoes lay eggs between the depression and the particles of the soil so that the sensitive eggs do not die by the process of drying in the process embryogenesis as well. Tor the mosquitoes of the *OC. caspius* and *Ae. vexans* which basically lay their eggs and breeds in the area of high flooding nature where the level of water frequently fluctuates, the egg-laying behavior should be appropriate as it is very crucial for the assurance of the successful development of the immature stages of the new born [14].

After the mosquito egg hatch it produces larvae which live in the water or moist habitat that develops into larvae stage that is second instar then it undergoes a metamorphosis and develops pupae which is a protective stage in which mosquito

goes through some morphological changes and then the last stages of the metamorphosis are also completes. The internal pressure is being increased with the help of the air swallowing that position and straight the abdomen in the horizontal position. The adult starts to emerge from the pupal skin when the cuticle of the cephalothoracic found inside the pupa splits along the ecdysial line. The appendages of the young emerging adults partially remain in the exuvia and the adults take precautions by moving so that they do not fall in the surface of water. The young adults remain very susceptible to the strong wind as well as the predators including the water strikes and the spiders [4].

The pupae belonging to the genus *Coquillettidia* remain fixed with the tissues of the plants inside the water body. To the last of their developing stages they have to float onto the water surface [25].

When males and females are emerging their sexual maturity also varies in the time period required. The males are not sexually mature at the time of emergence because they have to whirl their hypopygium by 180° and only then they are ready to mate and it normally takes about 1 day. So, the male population mostly emerge 1-2 days prior to the female population as they want to achieve their sexual maturity on the same time when the females are emerging. [4, 27, 28].

2.2 Medical Importance

Medically mosquitoes are responsible to transport different valuable pathogens and parasites like viruses, bacteria and nematodes that mostly produce lethal diseases like:

- Malaria.
- Dengue.
- Yellow Fever.
- Chikungunya fever.

- Encephalitis. [29–31].

The process of transmission can be in two ways:

i. Mechanical vector (e.g. Myxomatosis in rabits caused by Myxoma virus).

ii. Biological vector.

The latest one is more complicated due to following reasons:

a. It associates in necessary rather obligatory period of replication by the parasite in host.

b. Pathogen's development.

c. Parasitic containment by vector insect.

The pathogens that are vectored by insects are one of the most leading cause of the pandemics and epidemics, it also one of the leading cause of declining and fall of empires for example Roman Empire and Greece Empire. The malarial case study in the Roman Empire is best example of fall of Empire. The malaria was a big issue in latter days and the Roman marshy places were notorious for the "Malaria" (bad air). The blood sucking mosquito make them capable of attaining pathogens from one host and this behavior make them capable of passing it to others vertebrate hosts. The physiology of mosquitoes is applicable for the mechanism of transmitting. The efficient vectors are have a close association with their hosts and they should have enough long life span that it should be sufficient for them to make pathogen/parasite enable for the proliferation or to develop the infective stages in the vector. The successful transfer of parasite is dependent on the multiple blood meals. If we look into the stats of mortality and morbidity of vector-borne diseases the mosquitoes is the most fatal vector to the mankind. As the mosquito only threat 3 billion people of world alone in Sub-tropical or tropical areas and not only effects the human health but also the socio-economical factors and political factors as well [32].

2.2.1 Malaria

Until the mid of 20th century, Malaria scare the human life in the Europe. In Northern Europe and in Southern Europe malaria was found as lethal disease. It is also well-known that in Germany, when Napoleon invaded in the Upper Rhine they lost a large number of soldier due to malarial disease. *P. vivax* and *P. falciparum* were two main species that found in Europe. In Northern Europe, due to the modification in the climate the parasite has been *P. vivax* found. Parasites could sustain as hypnozoites within the liver of human during the cold phases for the process of transmitting. In recent days, *P.vivax* can cause lethal disease [4]. The most significant vector-born disease caused by protozoans (*Plasmodium spp.*) in the human is malaria. In 2006, it influences 3.3 billion people in more than 100 tropical countries. Annually, malaria is responsible for causing 300 million infections and more than 1 million deaths. Children in tropical Africa are more affected with age less than 5 (90%). Malaria becomes a dominant socio-economic stress due to following reasons the prodigious deficit of labours (lives and days), the expense for medication of patients, the bad effect on development. Almost 2 billion US$ cost were predicted for the malarial disease in tropical Africa [33, 34]. *Plasmodium* genus has four species that caused malaria and transferred lonely by the *Anopheline* mosquitoes.

i. *Plasmodium falciparum.*

ii. *Plasmodium vivax.*

iii. *Plasmodium ovale.*

iv. *Plasmodium malariae.*

Almost 40 species from total 400 species of *Anopheles* are significant vectors of human malaria. In Sub-Saharan Africa the most vital and the most capable vector that cause human malaria belonging to *Anopheles gambiae* Complex. In Africa, from the seven species of this complex, *Anopheles gambiae s.s.* and *Anopheles arabiensis* (species A and B) are the most deliberate vectors of *Plasmodium*

falciparum. These vectors showed higher vector capacity because of their anthropophilic behavior and physiological feasibilities.

It is difficult to recognize genotypic and phenotypic plasticity of the vector. So, some modern technologies such as PCR-assay performed to use genetic markers and recognize and to distinguish the species [34–36].

In Africa for a long period many reasons are involved against the battle of malaria which includes shortage of resources, nonappearance of proper infrastructure, deficiency of knowledge, deficiency of training, chloroquine resistance, resistance to other anti-malarial drugs, constrains of environment, pesticides usage[33, 34].

To diminish these shortcomings, some globally campaigns organized against malaria such as Roll Back Malaria (RBM).

2.2.2 Arboviruses

Arboviruses are the arthropod-born-viruses that firstly replicated in the arthropods than transmitted to vertebrates. During the process of viremia, the arthropods become infectious by feeding on the blood from other infected vertebrate. Mainly two types of transmission occurred in arbovirus:

i. Horizontal transmission.

ii. Vertical transmission.

The process of **horizontal transmission**, in which after proliferation in vector, virus transferred to other vertebrate host. In **vertical transmission**, virus can be passed from one generation to another Arthropoda generation. So, some of the viruses are able for wait out of winter season during egg stage of the vector such as some of the *Aedini* species [4]. In 1985, by Karabatsos more than 500 and in 1991, by Francki *et al.* more than 500 arboviruses are listed. In 1988, by Monath almost 40 infect the livestock and 100 viruses infect humans. Most vital viruses that are transferred by mosquitoes to other vertebrates or in humans are found in following three families:

- *Toga virus* having the genus *Alphavirus* (e.g. Chikungunya).
- *Flavi virus* have the genus *Flavivirus* (e.g. Yellow fever virus).
- *Bunyaviridae* have the genus *Bunyavirus Phlebovirus* (Rift Valley virus) (Murphy *et al.* 1995; Eldrige and Edman 2000).

Symptoms may be varied from mild to severe and can cause mortality especially in the tropical regions. There are some clinical symptoms on which aboviral disease are classified as encephalitis, febrile illness, rash and arthritis and hemorrhagic fever [4].

2.2.3 Chikungunya

Chikungunya is viral infection in humans occurred after an incubation period of 24 days with a sudden onset of symptoms including fever, chills, headache, photophobia, arthritis that effect multiple joints.
In 2007, Chikungunya virus dispersed in Asia and Africa. In Europe (Italy) less number of cases recorded first time. There are two main vectors of Chikungunya during epidemics that transmit disease from one human to another African tiger mosquito *Aedes aegypti* [*Stegomyia aegypti*], *Aedes albopictus* [*Stegomyia albopicta*] are one of these.
In 1990 in Italy, *Ae. albopictus* as it occurred in females was detected, showing that it was new epidemic pathogen and were transmitted by the Asian tiger mosquitoes. It is now being examined that this specie can transferred through vertical transmission process during the development of egg [4].
Some non-human primates such as monkey act as the host reservoir and it transfer virus to humans. The enzootic transmission also occurred in mosquito species other than the *Aedes* (*Stegomyia*) *spp.* In 2005 in Reunion (a French island) more than 300,000 people were victim of Chikungunyea. In 2007 in India more than 2 million people suffered from this virus. The first epidemic of this virus was spread in Italy. Sometime hemorrhaging can be occurred. Thus, aged people have low immunity and they can die due to this disease.

2.2.4 Dengue Virus

In 1779 to 1780 (Asia, Africa and North America), the first epidemic of dengue viruses reported. So it is indicated that from more than 200 years this virus is distributed worldwide. Until 1950s dengue was a normal fever but after the first case reported with DHF it becomes fatal in Thailand and Philippines. In recent days, DHF is a leading cause of the hospitalization with the dengue viral disease. In Europe (Athens) within 1927 to 1928, dengue fever was devastating. Almost, 1 million people were infected. In other countries (Spain, Italy, Austria, Europe Mediterranean sea) people suffered from this epidemic viruses [4].
There are two vectors of dengue:

- *Aedes aegypti* (primary vector in urban areas).
- *Aedes albopictus* (secondary vector in suburban/rural areas).

Dengue is a human disease caused due to mosquito. Dengue virus is provoked by four different serotypes:

i. DEN-1.

ii. DEN-2.

iii. DEN-3.

iv. DNE-4.

After the intrinsic incubation period, illness usually starts 5 to 7 days. The symptoms of dengue include fever with rashes, headache, pain behind the eyes, muscular and joint pains and diarrhea.
In some patients rashes spread out on the body while it firstly appear on chest and lower limbs. If a patient recovered from the infection by the one of these serotype than it produce such immunity that provide protection against the other serotypes. It is proof that the infection with different serotypes can increase the

possibility of producing Dengue hemorrhagic fever (DHF). Patient with infection from one serotype can produce different antibodies that neutralize the virus and such antibodies which are not capable for neutralization of other serotype by the other infective bite. Serotype of that virus can increase in number quickly in the epithelial cell of blood vessel and it make permeable and it can cause Dengue hemorrhagic fever. Slowly the blood pressure of patient become down. Dengue Hemorrhagic Fever is deadly disease that produces symptoms including the liver enlargement and failure of circulation system. After few days with high fever the condition of patient become fade, the temperature down, circulatory system failed and patient die within 1 day. Its fully recovery is possible but only under proper medical care [4].

DHF and dengue virus have become the international public problem and mostly it found around the whole world (tropical and subtropical). In more than 100 countries (Asia, Africa, Caribbean, Central, Western Pacific region, South America and Eastern Mediterranean) more than 40% of population live under the danger of Dengue viral infection. In America in 2007 almost 890,000 cases were reported and from this 26,000 cases were DHF. According to WHO recent estimation the annual risk of dengues infection is 50 million worldwide. In every year almost 500,000 people hospitalized due to DHF. 2.5% is fatality rate [4, 33, 34].

2.3 Microbiota of Insects

Insects are most successful group of animal kingdom in terms of survival and diversity both. It is estimated that insects contain 10 times more microbiome than that of their total body cells and 100 times more folds of microbial genome than that of their total genome [37]. Microorganisms found everywhere in insects yet they mostly colonize their gut through food and thus control many significant functions in insects like digestion and metabolism. Most of the microbes in insects are commensals or parasites yet few are also considered to be beneficial to hosts. Few microbes are vertically transmitted and their associations are mutually necessary

for insects e.g. *Buchnera sp.* in aphid flies [38]. It is evidenced that insects microbiota plays many important roles in controlling the biological processes of insects such as digestion, nutrition, sexual reproduction, development, refractoriness to pathogens [39]. Thus mostly studies focused on the understanding of the interactions of insects microbiome and host whether it is in symbiosis or is parasitic [40].

2.3.1 Microbiota of Mosquito

A mosquito's gut microbiota contains the prokaryotes, fungi and microbes. Mosquito gut microbiota is primarily acquired from the environment, its composition is highly dynamic, varying greatly with species, diet, stage of development of mosquitoes and geography [41, 42]. Sequencing of the 16S rRNA or18S rRNA hypervariable regions is used as a culture-independent tool for the study of mosquito microbiota composition [43]. Many of the mosquitoes are marine and terrestrial during their developmental periods as adults. Larvae primarily consume organic detritus, single-cell organisms and small invertebrates, while adults of both sexes usually eat extrafloral nectarines. Adult females also usually feed on vertebrate blood which provides nutrients for egg production but can result in the transmission of pathogens between hosts. Studies from the early 1900 's suggested that larval and adult stage mosquitoes harbor colonies of extracellular microbes in their digestive tract forming a gut microbiota. However, these microbial species and their roles in mosquito biology have only been studied more widely in the last 10 years. Results outlined in several recent studies suggest that adult mosquito gut microbiota may have both a positive and negative effect on vector competency, referring to the capacity of females to obtain, retain and transmit pathogen to vertebrates. Studies shows that the microbes form colonies in mosquitoes which influences there physiological and metabolic functions control. the mosquitoes have a community of microbes which includes bacteria, algae, fungi and viruses. These microbes live in close proximity causing the combined effect on the mosquito's physiology and metabolic functions.

2.3.2 Composition of Gut Microbiota of Mosquito

Most of the microbiota in the gut of the mosquitoes is demonstrated as being predominantly gram negative of facultative nature which actually belongs to four different phyla (*Proteobacteria*, *Firmicutes*, *Bacteroidetes* and *Actinobacteria*). In these phyla the bacteria are also one of the most prominent member of the community of insects growing, also from numerous novel orders and also gets the microbiota in the gut from one of their microenvironment. With the help of this, it could easily be stated that the insects are proliferating in a regular and smooth method. Moreover, multiple members of bacterial community are known as of the community of gut and were also extracted and cultured with success in the mosquitoes. Eukaryotes of unicellular nature are also known as the members of the community of the gut in multiple species related to the mosquitoes which have algae, fungi and also the *apicomplexes* but till now no such organism have been extracted and cultured in the mosquitoes. Mosquitoes viruses have also been categorized in survey based on series. Multiple genera belonging to this type is of small RNA genomic in nature and it include *Flaviviridae* and it includes those mosquitoes which have the activity of a pathogen as far as the vertebrates are considered. By comparison, the absence of *Bacteriophages* in published research indicates that either viruses infecting bacteria in the intestine are underrepresented, or that few *Bacteriophages* infect bacteria in the intestine of the mosquito [42]. It includes those mosquitoes which have the activity of a pathogen as far as the vertebrates are considered.

2.3.3 Gut Microbiota Acquisition by Larvae Mosquito

Some species obtain intestine microbiota directly from their parents or other individuals while others obtain their intestinal microbiota primarily from the climate. Three lines of evidence indicate that growing generation of mosquitoes reacquires

the gut microbiota mainly from the climate. Second, laboratory experiments indicate that the mosquito larvae hatch and in their intestines, without any extracellular bacteria. Second, gut-community composition studies suggest that the majority of microbes found in larvae correlate with those found in their aquatic environment. Third, mosquitoes host highly variable gut communities that would not be expected if the parents or congeners acquired those communities directly. Studies indicate that adult mosquito reproductive tracts contain multiple species of bacteria and that some of these bacteria are on the surface of laid female eggs, are exceptions to environmental acquisition. This can lead larvae to develop such microbes directly by ingesting egg shell fragments at hatching or inoculation of the aquatic environment in which larvae live. Many species of mosquitoes harbor intracellular bacteria that spread vertically in the genus *Wolbachia* and choose other genera found in eggs. Most viruses have also been shown to have a vertical transmission. Additionally, these species are not part of the extracellular microbial population which is the gut microbiota [42].

Cultural studies initially indicated that mosquito larvae remove their gut microbiota at metamorphosis in a meconium and that adults with little to no gut microbes emerge from the pupal stage. Such results have indicated that adults develop a gut microbiota by immersing water from the larval environment and/or feeding on resources such as extrafloral nectaries. However, controlled studies coupled with a study of the composition of the gut population provide clear evidence that *Aedes* and *Anopheles* larvae transfer a portion of their gut microbiota trans staidly to adults. However, subsequently the adult gut microbiota may change by consuming microbe-containing water, nectar, or other food sources. Vertebrate blood generally contains few to no bacteria, but some experiments indicate that the intake of a blood meal changes the composition of the gut microbiota persistently to transiently through alterations in redox status or metabolism. Infection by various vector-borne pathogens can also affect gut microbiota composition through unknown mechanisms [42].

One research identified 98 genera of bacteria in the *Anophelines*, the most common being *Pseudomonas*, *Aeromonas*, *Asaia*, *Comamonas*, *Elizabethkingia*, *Enterobacter*, *Klebsiella*, *Pantoea* and *Serratia*. Likewise, gram-negative bacteria also predominate in *Aedes spp.*

2.4 Microbial Variation in Gut

As a holobiont, mosquito undergoes a metamorphic transformation from larval stage to adult stage. Microbial mosquito residents and their larvae refer to the microbial communities that colonize within the target organism. In the adult mosquito the larvae-associated microflora is replaced by a new set of microbes. This microbiota variation is due to significant changes in the host mosquito depending on the changes in the environment and feeding habits. This microbial cleaning and acquisition process is termed gut sterilization. Mosquito mainly consume bacteria and planktons as nutritious resources during their larval stage. This paves the initial stage of invasion of bacteria that contributes to the inhabitants. Among the microbes, the bacteria colonize more in the midgut than in the reproductive organs and salivary glands [44–46]. Later during adult stages, mosquitoes begin to feed on nectar and blood which triggers the proliferation of some types of microbes and the decline of the other bacteria. Thus, the host diet and its developmental stage plays a crucial role in shaping the gut microbiome [47]. Mosquitoes then begin feeding on nectar and blood during adult stages, it then regulates some types of the proliferation of different microbes and decline of some other bacteria. Therefore the host diet and its level of growth play a key very vital role in the structuring of gut microbiome [48].

In the gut of mosquitoes, resident communities can vary from microscopic dominant bacteria to even Protista members. This resident consortium can be changed by the influx of new microbes from their natural habitat. Mosquitoes such as *Anopheles*, *Aedes* and *Culex* normally lay eggs in water that contains bacteria [47]. Aquatic plants presence affects the microbial populations when they act when a larval aid or provide signals for adult mosquito laid larvae many of these

plants' microbes are often transferred trans-steadily to adult gut [49–51]. These microbes have a significant impact on the characteristics of mosquito life such as fecundity, reproduction vector competency and immunity.

As per previous earlier studies, the general bacterial flora in mosquitoes includes gram-negative phylum *Proteobacteria* (*Gammaproteobacteria*, *Alphaproteobacteria*, *Betaproteobacteria*) phylum *Bacteroidetes*, gram-positive phylum *Firmicutes* including *Clostridia*, *Actinomycetes*, *Spirochetes* and other species. Naturally, a bacterial community in mosquito gut can reduce the development of *Plasmodium*, a human parasite (due to the presence of gram-negative bacteria). The outer membrane of the cell wall in these gram-negative bacteria contains lipopolysaccharides which acts as a physical barrier for harmful agents like hydrogen peroxide etc [52], while gram-positive bacteria have no such barrier. Furthermore, different gram-negative bacteria have varying effects against *Plasmodium*. These variations could reveal certain differences in the production of metabolites. *Plasmodium*, for example, is found to be effective against prodigiosin of red pigment produced by gram negative bacteria. One reason for this is the upregulation of antimicrobial peptide encoding immune genes (AMP) and a protein containing thioester which has an antiparasitic effect. Such bacteria that live in the gut can be pathogenic or symbiotic. The symbiotic microbes are beneficial for hosting in a number of ways. This requires nutritional supplementation, strengthening of the digestive system and tolerance to environmental perturbation and prevention against parasites.

The *Anopheline* gut microbiome is strongly influenced by microbes suspended in its natural habitat. This has been proved by the thorough gut analysis of mosquito larvae by Howland [53] who dissected over 1000 larvae of eight species, identified the algae present and ranked them by abundance in the food. She concluded that the abundance of algae in the larval food is correlated with algal abundance in the habitats. This has been also shown in another study on *Anopheles quadrimaculatus* larvae, a common vector of malaria in the Eastern United States [54] wherein the elimination of algae from a small pond with copper sulfate demonstrated its absence in their food. However, after recolonization the same pond, algal cells were again observed in the larval gut.

The *Anopheline* gut is dominated by resident bacteria of genus *Pantoea* and *Asaia*. These bacteria have shown stable association with *Anopheline* mosquitoes during different life stages. *Pantoea*, natural mosquito symbiont can cross-colonise several mosquito species and is readily transformed and cultured; this property of *Pantoea* has been proposed for paratransgenic applications [55, 56]. *Asaia* acts as an immunomodulator by producing antimicrobial peptides that interfere with the course of infection particularly its invasion to epithelial tissues and salivary gland [48].

Recent research on two *Anopheles* species *An. gambiae* and *An. coluzzii* from Ghana [57] compared the midgut microbiota of mosquitoes during rainy and dry seasons from urban and rural breeding sites using 454 pyrosequencing. The data suggested that *An. gambiae* and *An. coluzzi* do not differ significantly in their gut microenvironment. *Shewanellaceae* family was observed in both the species. Bacterial families *Enterobacteriaceae* and *Aeromonadaceae*, were also associated with *Anopheles* mosquitoes. The only difference observed was among *An. gambiae* collected from the different breeding site during summer. *Aeromonas*, *Shewanella* and *Thorsellia* were other bacterial genera found to be significantly varying in abundance according to the breeding sites. This indicates that larval breeding site has a significant impact on the adult mosquito midgut composition. The presence of *Enterobacter* and *Serratia* strain in *Anopheles* mosquito gut have an antiparasitic effect on mosquito. *Enterobacteriaceae* that survived during the rainy season is found to be more in number than that of during the dry season. Two members of this family include *Enterobacter* species and *Thorsellia Anopheles*. This gram-negative *Enterobacter* can directly act on *Plasmodium falciparum* and hinders the development of the parasite. *Thorsellia anophelis* was the dominant species in the midgut of *An. gambiae*. This symbiotic association with host mosquito vector attributes to its high tolerance for mosquito midgut alkalinity. *Serratia marcescens HB3*, isolated from laboratory-reared *An. stephensi* mosquitoes, inhibits *Plasmodium* development within the mosquito midgut by interrupting ookinete invasion through the midgut epithelial cells. Phenotypic variation at the cellular

and structural levels was observed and directly correlated with the ability to induce resistance against *Plasmodium* invasion [58]. The prevailing environmental conditions have a great influence on the gut microbiome and host- vector competence. One such parameter is the influence of chemicals in regulating the bacterial fauna in mosquito gut. For example, *Pseudomonas aeruginosa* boost the larval development of *Culex quinquefasciatus* in phosphate-rich medium [59].

2.5 Mosquito Mycobiome

A part of the mosquito gut microbiota is eukaryotic fungi including bacteria and influenza. Its position as commensal, mutualist or pathogenic in preserving the ecological balance of mosquitoes is inevitable. During the metamorphic transition, mosquitoes are exposed to fungi in the form of mosquito larvae in water, or by ingestion of fungi in sugar meals, or physical contact with conidia (adult mosquitoes) [60]. Filamentous fungi and yeast are the common fungal isolates present in the midgut and other tissues of mosquitoes. A filamentous fungus comprises some species of *Aspergillus* and *Penicillium* as pathogenic forms and some genera of fungi like *Beauveria* and *Metarhizium* as entomopathogenic forms [61]. Different genera of yeast like *Candida*, *Pichia* and *Wickerhamomyces* have been identified in *Aedes* and *Anopheles* mosquitoes through culture dependent and culture independent methods. Earlier explorations in mosquito myco diversity were based on these types of the culture-dependent method [47]. For example, a yeast strain *Wickerhamomyces anomalus* has been reported in the midgut and reproductive organ of *An. stephensi*, a primary vector of malaria [62]. Recently, with the advent of high throughout sequencing (HTS) technique, the knowledge about mosquito mycobiome has widened [63]. This HTS approach has been used to analyze the mycobials formation in *Ae. triseriatus*, from the Japanese E. The series documented the presence of 21 distinct taxonomic fungal operating units (OTUs), of which 15 were identified by both parties. *Ascomycota* phylum is the major fungal taxa among these two *Aedes* species. Although the existence of mycobiome in mosquito is evident, the tripartite connection between vector, pathogen and

fungus is less known. Hence, there are enough evidences of the fungi present in mosquitoes. These eukaryotic organisms are responsible for the masking of many signals in the organisms.

2.6 Mosquito Virome

Mosquito act as an exclusive host for a large group of virus which are insect-specific [64, 65]. A metagenomic approach was used to evaluate viral load by Shi *et. al.* [66] in two genera of mosquitoes *Aedes* and *Culex*. The comparison presented a striking difference in the virome of mosquitoes, where in genus *Aedes* showed a low viral diversity and less abundance than *Culex*. This metagenomic approach lead to the identification/discovery of different viral families in mosquitoes such as *Bunyaviridae*, *Rhabdoviridae*, *Orthomyxoviridae*, *Flaviviridae*, *Mesoviridae*, *Reoviridae*, unclassified *Chuvirus* and *Negevirus* groups. Most resident virome act as commensal microbe due to its inability to infect vertebrate cell lines, prolonged host infection and vertical transmission.

2.7 Microbes Influence on Host Vector Property

Vectorial capacity is a quantitative measure of several factors like cellular, biochemical, behavioural, immunological, genetic and environmental parameters which can influence vector density, longevity and vector competence [67]. All these factors are interrelated and can determine the pathogenicity and nonpathogenecity in mosquitoes.

Acetobacteria, a dominant member of gut microflora may interact directly or indirectly with invading pathogens. The indirect interaction is by activating innate immune response [68]. Usually pattern recognition receptors (PRRS) on the host cell recognize preserved surface determinants known as pathogen-associated molecular patterns (PAMPs) that are present/finded in microbes exclusively. Such linking activates immune signalling mechanisms such as the road toll or the route

to immune deficiency (IMD). A cascade of events leads to the degradation of IF- ranging from transcription factor (Cactus), nuclear translocation of NF- ranging from transcription factors (Dif and Dorsal) to antimicrobial peptide (AMP) genes being expressed, in the toll cell signaling pathway. This AMP, produced in the fat body, is secreted into haemolymph where it directly kills the invading microorganism. Genetic research showed that the AMP gene expresses are mainly regulated through the toll pathway and the IMD pathway. The toll pathway is mainly activated by gram-positive bacteria, human *P. falciparum* and DENV. The development of gram-negative bacteria stimulates the IMD pathway, which regulates the antibacterial peptide gene [69].

2.8 Applications of Microbiome of Insects

Microbiome study in the last few decades has led to an understanding of the potential microbial functions. The few examples of which are as follows [40]:

1. Hydrolysis of xylane.
2. Productions of Vitamins in *Glossina brevipalpis*.
3. Phenolic Metabolism and Nitrogen Fixation in Pine Beetle.
4. Resistance against Antibiotics in Gypsy Moth species.
5. Signal Mimics in Gypsy Moth species.

2.8.1 Impact of Microbiota on Mosquitoes

Mosquito microbiota plays critical roles in many mosquito biology processes including feeding, digestion, matting and sexual reproduction, development, immune response and refractory pathogeny [70].

2.8.2 Impact of Microbiota on Mosquito Physiology

Dong et al. (2009) compared transcriptom between septic and aseptic adult female mosquitoes fed various diets and observed that microbiota stimulates some genes involved in digestion and metabolic processes such as glycolysis, gluconeogenesis and sugar transport. Midgut microbiota, most especially *Enterobacter sp.* in *Aedes aegypti* and *Serratia sp.* Isolates have hemolytic activity that can contribute to lysis of the red blood cells (RBC) and hemoglobin release. Antibiotic treatment of female mosquitoes reduced RBC lysis and egg production within *Ae. aegypti* [71].

Yet not every bacterium supports the growth of the eggs. Various bacterial genera have been used to construct adult mosquitoes that have evolved from gnotobiotic larvae. Tests were carried out on five bacteria (*Aquitalea*, *Sphingobacterium*, *Chryseobacterium*, *Paenibacillus* and *Comamonas*) that assisted egg development in *A. aegypti*, *A. atropalpus* only assisted by *Comamonas* in the development of eggs [70].

2.9 Metabolic Detoxification Of Insecticides

Three major metabolic gene families are being involved in the mechanism of the detoxification of insecticides in mosquitoes: esterases, cytochrome P450s (P450s) and the S-transferases (GSTs) glutathione. Cytochrome P450s are among those genes families which have the most significant role in both biochemical as well as the physiological functions of the living organisms. Cytochrome P450s are the most critical and significant to detoxify and also to activate the endogenous compounds as well as the xenobiotics [72]. The largest quantity of the exogenous as well as the endogenous compounds in the metabolic detoxification and the excretion are GSTs which are dimeric protein having the property of the solubilization [73–75]. An important property of the GSTs and the P450s is the upregulation at the transcriptional level which in turn results in the formation of excessive production of proteins, hence, excessive enzymatic activity is being done. Moreover, it

also increases the detoxification of the insecticides with the help of oxidation and also the toxins of plants inside the insects and this further leads to the tolerance of the insecticides [72, 76] and the toxins of plants as well [77–80]. It was also stated that the production of the resistance against the insecticides [81–83] required that genes encoding P450s be amplified/duplicated. Tremendous number of organisms have the community of the esterase enzyme which is the community of heterogeneous community of enzymes. The overproduction of these enzymes have been studied extensively as the amplification and non-frequent over-expression of the genes of esterase enzymes have been proven to have increased detoxifying protein production [84–86].

With the help of the comparison of the toxicity level done with or even without the synergists, researchers are able to make the assumptions by drawing the conclusions related to the involved detoxification mechanisms in resistance development. Synergism research on resistance to pyrethroids in various species of mosquitoes strongly support the importance of mitochondrial detoxification in insecticide resistance. [87–93]. Nonetheless, the findings of synergistic studies must be interpreted with caution: while in many cases the use of synergists can correctly indicate the role of detoxification proteins in insecticide resistance, in some cases synergists may be imperfect inhibitors for some of the detoxification enzymes induced by the resistance [72]. Further work is required to support the synergistic studys findings. Metabolic Enzyme Activity Assays an alternative and separate diagnostic tool for detecting the possible involvement of a metabolic enzyme in resistance is to assess elevated levels of enzyme activity and/or an increase in insecticidal metabolism. For permithrin-resistant Cx, the metabolism of permithrin to 4-hydroxypermethrin by microsomal P450 monooxygenases was stated to be significantly greater. *Quinquefasciatus* mosquitoes than vulnerable counterparts thereof [81]. Elevated levels of cytochrome P450 monooxygenase, esterase or GST activities in insecticide-resistant mosquitoes of several species have also been reported; these include *An. albimanus* [18], *An. gambiae* [94], *An. stephensi* [89], *An. funestus* [95], *Ae. aegypti* [96, 97], *An. culicifacies*, *Anopheles annularis* [98]. Although these types of measurements suggest the critical function of metabolic

enzymes in the production of resistance, no specific evidence is available to determine the performance of the metabolic enzymes that can play the major role among several other enzymes generated by the metabolic gene superfamily in a single organism. Initiation of Individual Metabolic Gene/Protein Characterization Cloning and sequencing of partial or full-length individual metabolic genes as well as purification of individual metabolic proteins and screening of resistant mosquito-resistant cDNA libraries have helped to understand the molecular basis of metabolic-detoxification-mediated resistance. This area has been extensively studied in the 20 years since the publication of the first report on 17 partial CYP4 gene sequences from *An. albimanus* [99]. Shortly after the latter was followed by the first full-length CYP6E1 sequence from *Cx. quinquefasciatus* [81], which was calculated using techniques for reaction of the polymerase chain. The availability of individual partial or complete sequences has allowed researchers to identify gene expression and amplification and protein expression, revealing significant details of the metabolic enzyme characteristics associated with increased metabolic detoxification of insecticides in resistant mosquitoes via transcriptional up regulation/DNA amplification. Several P450 genes, including P450 genes from deltamethrin resistant *Anopheles minimus* [100], pyrethroid resistant have been individually reported to be overexpressed in resistant mosquito species/strains. *Funestus* [101], Cx-resistant to permithrin Quinquefasciatus [102] and *Cx.* resistant to deltamethrin [103] *Pippiens pallens*. Along with same methodologies, the over regulation of the GST genes specially GSTE-2 was also being identified in the mosquito resistant to the DDT which is *An. gambiae* [104] and also the over-production of the esterase genes with the help of the duplication and the amplification and it is being identified in the mosquito *Cx. Quinquefasciatus* [86, 105]. Certainly, there are tremendous amount of the studies done which suggests that the duplication and amplification of the detoxifying genes is very critical in the insecticide resistance phenomenon of the bacteria, still the single gene analysis do not show the complete complexity of this process which initiates the behavior of the specific genes against the insecticides. It is not yet clear that how so many of the genes responsible for the detoxification are directly or indirectly involved in

the insecticide resistance in so many of the mosquitoes species and what are the methods with which these genes are upregulated, there is no pathways which is completely showing that how this resistance is being done.

2.10 Methods to Study Microbiota of Insects

Insects gut descends from mouth to anus and is one of the largest organ in insect body. The major microbiome community is present in the insects gut. Thus, it is very important to carefully isolate the insects gut microbiota. For this purpose, no specific technique has been standardized up till now. Firstly, we have to carefully disinfect the insects body by a disinfecting buffer and make dissection to obtain the complete gut. The insects gut can be separated into three parts i.e. fore gut, mid gut and hind gut. After the collection of each part it is treated with extraction buffer and metagenomics DNA extraction is made. Cell lysis is a critical step in metagenomics DNA extraction thus it is carried out with the help of gentle means like lysis enzymes. The gut cells are lysed and the remaining gut microbial cellular community is washed. For this purpose, mechanical lysis can also be made like homogenization, bead beating and shocks to attain complete lysis [106].

2.10.1 Cultivation of Obtained Microbiome on the Culture

The obtained gut sample are then suspended in saline, phosphate buffers and then serially diluted to get cultured on the suitable growth medium. The culturing plates are then kept in incubator at 28°C for 48 hours. After this the morphological characteristics are carried for the characterization of bacterial colonies with at least three dilutions. Subsequently, enzyme activities are studied gene coding for enzymes are cloned and DNA is sequenced for genomic libraries [107]. The cultivated bacteria are then obtained and then used for the DNA extraction. Subsequently, enzyme activities are studied gene coding for enzymes are cloned and DNA is sequenced for genomic libraries [107]. The morphological characteristics are carried for the characterization of bacterial colonies once again to check.

2.10.2 Accessing Total Genome of Microbiota

Till no universally accepted literature has been published for the extraction of metagenomics DNA from insects. The major goal is to access unbiased microbial genome of whole communities along with the contamination and degradation of the genome should be taken under consideration. In the DNA isolation the sheering or DNA damage should be taken with care so that the DNA with high molecular weight can be obtained which can then be used to create DNA libraries through BAC vectors. The DNA should be free from downstream of the applications like cloning and PCR so, for this purpose no macromolecules should be attached to DNA [108].

2.10.3 Specified Gene Enrichment

In DNA, genes are the functional units, they control the phenotypes of a particular organism. For the quest of specific function, gene enrichment technique is used which in return increases the efficiency of cloning prospective and also leads to the discovery of uncharacterized genes from a microbial community. The typical methods for the enrichment are is to control the environment of the community by exposing them to pressure, temperature, pH, light or electric shock. This in return controls the phenotype of the genes. The enrichment techniques include suppressive subtractive hybridization phage display and affinity capture [109, 110].

2.11 Whole-Genome Sequence Analysis

With the advent of the field of genomics in the last decade the studies about the insecticide resistance has been revolutionized. With the help of the WGS analysis of the mosquitoes, mainly *Ae. aegypti* [111], *An. gambiae* [112], *Cx. quinquefasciatus* [113] and the *An. darlingi* [114], is one of the major milestone which have been achieved and have also boosted the development of the high-throughput analysis through the genomic studies and also have enhanced the knowledge of the basic

and most critical biological processes which are responsible for this resistance of the insecticides in the mosquitoes, also these high throughput techniques guarantees the most novel and innovative approaches for the control of the mosquitoes as the vector and hence reducing the mosquito borne-disease on the global scale [115]. The collective data on the EST known as expressed sequence tags and also some of the most very known and easily accessible techniques such as NGS (Next Generation Sequencing), oligonucleaotide microarray, applied quantitative trait loci analysis and suppression subtractive hybridization have the most significant impact on the studies related to the expression, these expression analysis have a very significant role in the making the blur picture of the role of the genes in the phenomenon of insecticide resistance on the genomic level very clear. With the help of these high-throughput techniques allows the researchers study the mechanism of the insecticide resistance on the whole genome level as well as on the individual scale as well, also very highly complex biological pathways have been developed with the help of the whole genome investigation of the mosquitoes. With the help of these new and novel techniques it is also been made possible for the researchers that they can study the characterization of the genes, their interaction with the insecticides and also their mechanism for the insecticide resistance. With the help of the analysis of the genome we have found enough knowledge on the complexities of the presence of the genes inside the genome of mosquitoes which in turn detoxifies the insecticides in the mosquito populations, examples are 31 GSTs, 51 esterase and also 111 P450s sequences of genes in the mosquito belonging to *An. gambiae* [116], 26 GSTs, 49 esterase and also 160 P450 sequences of genes in the mosquito belonging to *Ae. aegypti* [117], 35 GSTs, 71 esterase and 204 P450 genes sequences in the *Cx. quinquefasciatus* [118] and lastly 30 GSTs, 20 esterase and 89 of P450s sequences of genes in *An. darlingi* [114].

For itself, the cover interaction/expression relationship among the detoxification at the metabolic, multiple of the genes in the mosquitoes have been shown in multiple genera in which the DDT resistance is being done as well as pyrethroids, these genes include genes such as GSTs genes P450 genes which were overexpressing and was being interacting with the DDT and pyrethroids directly in the species such

as *Anopheles gambiae* [119–122], in *Ae. agypti* and *Anopheles funestus* and resistance is seen in pyrethroids [96, 117, 123], As such, the cover expression/interaction relationship of metabolic detoxification genes in individual mosquitoes has been explored in species resistant to DDT and pyrethroids, including multiple P450 and GST genes that are overexpressed or that interact in DDT/pyrethroid-resistant *An. gambiae* [119–122], pyrethroid-resistant *An. funestus* [82, 124], pyrethroid-resistant, *Ae. aegypti* [96, 117, 123]; multiple P450 genes that are overexpressed in pyrethroid-resistant *An. funestus* [82, 83], *An. gambiae* [125] and *Cx. quinquefasciatus* [118]; and multiple GST genes that are overexpressed in DDT and pyrethroid-resistant *Ae. aegypti* [126] and pyrethroid-resistant *An. gambiae* [127]. Genome-wide high-throughput technologies revealed, for the first time, duplication of P450s as a new mechanism contributing to mosquito resistance [82, 83, 123, 128]. Collectively, with the help of these explorations it is widely accepted that there are various genes which are regulating and interacting in the mechanism of the resistance in the mosquitoes. With the help of high throughput technologies, the researchers can also decode the expressing genes from the whole genome and researchers can also isolate the species of mosquitoes which were showing the resistance against the insecticides, also if any of a new mechanism of the resistance by the detoxification is shown that would also be made possible. With the help of novel technique of SSH/cDNA Liu *et al.* [129] discovered 22 new genes which were overexpressing in the *Cx. quinquefasciatus* for the pyrethroid resistance, also the genes for P450 was 2 in number, for EST genes 20 new genes and in all of these the genes responsible for the transduction of the signal was also being identified as well, all of these new genes were never ever related with the resistance of insecticides in the mosquitoes. Likewise, another high-throughput technique known as EST/cDNA microarray analysis have been unveiling the overexpression of the genes responsible for the DDT resistance, some of these genes belongs to those species in which they were not already known and they were directly involved in the mechanism of the resistance, these genes includes the genes which were encoding for the calcium/sodium and the peptidases also in the lipid implications and the metabolism of carbohydrates these genes are involved [130]. [130]. In their

study involving a functional characterization of upregulated metabolic genes in mosquitoes, the genes involved in the detoxification with the help of metabolism and some other genes which are identified newly has been proven to have a very significant role in the resistance against insecticides and the relationship among the phenotype of resistance and the overexpression of the genes, thought to have the most significant role, is yet not clear. Numerous strategies have been used for the validation of the overexpression of the genes and the resistance phenotype, to check the exact phenomenon of the resistance in the mosquitoes. These strategies include the in vitro protein metabolism assay, in vivo silencing of genes with the help of the RNAi techniques and also the modeling, these techniques are opted as they can fill up the gap between the conventional proteomics and genomics and the novel area of the field named as functional genomics. The in-vitro functional studies and the in-silico presentation functional validation is being done for the confirmation of the theory that overexpressed genes are involved in the metabolization of the insecticides in the mosquitoes or not, this is very important to determine as it will narrow down the number and names of genes which are actually involved in the insecticide resistance. Mitchell *et al.* [131] have performed a functional study on the DDTs metabolism with the help of the *An. gambiae* P450 reductase and recombinant CYP6M2. Same studies have also been done for the assessment of the abilities of the recombinant CYP6M2 [132] from the mosquitoes *An. gambiae* is used for the metabolism of pyrethroids and also the *An. funestus* have the recombinant CYP6P9a and also the CYP6P9b [82].

In an insect-*baculovirus* expression system, CYP6Z1 of *An. gambiae* and CYP6P7 and CYP6AA3 in *An. minimus* are also capable of metabolizing DDT [133] and pyrethroids [134], respectively. In silico 3-D homology modeling and molecular docking of metabolic enzymesubstrate interactions are new and effective tools for understanding the relationship between protein structures and substrates, which can provide reasonable explanations for substrate specificities and differences in metabolism [134]. Six regions of P450 proteins, designated substrate-recognition sites (SRS1 6; 46), contribute to the function of P450s, with SRS1, SRS4, SRS5

and SRS6 involved in the formation of catalytic sites and SRS2 and SRS3 participating in substrate access channel configuration [134]. With this new computer modeling system to complement highly complex functional metabolism studies, researchers can now confidently state that several mosquito P450s, including CYP6Z1,CYP6AA3, CYP6P7 and CYP6M2 are important in insecticide resistance. This approach explains both how the molecular structures (proteins and chemicals) interact and how changes in the insects metabolism are caused by allelic variation [132–134].

2.11.1 Metagenomics Expression Libraries

On the basis of functional genes metagenomics libraries are made by the help cloning vectors and the gene expressions are observed by functional assays. These gene expressions are then stored in metagenomics databases to help the researcher to access the previously unknown/uncharacterized genes. Furthermore, the characteristics of functional gene such as enzyme activities are expressed with a proficient vector. Heterologous expression of a gene in the host cells is impeded by various steps such as transcription, translation and post translational process or maturation [40]. Few metagenomics expression data of genes which are isolated from the functional expression library technique listed below in table 2.1.

TABLE 2.1: Table showing the examples of insects source with their enzymes and genes isolated by the metagenomics functional expression analysis

Insect Source	**Enzyme/Gene**	**Potential Application**	**Reference**
Reticulitermes flavipes	RfBGluc-1 beta-Glucosidase	Digestion of Lignocellulose	[135]
Rotschildia lebaeu (*Lepidoptera*)	Xylanase	Degradation of Xylane	[136]
Termites (*Nasutitermitidae*)	Endo-1, 4--xylanase	Degradation of Xylane	[137]
Nasutitermes ephratae	Glycosyl hydrolase	Digestion of Lignocellulose	[138]

2.11.2 Metagenomic Analysis of Microbiomes

16S rRNA sequencing became the standard and normal method of determining the structure of a human microbiome population. The V1V3 and V3V5 regions of the hypervariable 16S rRNA gene help to distinguish the taxonomic structure of different bacterial species. To check the composition of microbiota people divided this gene into taxonomic units of action (OTUs). Sanger sequencing was the primary instrument for sampling the entire amplicon range (16S rDNA). However, people discovered that species diversity can be classified utilizing shorter DNA stretches with higher sequence coverage and thus the developments of next generation sequencing (NGS), i.e. Roche 454 pyrosequencing, Illumina and Ion Torrent sequencing are also used for the meta-genomic sequencing. Numerous analytical methods for studying the 16S rRNA sequences of microbes were also developed later to better understand their biology in the microbials. Nonetheless, even though we have strong coverage and longer sequencing reads using 16S rRNA sequencing, it would still be challenging to access the genomic details of low-abundance species. Therefore, recent work has moved to the use of high-throughput data techniques to develop both the qualitative and quantitative microbiome DNA information, mRNA transcripts, metabolites and microbial community proteins. Meta-omic methods will help give a more detailed functional view of microorganisms and their functions within the microbiome [24].

Shotgun metagenomic sequencing was the first step in this direction in which the whole genomic DNA of human/environmental bacteria samples were analyzed with a view to identifying all species and recognizing the microbe's gene function potential [37]. Another example is the HMP Unified Metabolic Analysis Network (HUMAnN) which perform metabolic and functional metagenomic data reconstructions [139]. This technique was performed on 102 individuals at seven key locations in the human body namely diarrhea, dorsal tongue and anterior nares. For various sites, they established the main metabolic pathways, genes and functional modules that were distinct across individuals. Glycosaminoglycan degradation, phosphate and amino acid transport within this microbiota have been shown to be more involved in the vaginal microbiome these methods have also been applied

for insects microbiome [140].

Computational modeling strategies such as metabolic genome scale models (GEMs) have been developed to integrate and interpret data for research purpose based on the increased experimental data produced by the high-throughput strategies. Throughout recent years meta-omics results is used on a genome scale throughout tandem with metabolic models (GEMs). The genome size of metabolic models and metagenomic data were taken as feedback by using MAMBO (Metabolomic Analysis of Metagenomes using fBa and Optimisation). The use of in vitro, ex vivo and in situ laboratory evidence with in silico models serves as an outstanding testing tool for the discovery in human microbiomes of the elusive microbial microbe microbe and microbe-host relationships that suggest major therapeutic progresses. Each of the respective omic data types provides useful knowledge in characterizing the organism's working and certain data types are incorporated more directly into the modeling formalism than others. For example, *Vanee et al.* used a proteomics-derived model to describe the *Thermobifida fusca* microbe's metabolism functionalities where the growth rates seen in experimental and silico results were almost similar.

2.11.3 Homology Based Analysis of Metagenome Sequenced DNA

Compared to functional/expression analysis homology based metagenomics are more precise as they target the gene on the basis of the data present and existing conserved genomics databases. Sequence based screening methods depend on the existing conserved sequences and hence, may not help to identify brand new non-homologous enzymes [141].

The sequence-based search combined with powerful bioinformatics tools has led to a higher rate of identification of novel genes than function-based methods do. Bioinformatics tools for sequence mining have been developed, based not only on homology of the primary sequence but also on the predicted protein structures.

Gene function can be predicted with the improvement of the protein sorting and modeling tools, the putative active sites [142]. Some tools of Gene-finding such as MetaGene has been used in order to predict 90% of shotgun sequences [143].
Many recent publications identify metagenome sequence databases that look for genes and enzymes that would be useful for commercial development in prospecting. For example, 71 million base pairs of sequence data were created by sequencing a metagenome library of hindgut microbiota from the largest family of wood-feeding termmites. By detecting complete domains using global alignment, over 700 homologous domains of the glycoside hydrolase catalytic site corresponding to 45 different carbohydrateactive enzyme families were identified, including a rich diversity of putative cellulases and hemicellulases [138].

2.12 Insecticide resistance

Numerous studies have showing that the individual mosquito species are involved in multiple mechanisms of resistance [85, 93, 93, 102, 116, 129, 130, 144–149]. In particular, two mechanismsincreased metabolic detoxification of insecticides and reduced target protein sensitivity which is having the most critical part on which the insecticide acts and which is also known as the insensitivity of the target site have been studied very extensively and which have the most wide acceptance due to its extreme importance [145, 146, 149]. The relationship in between the genes related to the resistance on the regulation level of genes have provided with a very excellent example showing that how precisely these resistances develops in the insects. In the coding region, the over expression and the amplification of the gene having the mutations results in the structural differences insides the proteins are most often being linked with the resistance of the insecticides in the populations of mosquitoes, yet generally, the over expression at the transcriptional level of the genes present in the insects showing resistance to the insecticides, have been proven to be the most common and critical feature for the resistance development in the insects [85, 129, 130, 150]. Collectively it is very easy for the researchers to conclude that these resistances are not only being transmitted from one generation

to the other but also it is being regulated with the help of various regulation levels of the genes, especially the genes responsible for the resistance in the mosquitoes. Yet, it is not yet clear that which genes are directly or indirectly involved in the resistance and also that how many are involved in the phenomenon.

Chapter 3

Methodology

3.1 Block Diagram

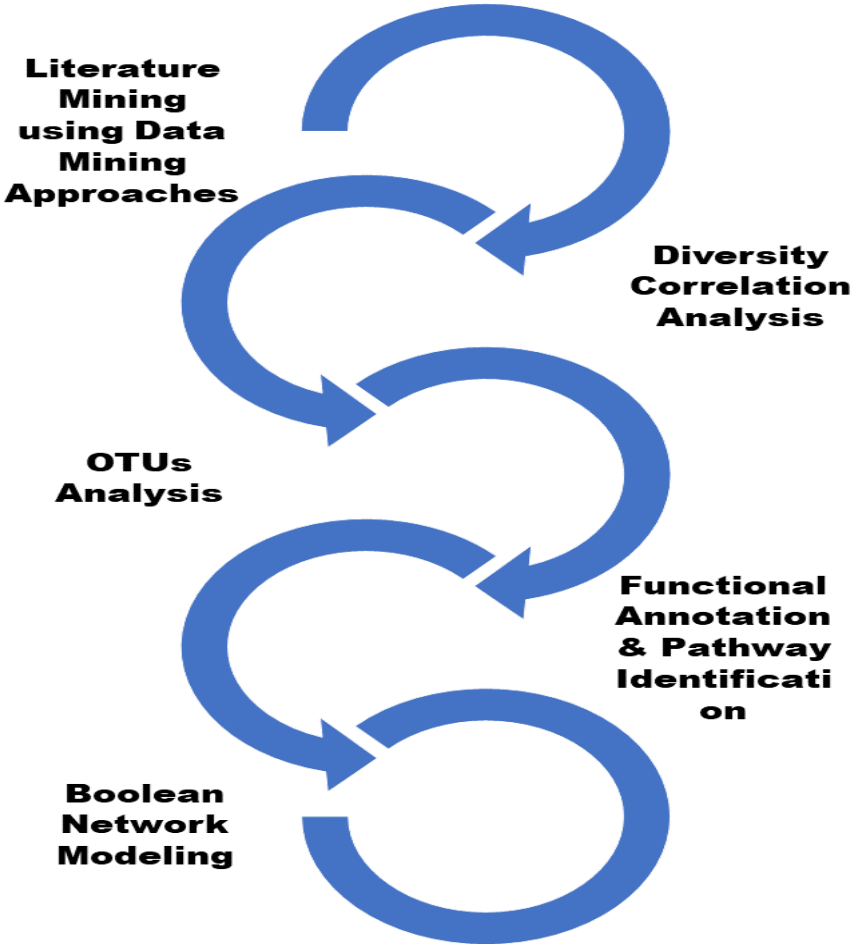

FIGURE 3.1: Flow Chart of Methodology Conducted for the Research.

3.2 Literature Survey for Acquiring Microbiota of *Anopheles*

In Pubmed the mosquito microbiota studies bloom in the last decade. Researchers started to investigate the most important disease vector and look inside it microbial community to search for the fact that specific interactions of microbes tend to control the phenotypic expressions of mosquitoes [42]. The first step in our research was the acqusition of microbiota information in the mosquito. For this purpose many data mining apporaches were used which greatly involved the Mesh keywords searches.

3.2.1 MESH Keywords

The MESH terms used were microbiota, microbiome, microbial, in the context of mosquito, *Anopheles* on various search engines. The relationship used for these keywords were AND, In, Gut microbiota. The Agilent Literature Search searched for the metadata and aliases as well. Then it provided the links of the pubmed data bases for the key words.

3.2.2 Agilent Literature Search

Agilent Literature Search 3.1.1 was used for the initial Pubmed search. It is a Cytoscape plugin which take keywords and context and search for those keyword in different data warehouses. Agilent mine the keywords in the abstract and title of the paper present in Pubmed and then displays the results. The output for Agilent Literature Search is links to the specific publications in the data warehouse [151]. The cytoscape can be downloaded from the following link *https://cytoscape.org/download.html* and the Agilent Literature Search can be used as the plugin in cytoscape.
In figure 3.2 the interface of Agilent Literature search showing the keywords on

the left side and the context in the right side. The output is displayed in the output window at bottom, showing the links to a specific paper in the Pubmed. The parameters used in agilent were as follows:

- Max Engine Matches = **20**.
- Use Aliases = **ON**.
- Use Context = **ON**.
- Concept Lexicon Restricts Search = **OFF**.

The keywords and context is already discussed in section 3.2.1. In the result of Agilent Literature Search the initial related papers present in Pubmed till date extracted. Agilent results contain 64 papers which included these mesh keywords and context in the relationship already discussed in the section 3.2.1. After that the manual curation was done on the inclusive criteria.

3.2.3 Manual Search

The manual search was also conducted to extract the latest research done on this topic. For this purpose many search engines were used which are "Pubmed, Science Direct, Elsvier, Google Scholar". On the basis of manual curation the papers were extracted which include our set criteria.

3.2.4 Inclusive Criteria

After the retrieval of these results the articles were manually curated according to the required data set. The articles were manually mined and the data obtained by these articles were maintained in a file. The information obtained from the articles was as followed:

- Species of *Anopheles*

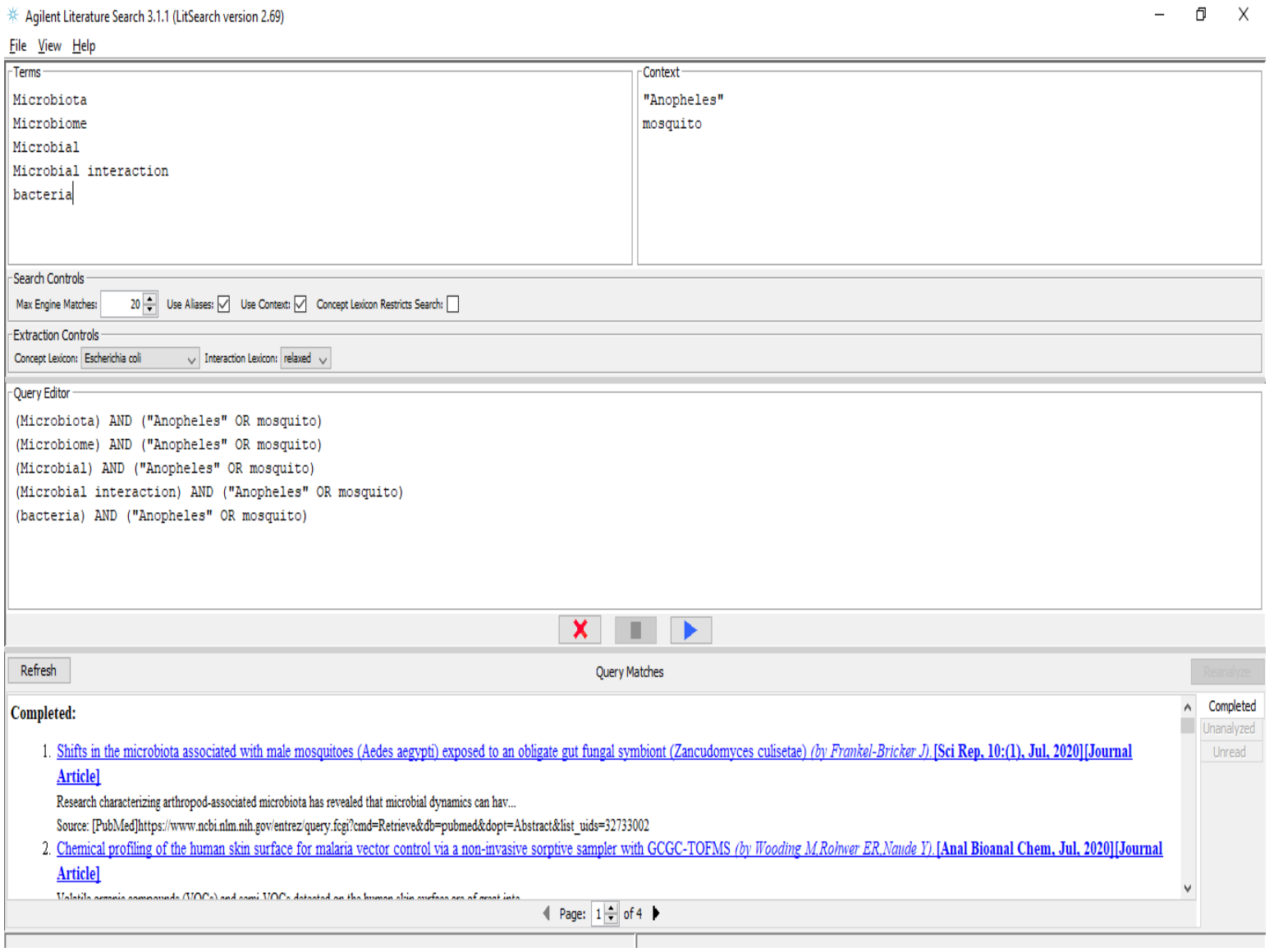

FIGURE 3.2: Figure Showing Agilent Literature Search Tool Results Against the Specific MESH Terms.

- Microbial phylum.
- Microbial class.
- Microbial family.
- Microbial genus.
- Method of their isolation.
- Examples of microbes.
- Conditions from they were collected.
- Developmental Stages.

3.3 Sequences Extraction

3.3.1 16s rRNA Sequences Extraction

After the literature mining the first step for other analysis is extraction of the sequence data on the microbial genera collected through the literature mining results. For this purpose NCBI Genomes utility was used (https://www.ncbi.nlm.nih.gov/genome/). NCBI Genome is a biological repository which includes all the reference genome sequecnes and as well as non-annotated genome sequences. It also contain the genomic information about that genome [152]. From NCBI Genome search 74 genera 16s rRNA sequence data was collected in FASTA format. This data was further used for the OTU analysis.

3.3.2 Whole Genome Sequences Extraction

In the second step we extracted whole genome sequences for the genera extracted from literature mining using NCBI Genome utility to process that WGS for the functional annotation for the pathway identification. The criteria for the selection

of whole genome sequences was the total coverage more than 50% if the sequence is less than 70% it was excluded from the search.

3.4 OTU Analysis

The universal gene present in all the bacteria is 16s rRNA. 16s rRNA is highly conserved yet it share few variable region in them that help to distinguish between species. Thus to cluster these sequences into bin a term used is called "Operational Taxonomic Unit (OTU)". The most common benefit of OTU clustering is that it is computational. Clustering allows rapid analysis of amplicon. [153].

3.4.1 Multiple Sequence Alignment (MSA)

The most important downstream process in OTU clustering is performing MSA. In MSA all the sequences are aligned in single multiple alignment. The dissimilarity between a pair of sequences is defined as the percentage of non-gapped sites that disagree in the induced pairwise alignment [153]. The MSA generates a distance matrix on the basis of which the OTU correlational analysis can be done.
The Molecular Evolutionary Genetics Analysis (MEGAX) software was used for the MSA analysis. All the retrieved 16s rRNA sequences were uploaded into the MEGAX as an alignment file. Then the MUSCLES algorithm was used for the MSA analysis because it is more preferable than Clustal [154]. Default settings were used for the construction of MSA. It gives two options about the sequences while aligning them which are: Align DNA or Align Codon, if the sequences is protein coding choose Align Codon because it is more realistic to align the DNA according to codon than direct DNA alignment because it will prevent in adding gaps into position that may cause frame shift in the real sequences [154]. In the result of MSA the sequences are aligned and a distance matrix is generated which can be used further for the phylogenetic analysis and correlation studies.

3.4.2 Diversity of Microbiota among Species of *Anopheles*

In 2009 Hamady and Knight presented the idea of core in nature; the core can be substantial (one in which large population of microbial taxa are shared) or may be minimal, gradient or not existent. This idea provided the starting point for the analysis of large microbial population and their association. With the passage of time the core idea flourished and studied widely. For this, a typical approach is followed to report number of species present in a habitat. This approach can be obtained by making venn diagramin by which different microbial species can be visualized in Anopheles species [155].

Venn diagram is a power full visualization technique that can help research to view full containment, partial intersection or total disjunctness of a data set to the other at a glance. Simple Venn diagrams are already used in the biological studies. It can be used to study the genes coverage or to study RNA coverage [156]. In our study to visualize the microbial *Anopheles* we used Venn diagram. It was created using Bioinformatics & Evolutionary Genomics Venn Diagram Tool. The limitation for this tool was that it can draw venn diagram only for 8 data sets yet in our studies 11 *Anopheles* species were identified which means 11 data sets. Instead of a visualization it provided us the results in tabular format which was then used manually to make a figure using manually drawing methods. This tool can be access using http://bioinformatics.psb.ugent.be/webtools/Venn/ link. It takes in the list or data set along the name of data set. The data set contain microbial genera in each *Anopheles* species.

3.4.3 Phylogenetic Analysis

The goal for phylogenetic analysis is to construct a phylogenetic tree. There are multiple technique by which we can generate a phylogenetic tree like Neighbor Joining, UPGMA, Maximum Parsimony and Maximum Likelihood (ML) but in our studies we used Maximum Likelihood method because ML uses many substitution methods to study the changes occur at same site in an evolutionary history

of sequence [154]. The parameters for ML used in our work were:

- Test of Phylogeny: **Bootstap**.
- Substitution Type: **Nucleotide**.
- Model/Method: **Tamura-Nei model**.
- Rates among Sites: **Gamma Distributed With Invariant Sites (G+I)**.
- No. of Discrete Gama Categories: **2**.
- Gaps/Missing Data Treatment: **Complete Deletion**.
- Branch Swap filter: **Very Strong**.
- Number of Threads: 3.

3.4.3.1 Bootstrap Method

The bootstrap is a computer based technique to assess the accuracy of any statistical estimation. It is more useful in complicated non-parametric methods where analytical methods are not so useful. Felsenstein introduced this method in phylogeny for the phylogenetic tree estimation. This method is used for the "confidence" for each clade in an observed phylogenetic tree, based on bootstrap tree clade at the same time [157].

3.4.3.2 Tamura-Nei Model

In Tamura-Nei (1993) substitution model rates of transversional and transitional substitution are considered separately. This is done by taking in account the unequal frequencies of four nucleotides [158].

3.4.4 Diversity and Correlation Analysis

The distance matrix obtained from the MSA used to perform the correlational analysis. The correlational analysis was done using Orange3 a python based statistical tool. The input for this tool is distance matrix in csv format. Orang3 is dynamic tool in which all type of statistical analysis can be performed using visualization aid [159].

3.4.4.1 Distance Matrix Visualization

The distance matrix obtained by MSA was firstly visualized to see the relationship between two genomes. This was done by the Distance Matrix utility in the Orange3 tool box. The visualization helps the researcher to initially interpret the similarity between two sequences. The more clear visualization was obtained by using Heatmap utility of the Orang3 which helped researchers to view the expressional features between 2 sequences.

3.4.4.2 Pearson's Correlation

The Pearson's Correlation also referred as the Pearson's r is a statistical test that measure the linear relationship between two variables x and y. Thus it is also known as bivariate correlation. The value of Pearson's correlation ranges between -1 to $+1$. The positive linearity correlation is displayed in terms of $+1$ and the no correlation is depicted in terms of 0. Then the negative correlation is displayed in -1 [160] [161]. The Pearsons correlation among the species were calculated using the Orang3 Tool. It takes in the similarity data in the matrix form and creates the correlational results and then displays it in the scattered plot. The distance matrix generated previously by MSA was used for this purpose. The Correlation Co-efficient for each genera was calculated and then used to construct a scattered plot. The analysis help us to infers that the correlated genera are more related to each other.

3.4.4.3 PCA Analysis

Principal Component Analysis (PCA) is the statistical analysis in which we compute the principal component and utilize them in performing the change which is based on the data. Few times few first principal component are used and rest are left.

Principal components is collection of dataset point in a real space where each points of the data set are displayed in the vectors. It is set of p direction vectors and the i^{th} vector is in the direction of best fits to the orthogonal to first $i-1$ vectors [161].

PCA among the species were calculated using the Orang3 Tool. It takes in the similarity data in the matrix form and creates the PCA results on default settings and then displays it in the scattered plot.

3.4.5 K-Mean Clustering

K-means is a method of clustering by which the vector quantization could be done, this is originally from the processing of the signaling which in turn aims for the observation and partition of this quantization into the K-clusters and inside of it every observation is belonging to its nearest clusters and its mean value which again in turn serve as a cluster of the prototypes. With the help of this results it could be judged as that the space of data is into the Voronoi cells. It is one of the most popular method for the data mining in the analysis of the clusters [162].

3.5 Identifying Target Pathway

In systems biology the individual entity or molecule is not considered. The systems are analysis on the basis of systems. The biological systems are so complex that each molecule interact with one and other and produces a combined response. Thus the identification of correct pathway for the interaction studies is necessary in biological process. In our study we used RAST annotation pipeline which

can be access by https://rast.nmpdr.org/ for the identification of functional pathways. The whole genome sequences were uploaded on RAST with the taxonomic Id. RAST provides a detail view on the systems and subsystems present in that genome. In our study we selected 64 genomes to be uploaded on RAST. The RAST provided their information and displayed all the information present in those systems [163]. Those systems were selected which were involved in the metabolism of Aromatic compounds. Then they were further cut down to subsystem level and only those microbes were selected which contains Biphenyl degradation system because biphenyl is the core pathway in xenobiotics pathway of degradation.

3.6 Boolean Network Modeling

Boolean Network provide researchers an easy approach to study the attractors which control the pathway. This can be done by making pathway on the boolean rules. The future value of a node is controlled by the past node. If a node gets inhibited the future nodes also inhibits. If a node is dependent on more than one node than its future value will be dependent on all of those nodes. The rules for boolean network can be &&, ||, !. The modeling of the pathway is based on these rules. The value 1 represents ON and 0 represents OFF. The equation 3.1 shows simple boolean relationship which depict that the A is dependent in AND relationship with the B and C. Means if B and C are True A will be true or if any of B and C will be false A will be false [164].

$$A = B\&\&C \tag{3.1}$$

The subsystem identified by the RAST is then searched using KEGG pathways database. This pathway then draws on the Boolesim for the Boolean network simulations [165] this can be accessed by the following link https://rumo.biologie.hu-berlin.de/boolesim/. The effects of switching on and off for the certain environmental conditions were studied. The dynamic Boolean network analysis can be completed using Boolean network rules. The rules are based on the Boolean logics

which are representing the pathway in Boolean operators like AND, OR, NOT. This type of analysis help us to find the attractors for the specific pathways. Boolean network based on two states either a node is off or on. The logical relations represents the network in either 1 or 0 states which helps us to find the relations and core molecule that controls that pathway.
The rules for this analysis used were as follows.

- bphAa == biphenyl.
- bphB == C06589 && bphAa.
- bphC == C02526 && bphB.
- bphD == C01273 && bphC.
- Benzoate == bphD.
- Benzoate Degradation == Benzoate.

Chapter 4

Results and Discussions

In this chapter the results obtained from the implementation of the methodology as mentioned in Chapter 3 are discussed in details with the comparison of the existing work present for this metagenomics analysis and modeling of the pathway which are linked to this metagenomics.

4.1 Microbiota Retrieval from Literature

4.1.1 Data Extraction from Literature

The table 1 illustrate all the microbial community present in the *Anopheles* mosquitoes. The major constitution of microbiota in *Anopheles* belongs to 4 phyla of Bacteria, which are as follow *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and rest are others. The table 1 illustrate that 96 genera are found in the *Anopheles* mosquitoes. Figure 4.1 showing the distribution of genera according to the phyla found in *Anopheles*. Out of 96 genera 61 belongs to phylum *Proteobacteria*, 9 belongs to *Actinobacteria*, *Bacteroidetes*, *Firmicutes* each and 10 belongs to other which includes the following:

- *Bacillariophyta*.

- *Chlorophyta.*
- *Calothrix.*
- *Deinococcus.*
- *Mycoplasma.*
- *Spiroplasma.*
- *Cyanobacteria (Gpl).*
- *Cyanobacteria (Gplla).*
- *Cynobacteria (GpV).*
- *Fusobacterium.*

Table 1 also depicts the distribution of microbiota among the developmental stages of mosquitoes, along with the isolation methods by which they are extracted. Figure 4.3 depicting the acquisition of microbiota in the life stages of mosquito. The most microbiota is acquired by mosquito in adult stage and followed by the larval stage. As the pupal stage is semi-dormant stage thus the acquisition rate in pupal stage is found to be very low. 71 bacterial genus found in Adult stage while 43 in larval stages and 7 in pupal stage which shows the relationship that the bacteria acquired in a particular stage can also be passed on to the next developmental stage. Hence this support the fact that the acquisition at larval stage in natural field environments can be passed on to the later laboratory investigations.

The ecosystem of the mosquito gut accommodates the closely linked and complex microbiome. It is evidenced that the gut microbiota influences large variety of host functions like immunity, growth, fitness and nutrition. To understand the microbiome dynamics and structure in the whole life of mosquito it is to comprehend the symbiosis of mosquito and its gut microbiota. The variations in the gut microbiota was observed during the developmental stages like adult stage, larval stage and pupal stage in *Anopheles gambiae*, this study was done in the Kenya by 16s rRNA pyrosequencing. The adults and immature showed very distinctive

microbial community structure. In the larval and pupal stage the *Cyanobacteria* a photosynthetic bacteria while in the adults the *Proteobacteria* and *Bacteroidetes* dominated with core taxa of *Enterobacteriaceae* and *Flavobacteriaceae* [166].

The table 1 also depicts the sources by which the researchers gathered these microbiotas, three techniques were found by which these microbes were isolated which includes deep sequencing by 16s rRNA, culturing technique and non-culturing technique which includes techniques like antibodies assays, antimicrobial assays.

The gut microbial community has a link between its genetic functionality and controlling of host traits. The fact that at adult stage the food of adult (i.e majorly nectar and blood) controls the significant affect in microbial structure. Interestingly the blood meal reduces the gut microbiota drastically in the adults and favor *Enteric* bacteria. Blood meal is necessary for the mosquito development and the transmission of pathogens from a host to other in mosquito borne diseases. The interesting fact is that the high impact of blood meal displays beneficial symbiosis in the gut microbiota ecosystem as it induces the antioxidant capacity. Looking into ecological point of view the microbial proper functionality is the fact of synchronized composition of the microbial consortium. The avenue of intentionally disturbing the microbial community structure for the symbiotic benefits is unexplored and should be developed for the better mosquito control strategies [167].

Comparative genomic analysis revealed that the enriched *enteric* bacteria possess large genetic redox capacity of coping with oxidative and nitrosative stresses that are associated with the catabolism of blood meal, suggesting a beneficial role in maintaining gut redox homeostasis. The blood fed gut has been shown to be a reducing environment [168].

The presence of an expanded bacterial redox reservoir during blood digestion could be one of the essential factors maintaining gut redox homeostasis. Mainly, in the gut of the mosquitoes which is fed by the blood, the proliferation of the bacteria is mainly enhanced with the help of the dityrosine network which is genuinely formed by the dual nature of the peroxidase/oxidase involved in these of the mechanism. The cross-linking of the mucosal layer present onto the epithelial luminal side basically reduce the permeability for the immune elicitors of the bacteria, by this

the hosts anti-microbial response could easily be evaded and the microbes could easily grow inside the endoperitrophic space [52].

Steadily, with the help of most recent evidences it can be concluded that the mosquitoes related to the *Aedes* genus having the blood as in their energy relation releases the heme and this will have a decreasing effect on the ROS production inside the cells of the mid-gut, this is done parallel with the guts expansion and the load of bacteria [169]. Larger microbiota diversity found in an *An. gambiae s.l.* of African nature and *Anopheles funestus*have one of the greatest infection rate in the *P. falciparum* than those which were not infected [169]. The frequency

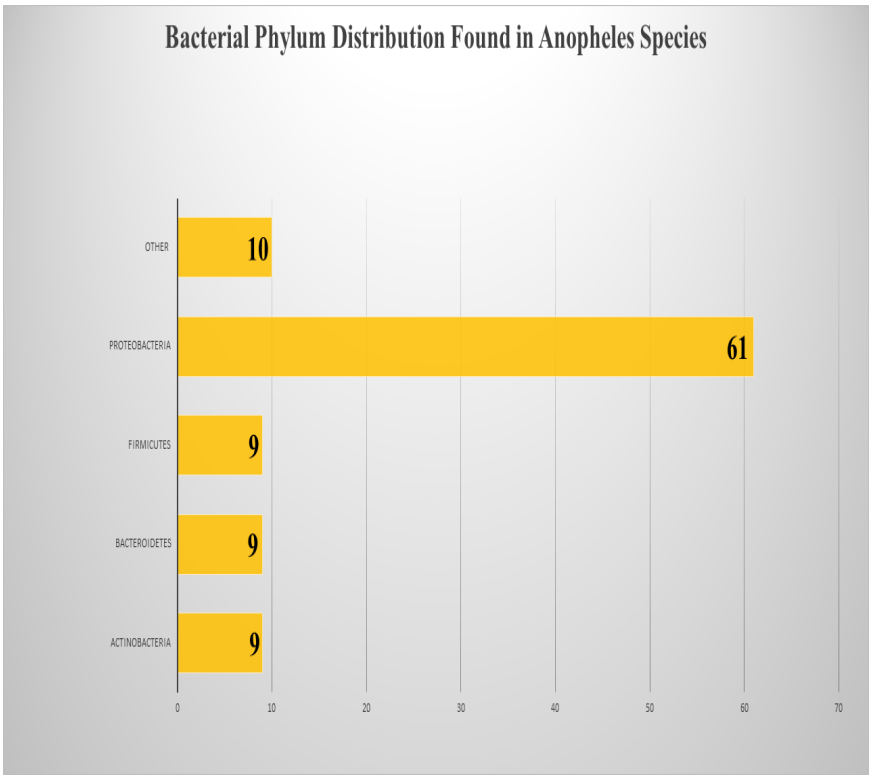

FIGURE 4.1: Bacterial Phylum Distribution Found In The Case of *Anopheles* Mosquitoes.

of *Proteobacterial* species in mosquito is explanatory that major controlling of the functions through the microbial community is controlled by the *Proteobacterial* species figure 4.1 refers to this distribution result. The 41 genera out of 96 were reported in more than one *Anopheles* species and 9 were reported in 7 species. This concludes that the bacterial genera are frequently found in the species. The *Pseudomonas* which belongs to *Proteobacteria*, class *Gammaproteobacteria* and

family *Pseudomonadaceae* is the most frequently found genus among the Anopheles species as it is found in the 7 species of *Anopheles* those are as follows:

- *albimanus.*
- *darlingi.*
- *funestus.*
- *gambie.*
- *maculipennis.*
- *quadrimaculatus.*
- *stephensi.*

The study of conditions in which the microbes are isolated suggests that the most bacteria found in laboratory setups are also found in the natural or semi-natural setups, which can be concluded that the acquired microbes in natural setups persists in to the laboratory setups as well. The 11 *Anopheles* species are studied till date for the microbial studies. The distribution of microbial genera across the *Anopheles* species can be seen in figure 4.3 as the most genera is found in the *Anopheles gambiae* and the least genera are present in the *Anopheles dureni.* This is due to the fact that *Anopheles gambiae* is most frequently found *Anopheles* specie in the world [170].

The bacterial biodiversity in nine species of field-collected *Anopheles* in Thailand and Vietnam demonstrated complex microbiota in the mosquito midgut and abdomen, primarily Gram-negative bacterial rods, including *Serratia marcescens*, *Klebsiella ozaenae*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Enterobacter spp.* [171]. Other studies have reported the majority of adult mosquito midgut microbiota were Gram-negative species in the phylum *Proteobacteria* [172, 173].

It has been described that a group of phyla formed by *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* constitutes more than 99% of the total microbiota community in adult mosquitoes [174].In Andrea *et.al. 2020* study, this group represented more than 96% of the total bacteria.

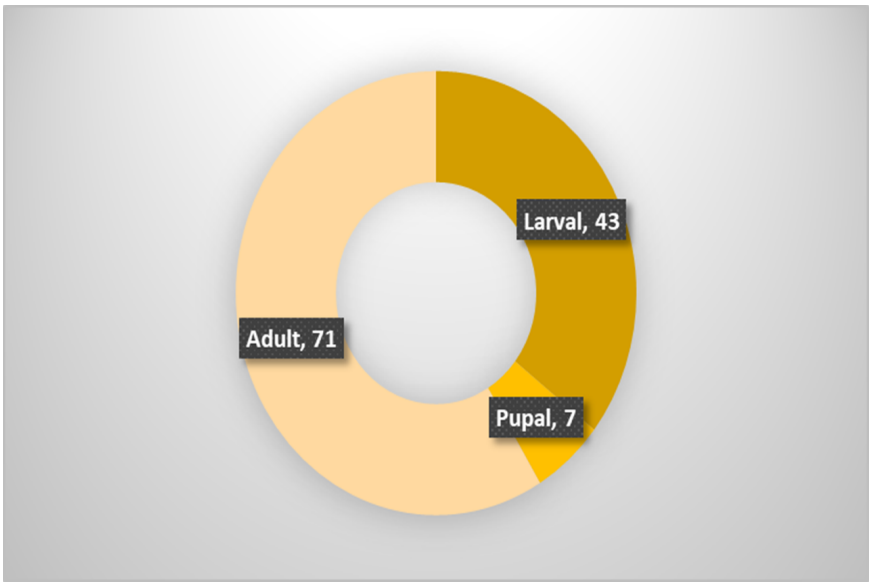

FIGURE 4.2: Distribution of the Acquisition of Microbiota In The Life Stages of *Anopheles*

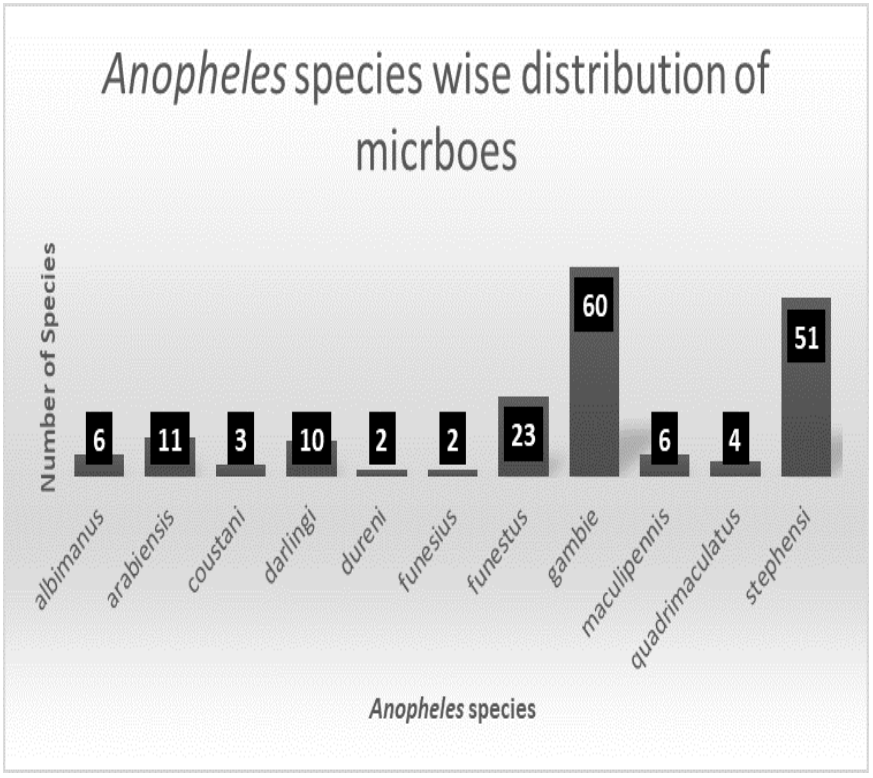

FIGURE 4.3: The microbial Phylum Distribution Across the *Anopheles* Species Depicting That The Most Species Are Found in *Anopheles gambiae*.

These common bacteria constitute the "core microbiota in adult mosquitoes because they have been consistently found [11, 174–177], especially in the midgut of *Ae. aegypti* [178–181]. *Proteobacteria* was the most abundant phylum, which

was consistent with other studies [177, 180, 181]. This has also been shown in other species; there was a similar composition in the bacterial communities among adults of Anopheles coluzzii and An. gambiae [182], and between larvae of Ae. aegypti and *An. gambiae* [12]. Some authors have proposed that the similarity in bacterial communities may be due to the conditions under which mosquitoes are reared (laboratory colonization or field-collected), suggesting that environmental or host factors could shape the microbial community structure of mosquitoes [12, 183–185]. However, similarity at the phylum, class, or family levels has been observed independent of environmental conditions or host factors [177, 186, 187]; therefore, the reason for the similarity in bacterial communities among *Ae. aegypti* populations is not yet clear [174]. The bacterial diversity of our populations does not seem to differ significantly based on geographical origin, temperature, climatic factors, or elevation. Studies with Ae. aegypti adults and larvae demonstrated that bacterial diversity was not affected by geographic area and larval habitat characteristics such as water temperature and pH, in agreement. It is possible that other factors, including microbial interactions, mosquito genotype, amino acid metabolic pathways, could shape mosquito microbiome communities [177, 186, 188, 189].

Although similarity at the population level is commonly found in the mosquito microbiota, several studies in *Ae. aegypti*, *Ae. albopictus*, *An. gambiae*, *An. coluzzii*, *Culex quinquefasciatus*, and *Mansonia uniformis* have described high inter-individual variation [177, 182, 183, 187, 189], where certain bacteria members are prevalent in one individual but are rare or absent from others; this is found especially at lower classification levels [174, 183, 187] and it was observed at the family level. It has been suggested that this condition may be important for metabolite production [190], or vector competence for the transmission of pathogens [183]. The role of inter-individual variability in mosquitoes is not well understood, but it could have a potential effect in resistance of field populations where insecticide pressure could shape bacterial communities, as seen in the *RiptortusBurkholderia* symbiotic system, where the abundance of fenitrothion-degrading bacteria increased with the spraying of the pesticide [21**?**].

4.2 Operational Taxonomic Unit

4.2.1 Diversity of Microbes among *Anopheles*

The table 4.1 illustrates that the 7 *Anopheles* species that are *Anopheles albimanus*, *Anopheles darlingi*, *Anopheles funestus*, *Anopheles gambiae*, *Anopheles maculipennis*, *Anopheles quadrimaculatus*, *Anopheles stephensi* share a common specie i.e. *Pseudomonas*. The *Anopheles gambiae* specie has the most unique genera set i.e 29 genera are present in the *Anopheles gambiae* which are not present in any other specie, while at the second number 19 unique species are present in *Anopheles stephensi*.

TABLE 4.1: Correlation of Anopheles species with microbial phylums. (http://bioinformatics.psb.ugent.be/webtools/Venn/)

***Anopheles* Species**	**Total Number of Microbial Phylum**	**Microbial Phylum**
albimanus	1	*Pseudomonas*
darling		
funestus		
gambie		
maculipennis,		
quadrimaculatus		
stephensi		
arabiensis	1	*Aeromonas*
coustani		
darlingi		
gambie		
maculipennis		
stephensi		
albimanus	1	*Enterobacter*

Continued on next page

Table 4.1 – Continued From Previous Page

***Anopheles* Species**	**Total Number of Microbial Phylum**	**Microbial Phylum**
darlingi		
funestus		
gambie		
stephensi		
coustani	1	*Asaia*
funestus		
gambie		
maculipennis		
stephensi		
funestus	1	*Staphylococcuss*
gambie		
maculipennis		
quadrimaculatus		
stephensi		
albimanus	1	*Schlegelellea*
dureni		
gambie		
maculipennis		
quadrimaculatus		
darlingi		*Klebsiella*
funestus		
gambie		
stephensi		

Continued on next page

Table 4.1 – Continued From Previous Page

***Anopheles* Species**	**Total Number of Microbial Phylum**	**Microbial Phylum**
albimanus *darlingi* *gambie* *stephensi*	1	*Novosphingobium*
arabiensis *funestus* *gambie* *stephensi*	1	*Bacillus*
coustani *funestus* *gambie* *stephensi*	1	*Chryseobacterium*
albimanus *funestus* *gambie* *stephensi*	2	*Flavobacterium* *Acinetobacter*
abaiensis *funestus* *gambie* *stephensi*	1	*Escherichia* *Shigella*

Continued on next page

Table 4.1 – Continued From Previous Page

***Anopheles* Species**	**Total Number of Microbial Phylum**	**Microbial Phylum**
dureni	1	*Comamonas*
funestus		
quadrimaculatus		
stephensi		
darlingi	1	*Erwinia*
funestus		
gambie		
funestus	4	*Streptococcus*
gambie		*Enterococcus*
stephensi		*Sphingobium*
		Cedecea
arabiensis	1	*Sphingomonas*
funestus		
gambie		
gambie	8	*Micrococcus*
stephensi		*Hydrogenophaga*
		Burkholderia
		Herbaspirillum
		Elizabethkingia
		Microbacterium
		Stenotrophomonas
		Lactobacillus
funesius	2	*Paenibacillus*
gambie		*Rhodococcus*

Continued on next page

Table 4.1 – Continued From Previous Page

***Anopheles* Species**	**Total Number of Microbial Phylum**	**Microbial Phylum**
funestus	2	*Methylobacterium*
gambie		*Yersinia*
darlingi	1	*Citrobacter*
stephensi		
arabiensis	2	*Paenibacillus*
stephensi		*Rhodococcus*
funestus	3	*Brevundimonas*
stephensi		*Fluconobacter*
		Alcaligenes
maculipennis	1	*Lysinibacillus*
stephensi		
gambie	29	*Cyanobacteria (Gplla)*
		Prophyrobacter
		Methylophilus
		Chlorophyta
		Salmonella
		Phytobacter
		Raoultella
		Pelagibacter
		Gluconacetobacter
		Neisseria
		Aquabacterium
		Ralstonia
		Sphingobacterium

Continued on next page

Table 4.1 – Continued From Previous Page

***Anopheles* Species**	**Total Number of Microbial Phylum**	**Microbial Phylum**
		Delftia
		Calothrix
		Prevotella
		Rhizobium
		Shewanella
		Bacillariophyta
		Clostridium
		Phenylobacterium
		Cynobacteria (Gpv)
		Morganella
		Fusobacterium
		Bradyrhizobium
		Agromyces
		Sediminibacterium
		Pantoea
		Cyanobacteria (Gpl)
darlingi	3	*Ehrlichia*
		Buttiauxella
		Xenorhabdus
stephensi	19	*Leptothrix*
		Acetobacter
		Ignatzshineria
		Azoarcus
		Leminorella
		Bordetella
		Brevibaterium

Continued on next page

Table 4.1 – Continued From Previous Page

***Anopheles* Species**	**Total Number of Microbial Phylum**	**Microbial Phylum**
		Deinococcus
		Serratia
		Myroides
		Kocuria
		Dysgonomonas
		Agrobacterium
		Vibrio
		Achromobacter
		Exiguobacterium
		Flexibacteraceae
		Ewingella
		Rahnella
arabiensis	5	Janibacter
		Acidovorax
		Anaplasma
		Thorsellia
		Mycoplasma
Funestus	1	Spiroplasma

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Figure 4.4 depicts the distribution of microbes in the *Anopheles* species. Each color represents separate genera. The labeling alphabetical labeling represents the *Anopheles* species. In this fig it can be seen that yellow color appears for 7 times which represents *Pseudomonas* that was found in 7 *Anopheles* species. The

figure 4.4 also represents that the *Anopheles gambiae* (labeled as A) has the largest number of species among all while the *Anopheles stephensi* following it with the 2^{nd} highest number. Figure 4.4 also depicts that the *Anopheles gambiae* has the most unique number of species i.e. 29 and the *Anopheles stephensi* i.e. 19. The least number of species are in label J which belongs to *Anopheles Dureni* which is 2 and that are also found in other species. The correlational analysis helped us to find out the shared species among the *Anopheles* species which works as the 1^{st} step in OTU formulation. The study of distribution of microbial community among the species concluded that the few species plays a vital role in the *Anopheles* functions and governs major changes in their functionalities.

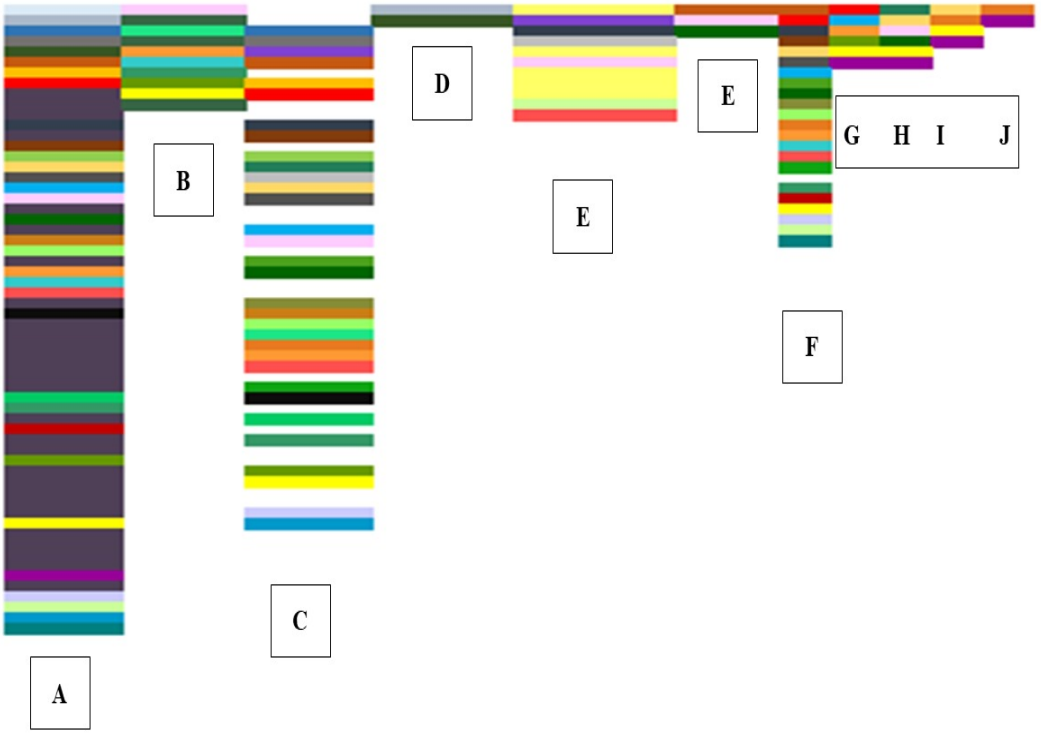

FIGURE 4.4: Alpha Diversity of Microbes in *Anopheles* Species.

4.2.2 Phylogenetic Analysis

The phylogenetic tree was obtained shows the relationship between different phyla of microbes. The phylogenetic tree displays relative divergence of species from

ancestors. It is seen in the phylogeny that the microbes divert according to their genome. In figure 4.5 the ancestral relationship shows that the microbes greatly divert from their phylum species like *Janibacter* is closely related to *Bacillus* but the phyla of both the species are different. Phylogenetic analysis also represents the functional and ecological properties of species. The groups also shows that the closely related species also shares the common genome and this similarity also helps to determine the function of the species.

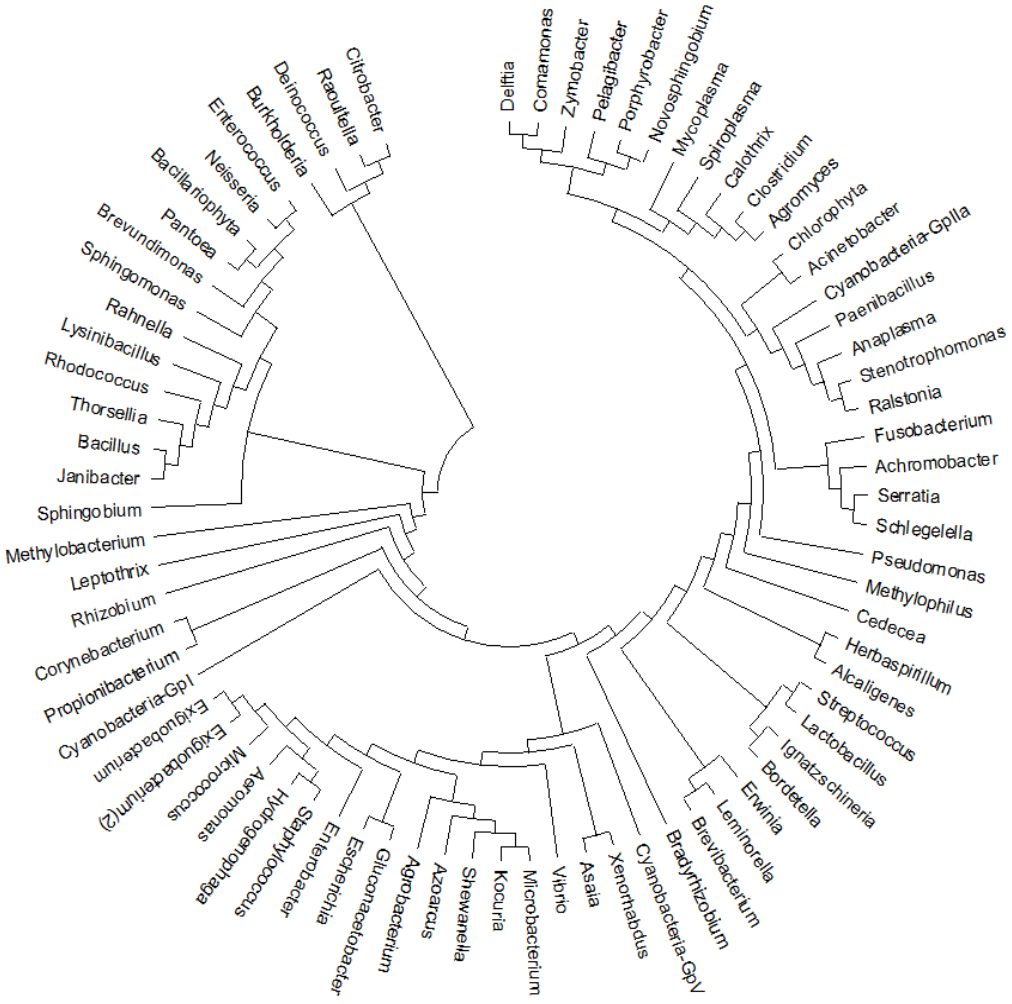

FIGURE 4.5: Phylogenetic Tree Formulated For Falling The Phyla In OTUs.

The relative branch length in figure 4.5 shows phyla diversity in the data. It also shows the relative closeness of two phyla like at 124 level two phyla *Proteobacteria* and *Actinobacteria* shows similarity in genome while this result depict that these two phyla share common portion in their genome and have evolved from closely.

This phylogenetic result revealed the descriptive OTU formulation of the microbial species. The species are fallen into the operational units. The operational units on the basis of genome also predict the functional distribution in the species. In figure 4.5 the neighbors displays relative functional similarity with a slight change. The figure 4.5 also shows that the *Proteobacteria* phylum almost link with all other phyla. The parent clad contains *Chlorophyta*, *Bordetella*, *Xenorhabdus*, *Kocuria*, *Schlegelellia* and *Microbacterium*. All other clads diverges from this clad. It contains 3 phyla *Actinobacteria*, *Proteobacteria* and others species. All other species diverges from this parent clad.

This phylogenetic tree also depict that the species minimum time to diverge from parent clad of a specie was 0.42 and the minimum time it took was 0.00 and the maximum time was 72. This time is based on the branch length as the maximum branch length depicts the longer time was required for a phylum to diverge.

After the bootstrap analysis of the phylogenetic tree it was found that theses species contain one Out group. The out group in figure 4.7 depicts that from where the tree is originated and what are the roots of tree. The out group in this case contains: *Deinococcus*, *Burkholderia*, *Citrobacter*, *Raoutella*, *Brevibacterium*, *Leminorella*, *Erwinia*. These serves as the root for all other species.

4.2.3 Correlational Analysis

4.2.3.1 Heat Map of Similarity between Species

The figure 4.9 depicts that the few species shows close relatedness and these species are based on the distance matrix generated with the help of MSA. The heat map showing the relation between two speices on the basis of two color schemes green and red. The red colour shows that the species are closely related to each other while the green color shows that there is no similarity in species. The black color shows that these species share few similarities between them. This result also shows that few species share mild closeness with each other in light red color. The bright red diagnol line displays the correlation of species with themselves.

The value of approaches to 0 are represented in red while the maximum dissimilar species or values ¿0 are represented in shades approaching to green. If we look on the fig it is been observed that the *Zymobacter* with *Zymobacter* showing bright red color while the *Zymobacter* with *Janibacter* displays green color.

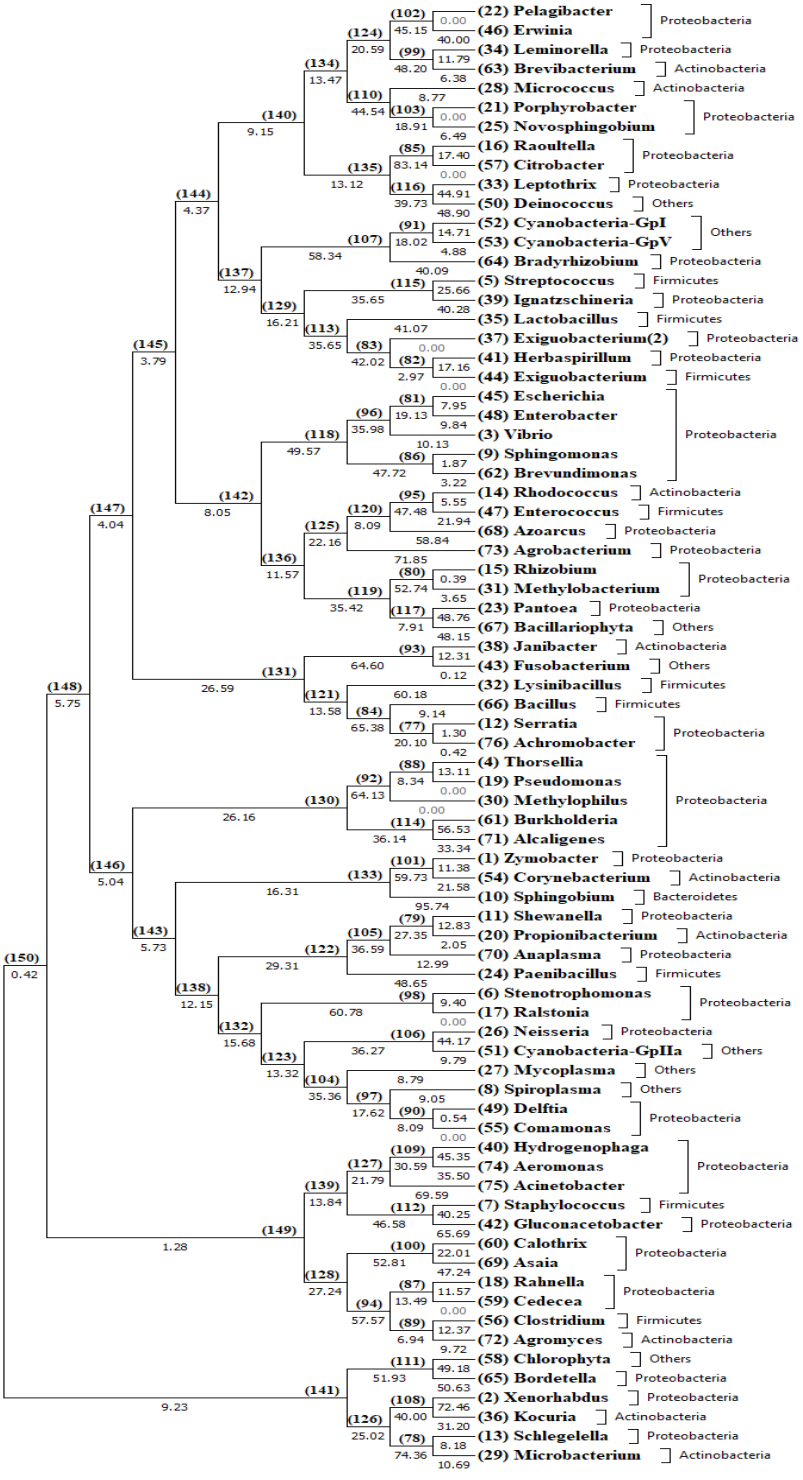

FIGURE 4.6: Phylogenetic Tree Formulated For Falling The Phyla In OTUs.

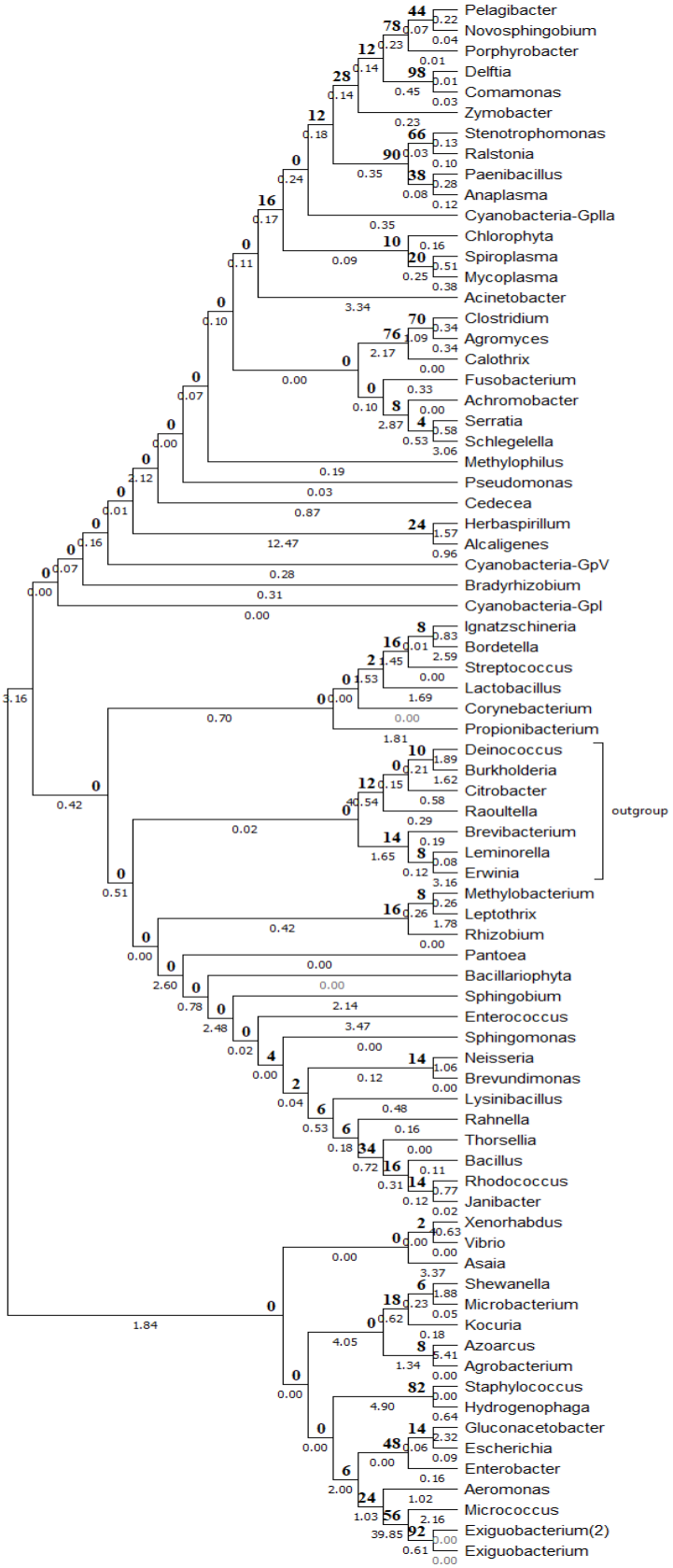

FIGURE 4.7: Phylogenetic Tree Outgroup Formulated For Falling The Phyla In OTUs.

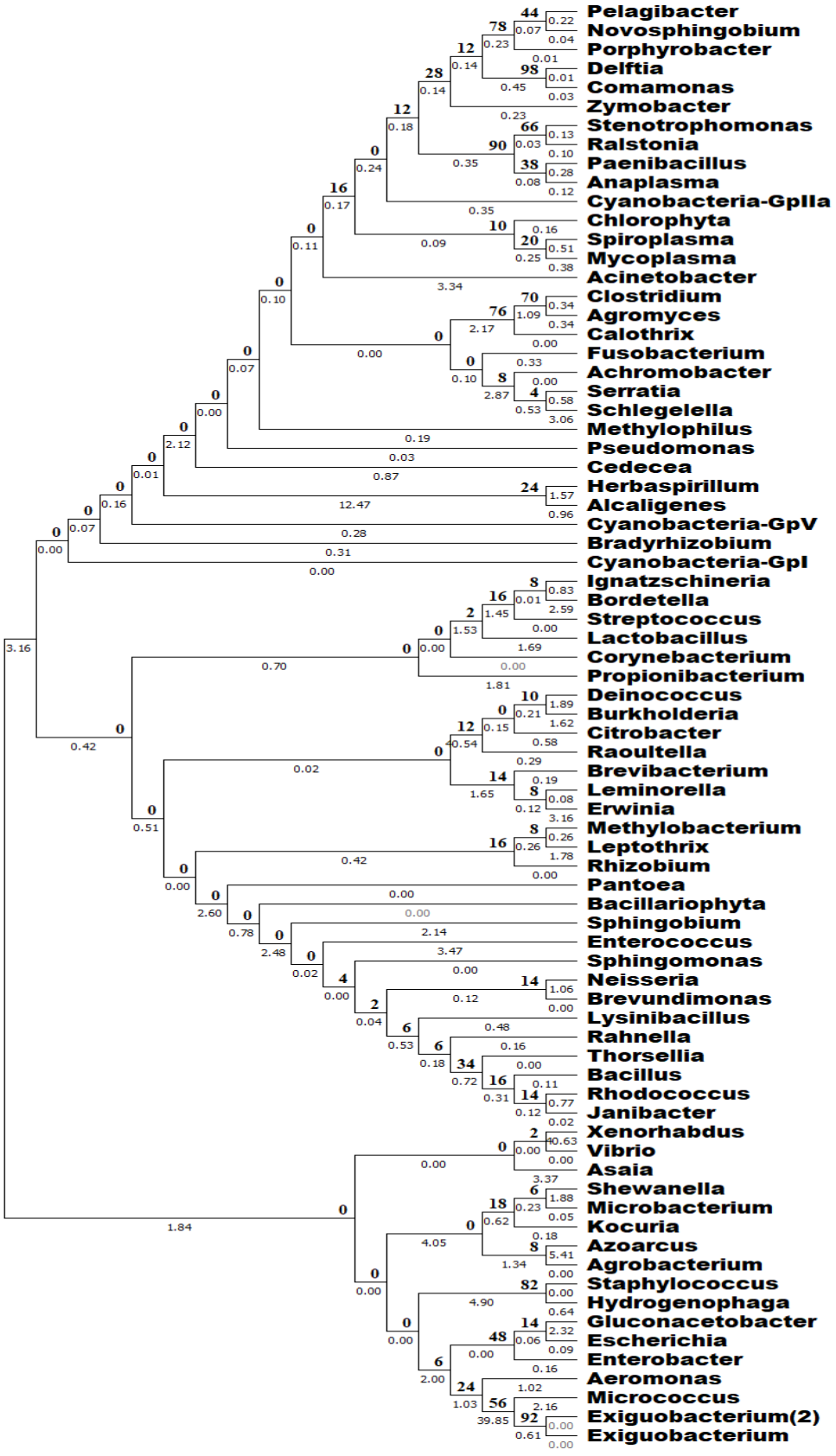

FIGURE 4.8: Phylogenetic Tree Bootstrap Formulated For Falling The Phyla In OTUs.

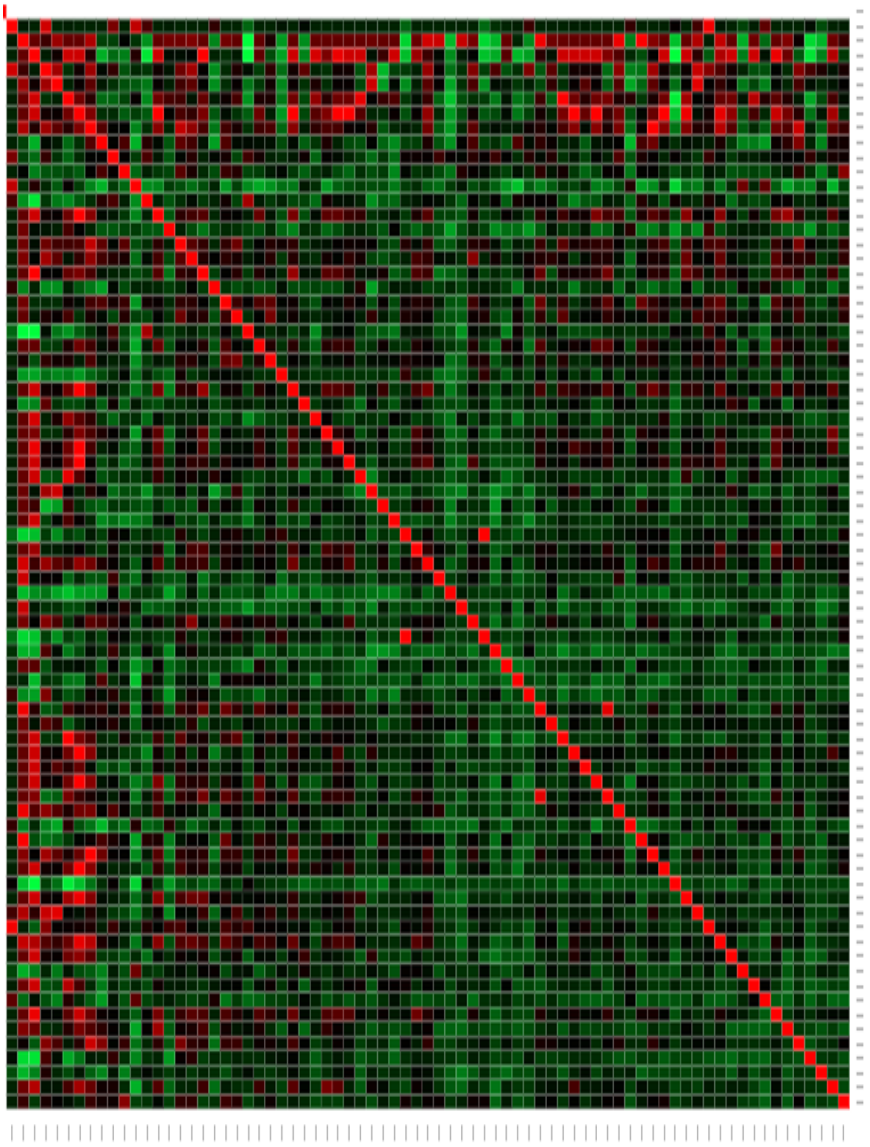

FIGURE 4.9: Expression Analysis Of The Distance Matrix Generated As A Result Of The Phylogenetic Analysis

4.2.4 Clustering Analysis of Species

The clustering of species was done on the basis of Pearsons correlation, K-mean clustering and PCA. The figure 4.10 shows that the species are greatly clustered in center region while few species like *Enterococcus* are diverging from the cluster. This trend of species can also be seen in phylogenetic tree as these species are the most diverged species in the tree showing the most divergence and the branch length is maximum in this case. The phylum of this genus is *Firmicutes* and

beside this many species of *Proteobacteria* also observed to show the divergence pattern. The *Zymobacter* is seen to be most diversed in a clad which belongs to *Proteobacteria*, its branch is 101 in phylogenetic tree indicating its diversity from its ancestor and sister clad.

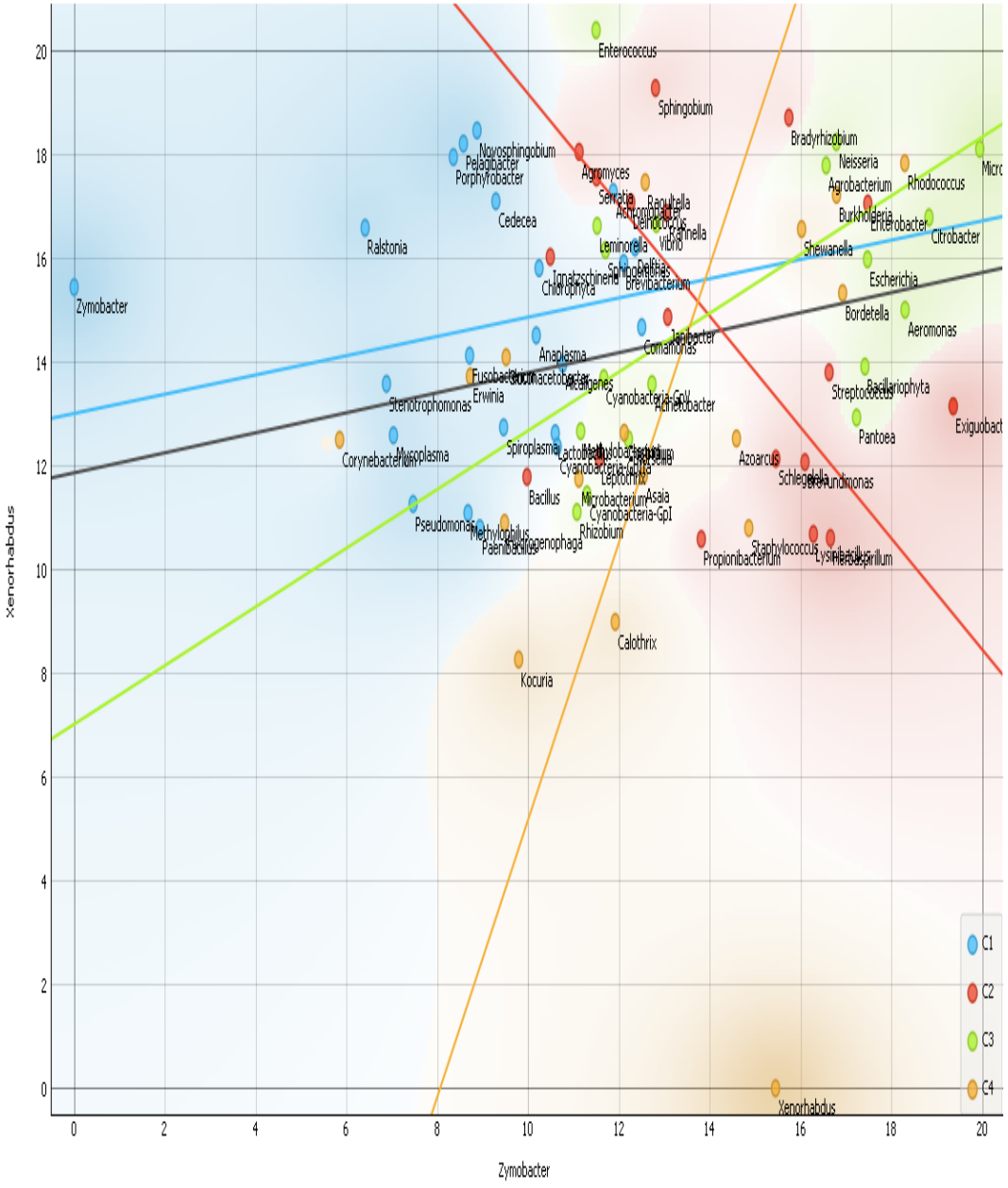

FIGURE 4.10: Clustering Of The Phyla

In the K-Mean clustering and Pearson correlation both the *Zymobacter* shows most divergence from the other species. Figure 4.11 showing the divergences of

the *Zymobacter* species from the mean cluster. In the phylogenetic tree it is observed that the this phylum undergoes 5 time divergence and due to this the genome of this bacteria shows dissimilarity patterns.

In the figure 4.12 the scattered plot of the MDS is presented which represent that the phylum *Enterococcus* and *Micrococcus* species which belong to *Firmicutes* and *Actinobacteria* phylum shows no relation with the rest of cluster. This MDS result depict that these species are not correlated with other species and represents their individual identity. The pairwise distance between them and rest of the dataset is larger thus these species show no relationship with rest of species within the same phylum or genus which conclude them to be important species.

Figure 4.13 showing the PCA analysis of the species which is used for the Principle component. It displays that the species concentration around the mean line is more as compared to the axis. It also indicates that the *Anaplasma* species are lying at mean line. The *Zymobacter* species are more diverged and present at the axis. Beside this *Enterococcus* species are also present at a distance from the mean. The *Xenorhabus* species are also present opposite to the mean position. This trend can be observed in phylogenetic tree as well as the diverged species are also displayed in the diverging branches. This revealed that the result of phylogenetics analysis are in consistency with the clustering, PCA analysis. PCA result indicate that the microbiome of arthropods functions as discrete groups [191]. Variation in microbiome trait is determined largely by environmental factors [192]. The higher variation indicate that none of the bacterial species are redundant for a certain function [193].

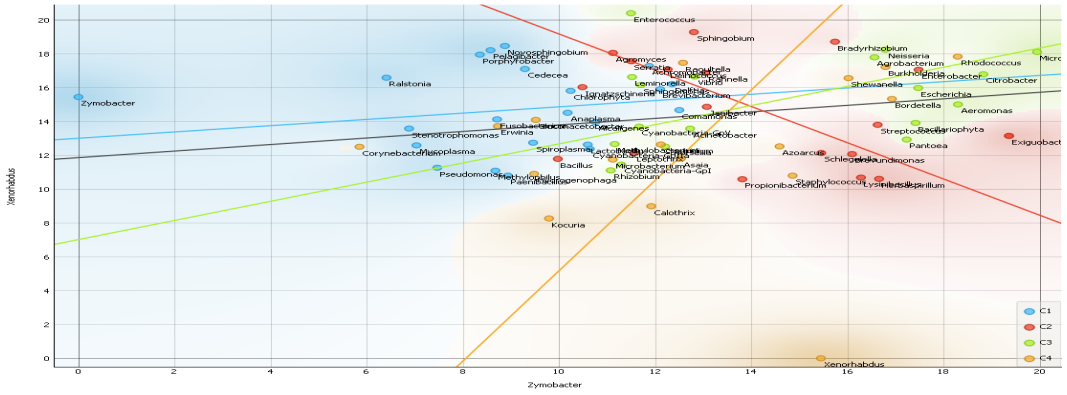

FIGURE 4.11: Clustering Of The Genera Using Correlation Analysis.

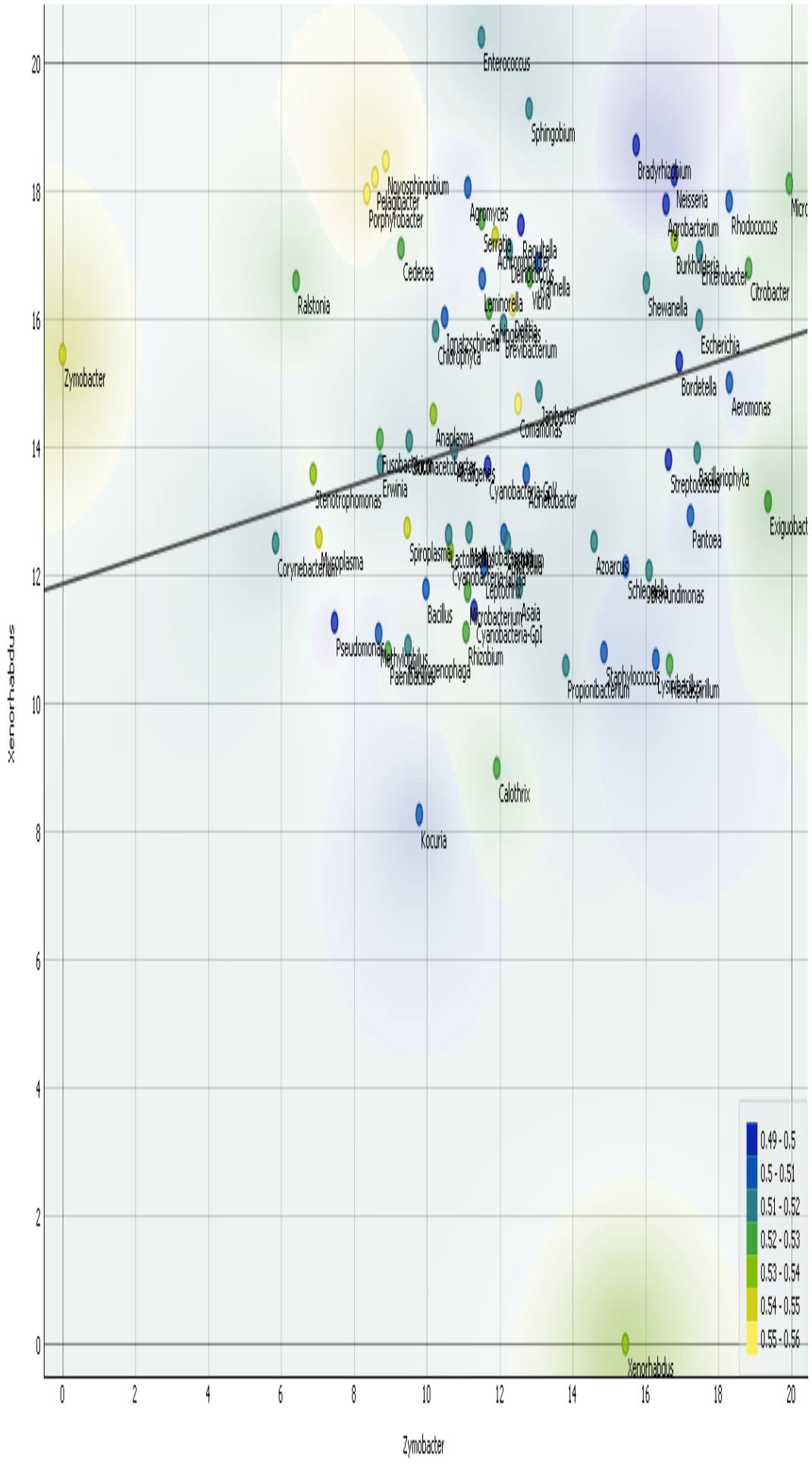

FIGURE 4.12: K-Mean Clustering Of The Phylum Using K-Mean

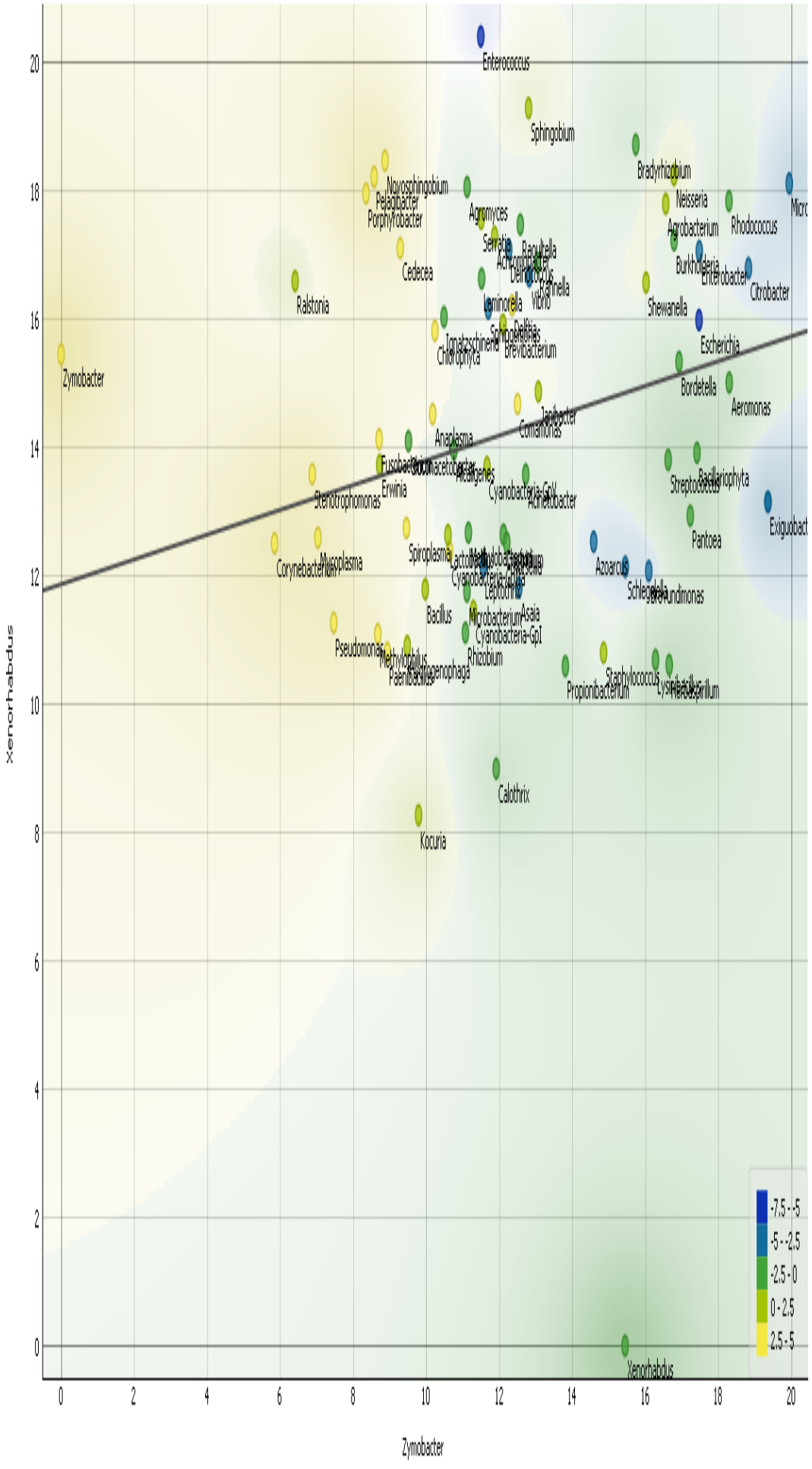

FIGURE 4.13: Principle Component Analysis of Microbes

4.3 Identification of Target Pathway

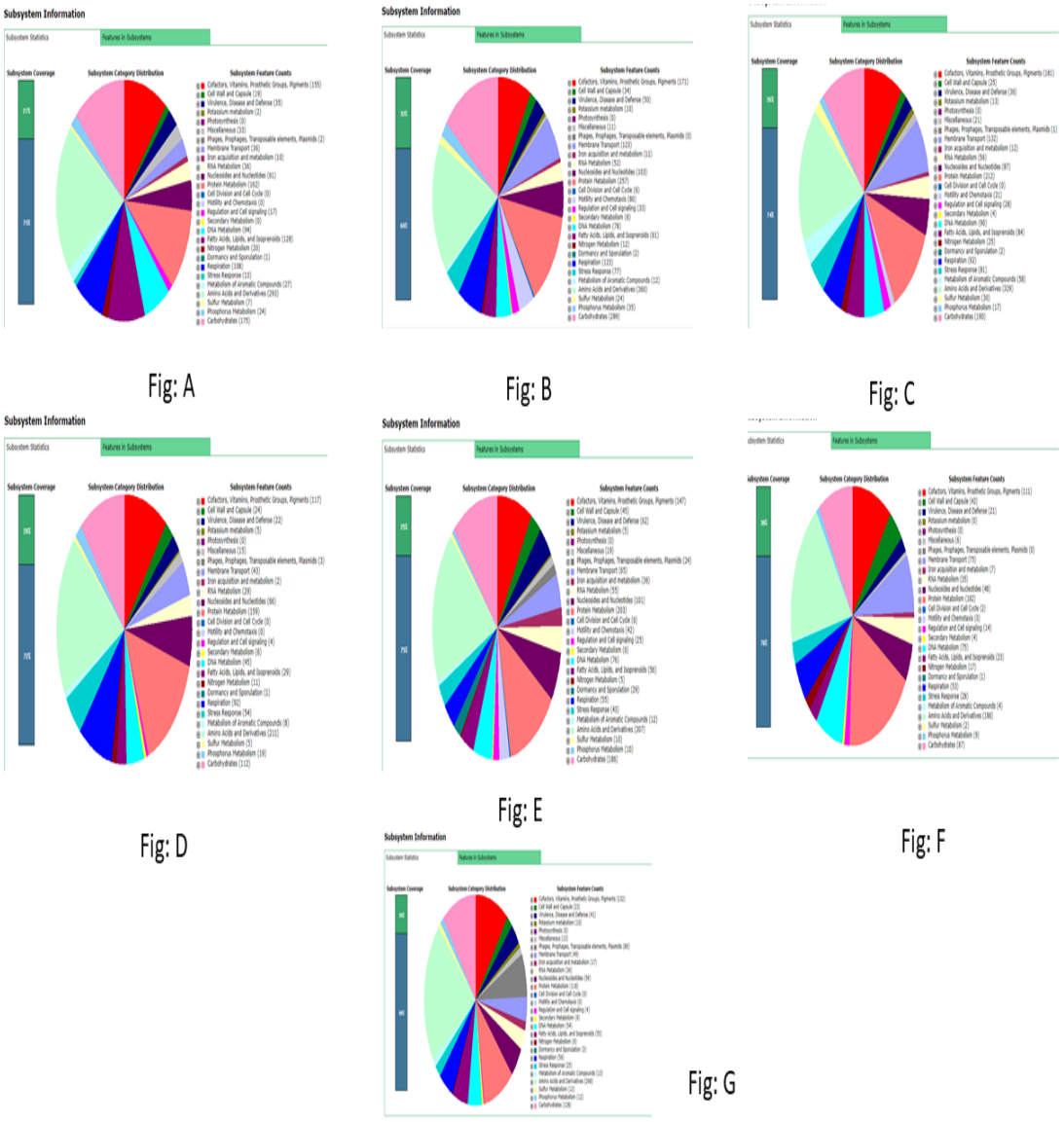

FIGURE 4.14: Figure Showing The Sub Systems Details Obtained By RAST Pipeline. In the figure "A" Is *Janibacter*, "B" Is *Aeromonas*, "C" Is *Acidovorax*, "D" Is *Acetobacter*, "E" Is *Lysinibacillus*, "F" Is *Neisseria* and "G" Is *Elizabethkingia*. These Phylums Are Directly Involved In The Biphenyl Pathway.

The RAST is a rapid functional genomics pipeline which gives us the information about the genome and provide a step for genome wide association. Fig 4.14 represents the genera which contains the Xenobiotic pathway involved in the degradation of the Aromatic compounds. The Aromatic compounds are the ones, which

are involved in insecticides. Figure 4.15 shows the target pathway which is involved in the benzoate degradation which is the core component of the insecticide resistant.The RAST results shows that the 07 genera were identified to be involved in the Biphenyl pathway which is core pathway involved in the benzoate degradation. These genera were as follow:

- *Janibacter* Figure 4.14 A.
- *Aeromonas* Figure 4.14 B.
- *Acidovorax* Figure 4.14 C.
- *Actobacter* Figure 4.14 D.
- *Lysinibacillus* Figure 4.14 E.
- *Neisseria* Figure 4.14 F.
- *Elizabethkingia* Figure 4.14 G.

If we link these result with the OTUs it is observed that the *Janibacter*, *Neisseria*, *Lysinibacillus* are linked with one clad and are neighbours of each other. Which support the fact that the genomes in these species share common properties. If we look back in 2 divergence the *Aeromona* is seen which make it the neighbour of the *Janibacter*, *Neisseria* and *Lysinibacillus*. The branches are of this clad share a common property which makes it important in the xenobiotic pathway as the members of this clad share common genome and likely is responsible in the degradation of phenyl compounds.

Further analysis of whole genome of each genera using RAST showed that the involvement of each genera revealed the subsystem coverage in terms of function. Among 64 the 47 genera were identified in the aromatic metabolism using different pathways like Quinate degradation, benzoate degradation, Salicylate and gentisate catabolism e.t.c out of which 7 genera were involved in the biphenyl pathway which is benzoate degrading.

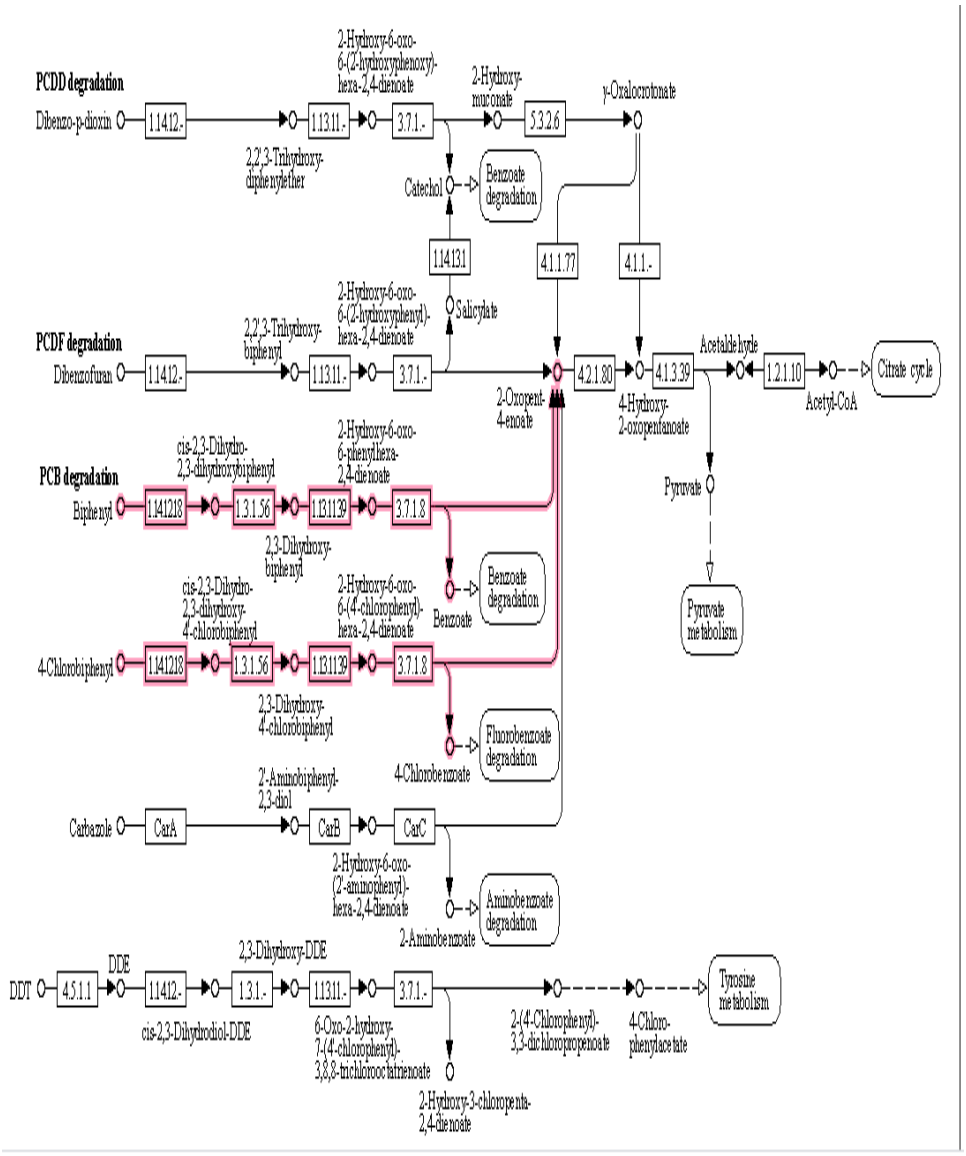

FIGURE 4.15: Biphenyl Pathway Extracted From KEGG Using The Subsystem Information From The RAST.

In the polystyrene degradation the gut microbiota are also involved in Tenebrio molitor [194]. Several genes are identified in the pine weevil, Hylobius abietis using metagenomics survey which have the ability to degrade Norway spruces dipterpene acids [195].

The insecticidal resistance induced by the bacteria has been reported in Rhagoletis pomonella (Apple maggot) [196], Riptortus pedestris (Bean bug) and their aliases [18], Plutella xylostella (Diamond black moth) [19], Bactrocera dorsalis (oriental fruit fly) [197] and other insects [198], this degrading of insecticides result were

deduced by the gut isolated bacteria.

Currently all categories (Pyrethroid, Organophosphate, Organochloride, Neonicotinoids) of insecticides in the form of mats, coils, sprays against the mosquito include aromatic compound.

Recently, an association was established between *Ae. aegypti* midgut bacteria and esterases and CYP450 activities [184]; there are midgut bacterial communities in *Ae. aegypti* associated with the detoxification metabolism of insecticides such as the carbamate propoxur and the organophosphate naled [184]. The elimination of bacteria in larvae with antibiotic treatment reduced esterases and CYP450 activities, consequently decreasing the metabolic detoxification of propoxur and naled. It is not known exactly which bacterial communities are associated with these phenotypes but these results highlight the importance of the microbiota in the metabolic detoxification of carbamates and organophosphates. we found that the gut microbiome reveals different relative abundances of groups of bacteria between lambda-cyhalothrin resistant and susceptible populations. At the species level, our analysis revealed the presence of *Pseudomonas viridiflava* in resistant populations from *Acacias*, *Neiva* and *Puerto Bogota*, but not in the susceptible population from *Bello*. The genus *Pseudomonas* has been found previously in low frequencies in the mosquito midgut. This bacterium is involved in the efficient degradation of fenvalerate, a type II pyrethroid principally used in agriculture, but also used in homes and gardens for insect control [199, 200].

The abundance of *Clostridium ramosum* was significantly increased in all resistant populations, *Clostridium clostridioforme* was unique to *Neiva* and *Honda*. *Clostridium* is associated with the degradation of fenpropathrin [201–203], a relatively new synthetic pyrethroid for controlling insect pests in agriculture and households, which has not been classified in the traditional pyrethroid classifications [204]. Another important genus was *Rhizobium*, which is related to the degradation of the insecticides malathion, an organophosphate [205], imidacloprid, a neonicotinoid compound with high activity against a wide range of pests [206]. This genus was associated with resistant populations (*Acacias*, *Neiva* and *Puerto Bogota*) in Andrea *et. al.,2020* study with the presence of the species *Rhizobium*

daejeonense. These results are novel for *Ae. aegypti* populations [200].

Microorganisms play a significant role in degrading and detoxifying pyrethroids [202]. Many pyrethroid-degrading bacteria have been isolated and characterized: *Micrococcus sp.*, *Streptomyces aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *P. stutzeri*, *Serratia sp.*, *Catellibacterium sp.* and *Enterobacter asuburiae*, which biodegrade cypermethrin [200, 202, 203, 207–209]; *Klebsiella sp.*, *Pseudomonas oleovorans*, *P. stutzeri* and *Bacillus thuringiensis* which participate in the biodegradation of lambda-cyhalothrin [21, 200, 202, 210]; *Brevibacterium aureum*, *Catellibacterium sp.*, *P. aeruginosa*, *Serratia marcescens*, *Sphingobium sp.*, *B. thuringiensis* and *Arthrobacter nicotinovorans* which degraded deltamethrin [202, 203, 210]; *Bacillus cereus*, *Stenotrophomonas sp.* and *Pseudomonas viridiflava*, reported in the present study, which have a role in the degradation of fenvalerate [199, 200, 202, 203]

The bacterial load of a particular species may influence insecticide resistance [211]. This load, in turn, could be determined by selection pressures [22, 211] or the presence of resistance mutations [211]. A study in mosquitoes has demonstrated differing composition of the microbiota and its function between fenitrothion-susceptible and resistant strains of *An. albimanus*. Lower bacterial diversity and significant enrichment of organophosphate-degrading bacteria were observed in the resistant population, suggesting the enrichment of bacterial taxa with a competitive advantage in response to insecticide selection pressure [22].

4.4 Boolean Networks

The Boolean network of biphenyl pathway shows that if the biphenyl is ON and C06589 is OFF the whole pathway shutdowns and the benzoate degradation is not triggered. If the C06589 and biphenyl is OFF the pathway also shutdowns. Figure 4.16 and 4.17 refers to these states the blue color in the figure 4.16 and 4.17 shows the OFF state and the Yellow state to be ON. Figure 4.18 refers to the biphenyl ON and C06589 to ON which initiates the pathway even the state of BphAa is OFF.

Figure 4.19 refers to the state when BphAa is OFF and C02525 is ON the pathway triggers as the BphAa is ON due to the ON state of previous nodes i.e. biphenyl and C06589. If the state of BphAa is ON and the C06589 is OFF it stops the pathway at the BphAa node as the these two constitute the BphC which can be observed in Figure 4.20.

Fig 4.21 refers to the state when the BphC is ON and C01273 is OFF the pathway stops at BphC node as the C01273 is essential compouond for the production of BphD. It is observed in figure 4.22 that if we OFF the BphC the pathway will run and the aminobenzoate degradation will occur as the BphC get its signal from previous nodes.

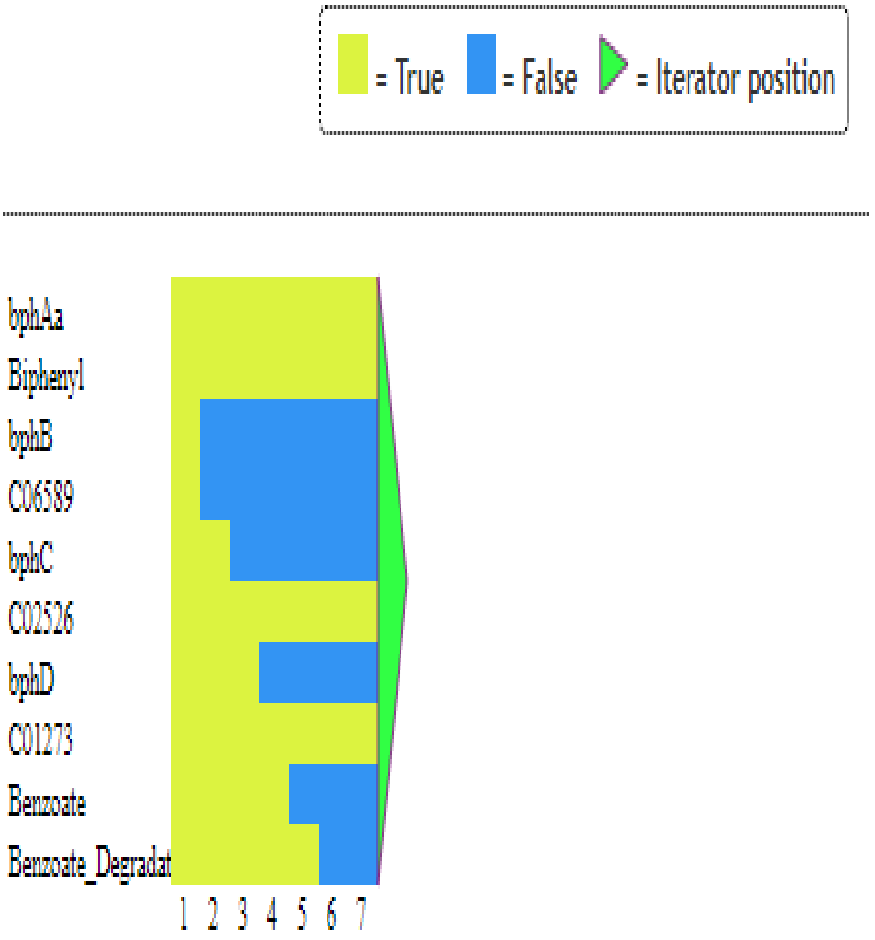

FIGURE 4.16: Boolean Network Simulation When The Biphenyl=ON, BphAa = OFF and C06589=OFF.

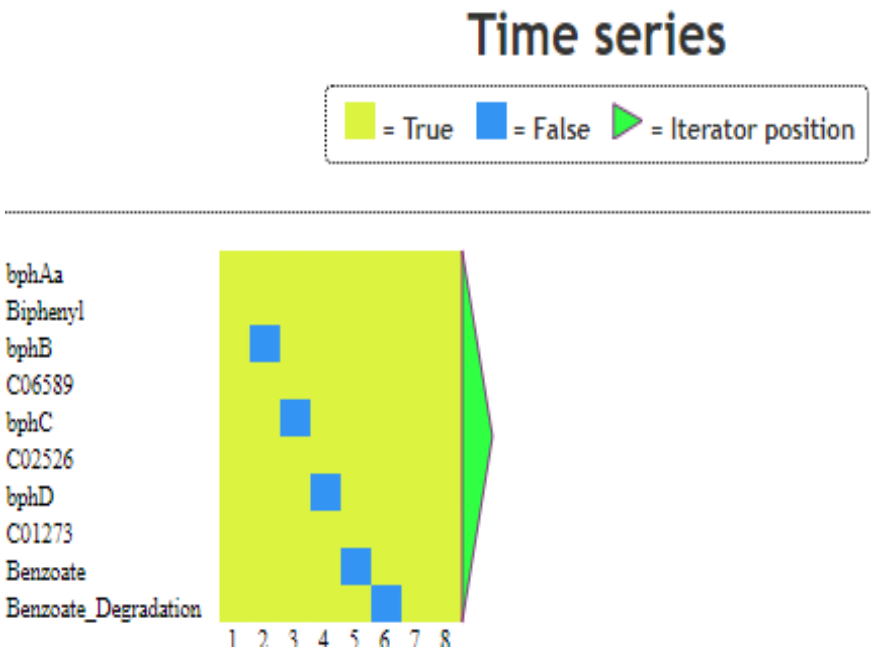

FIGURE 4.17: Boolean Network Simulation When The Biphenyl=ON, BphAa=OFF and C06589 =ON.

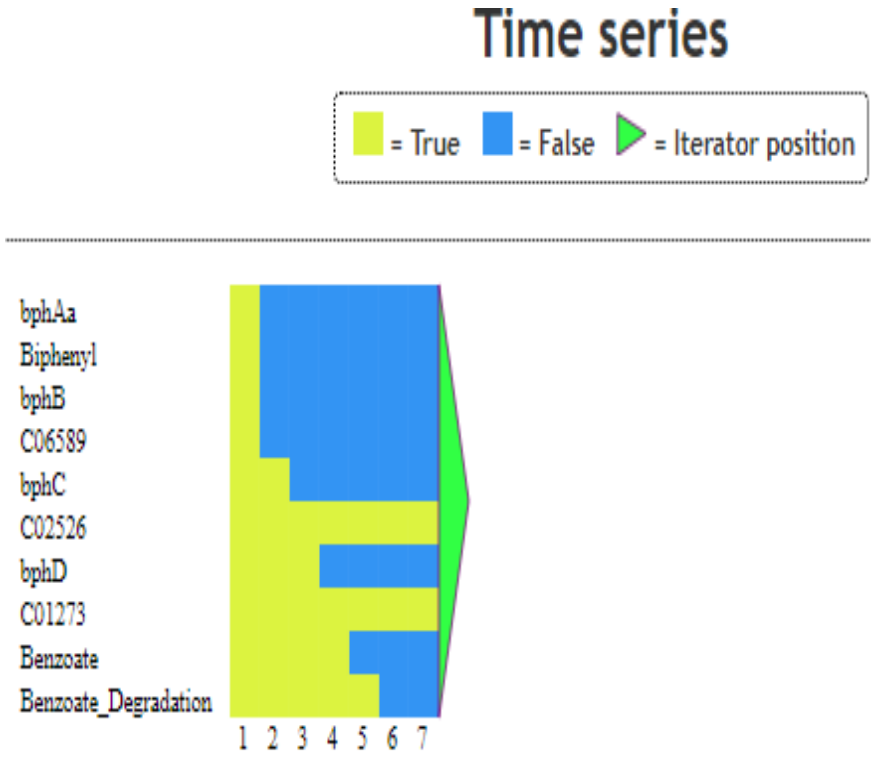

FIGURE 4.18: Boolean Network Simulation When Biphenyl=OFF, BphAa=ON and C06589=OFF.

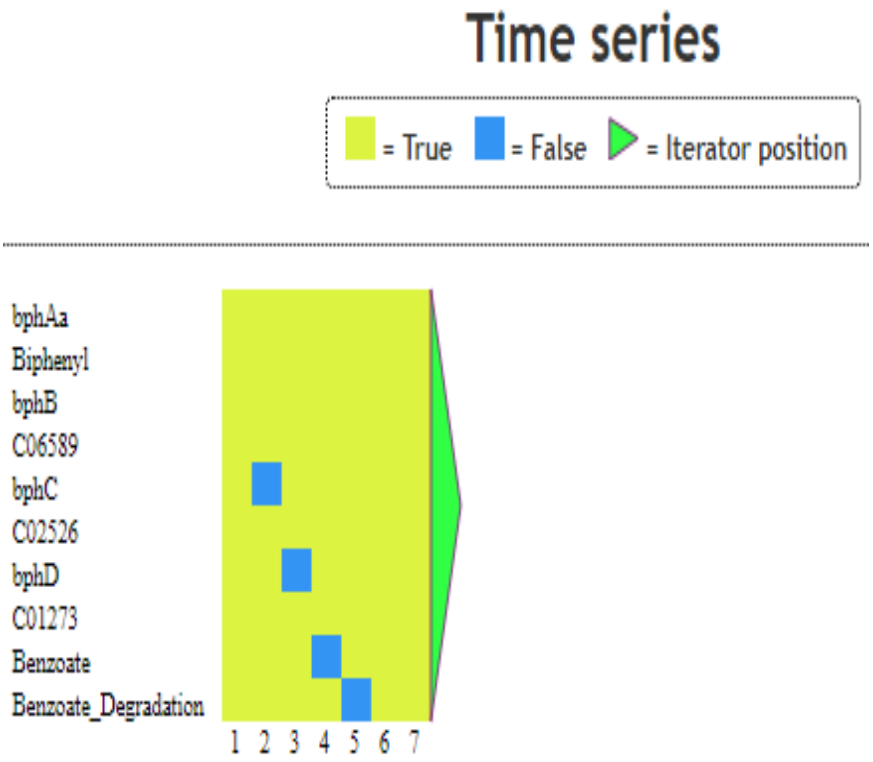

FIGURE 4.19: Boolean Network Simulation When bphB=OFF and C02525=ON

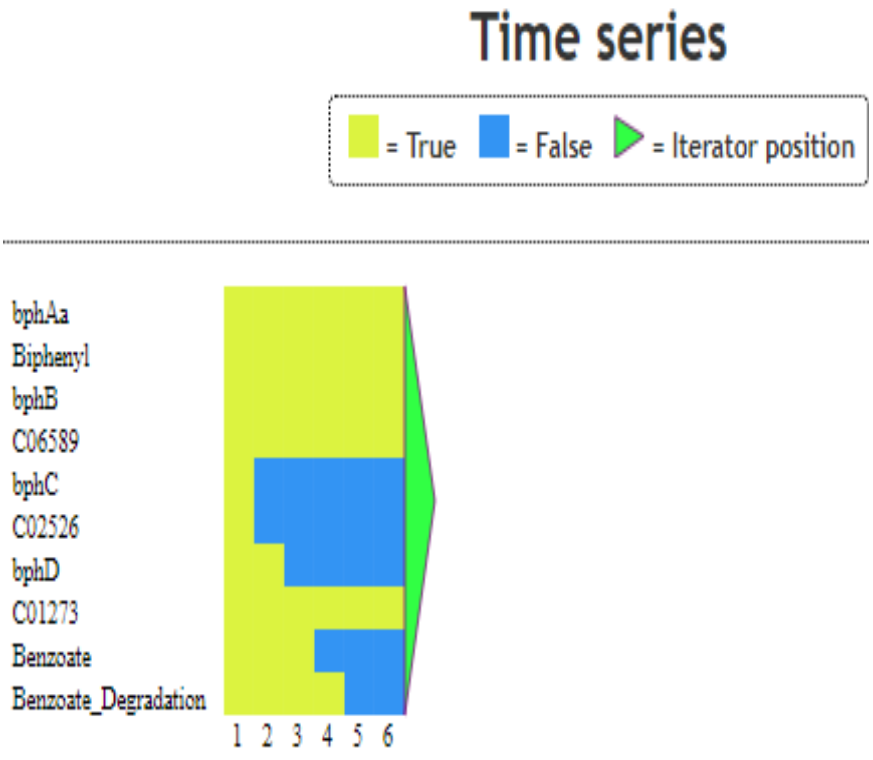

FIGURE 4.20: Boolean Network Simulation When bphB=ON and C02525=OFF.

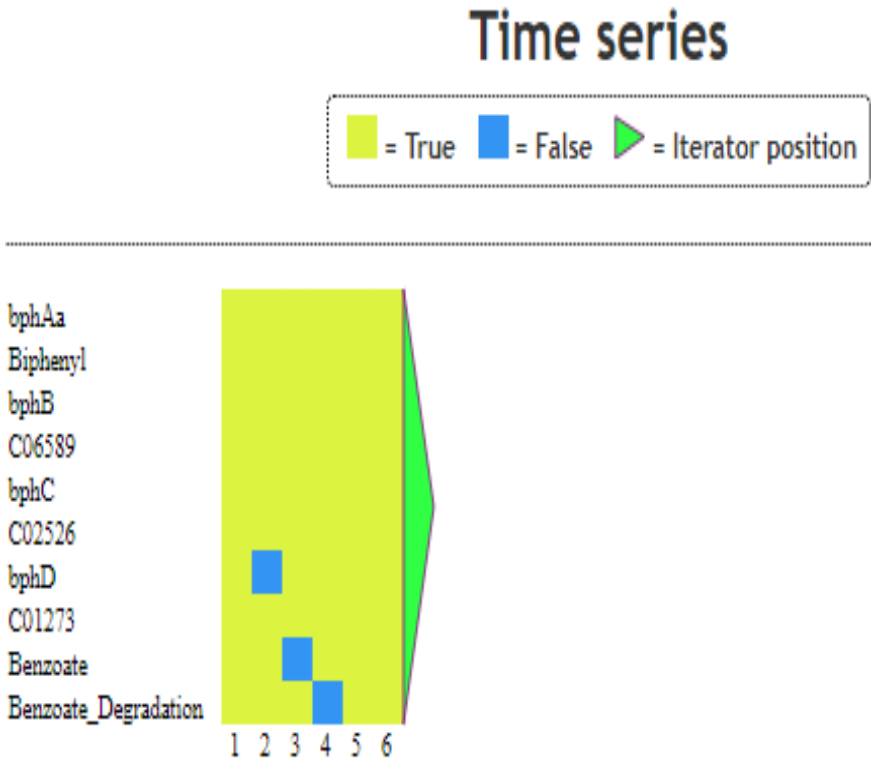

FIGURE 4.21: Boolean Network Simulation When bphC=ON and C01273 = OFF.

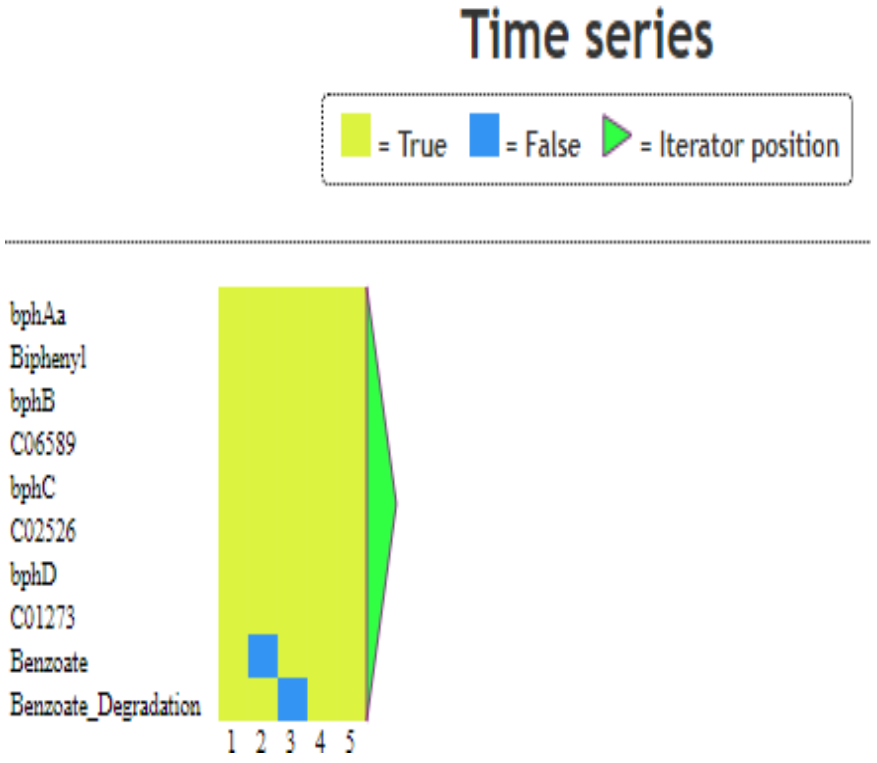

FIGURE 4.22: Boolean Network Simulation When bph=OFF.

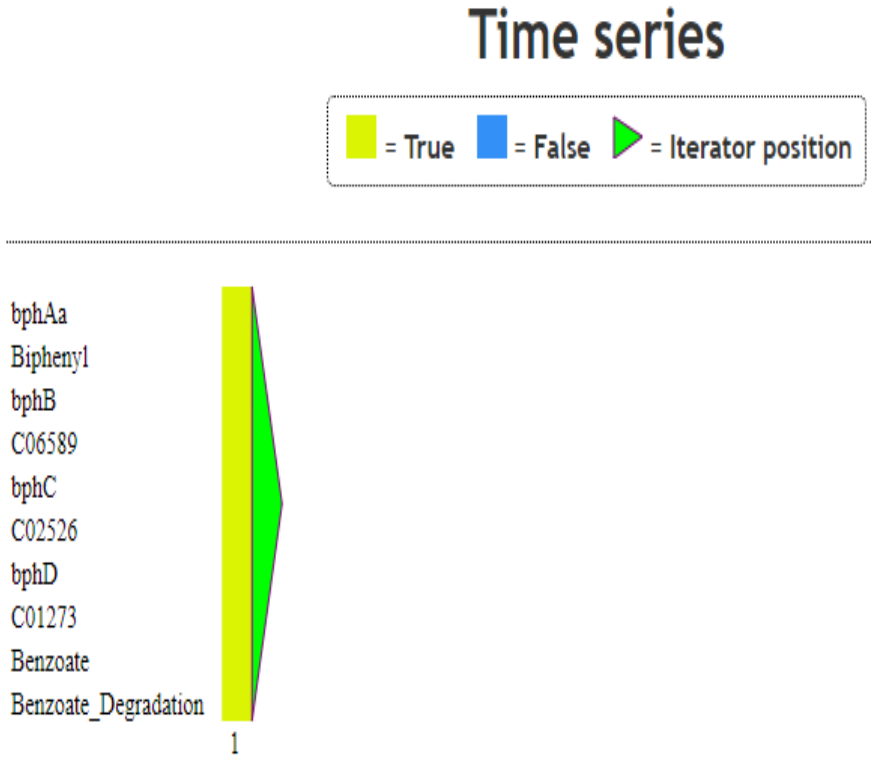

FIGURE 4.23: Boolean Network Simulation When All States Are True.

Figure 4.23 refers to the state when BphD is OFF but nothing happens to the pathway as it runs according to it normal path.

These results are suggestive that the compound C06589, C02525, C01273 are the core constitutes along with biphenyl as when their state is disturbed the pathway stops its function. It also concluded that if we stops the production of these compounds the amino benzoate pathway will stop its working which will help to develop better insecticides for the Anopheles species.

The key compound to study the bacterial aromatic catabolism is benzoate [212, 213]. The activation of benzoate degradation pathway by strict or facultative anaerobes is initiated by the benzyl-CoA this is aided by the an ATP-dependent benzoate-CoA ligase. Benzyl-CoA is then directed to the aromatic ring reduction and a modified β-oxidation pathway is triggered which finish at an aliphatic C7-dicarboxyl-CoA derivative [212–214]. In contrast to this the conventional aerobic pathway depends on the hydroxylation of aeromatic ring and produces the catechol this ring is cleaved by the dioxygenase [215]. The third method of degradation of

benzoate is box pathway which is initiated by the activate benzoate to benzoyl-CoA this is done by benzoate-CoA ligase (Bc1A). Then the BoxAB and a BoxC dihydrolase are responsible for the cleavage of aromatic ring [216, 217].

The increase tolerance of insect to insecticides by microbial symbionts let to the suggestions that microbial symbionts may also contribute to the evolution of insect resistance to insecticide.

TABLE 4.2: The Results Of Boolean Network Simulation In Which 1 Shows The ON State And 0 Shows The OFF State Of A Node. The Attractors Are In Columns And The Nodes Are In Rows. This Was Build Using Boolesim Web Based Python Tool Which Can Be Access From (https://rumo.biologie.hu-berlin.de/boolesim/)

Nodes	**Biphenyl = On bphAa = OFF C06589 = OFF**	**Biphenyl = On bphAa = OFF C06589 = ON**	**Biphenyl = OFF bphAa = ON C06589 = OFF**	**bphB=OFF C02525 = ON**	**bphB = ON C02525 =OFF**	**bphC=ON C01273 =OFF**	**bphC=OFF C01273 =ON**	**bphD = OFF**
Biphenyl	1	1	0	1	1	1	1	1
bphAa	1	1	0	1	1	1	1	1
C06589	0	1	0	1	1	1	1	1
bphB	0	1	0	1	1	1	1	1
C02526	1	1	1	1	0	1	1	1
bphC	0	1	0	1	0	1	1	1
C01273	1	1	1	1	1	0	1	1
bphD	0	1	0	1	0	0	1	1
Benzoate	0	1	0	1	0	0	1	1

Chapter 5

Conclusion & Future Perspective

The microbiota of mosquito has an key impact in the host characteristics like nutrition, development, reproduction, growth, vector competence, interactions with parasites and the present day studies provide an evidence that microbiota are also involved in the mosquito resistance to insecticides. The results presented shows differential composition and function of microbiota. The microbiota in *Anopheles* is abundant and controlling many functionalities in the mosquito. The literature mining helped us to gathered the information on these microbiome. It was found that the 96 genera were found in the 11 *Anopheles* species. These genera were belong to 4 major phylum i.e. *Actinobacteria*, *Proteobacteria*, *Brodettella*, *Firmicutes*. These microbes were identified to control major function in the mosquitoes. The *Pseudomonas* species were identified to be found in 9 *Anopheles* species. The distribution of microbes was found to be greater in *Proteobacteria*. These microbes were when studied in the life stages found more in the Adult stage second by larvae stage and the pupal stage stood last. The *Anopheles gambiae* contains 64 species and the *Anopheles stephensi* have 51 species. The least amount of species were present in *Anopheles funestus*. After this the OTU analysis was conducted. The microbes were fallen into the operational units. The first step was to make a distance matrix by MSA. The phylogenetic tree was formed. The phylogenetic tree shows that the genera diverge from each other at a very random rate. Tree also explained that the genera belongs to *Proteobacteria* are linked with almost each

phylum. This was suggestive that all other phylum may be diverged from the *Proteobacteria* phylum. Then the correlational analysis was performed on the basis of the distance matrix generated through MSA. The correlational analysis suggests that few species are clustered in the center which represents the relative functional similarity while few species are away from the center to the axis like *Zymobacter* which indicates their divergence from the rest of cluster. This study indicated that almost 90% of the bacterial species were closely related to each other and have some common functional points. After this major part was to identify the microbes that are involved in triggering the benzoate degradation pathway which is insecticide degrading, 7 genera were identified which were directly involved in the biphenyl degradation which is a core pathway to start the benzoate degradation. Then the Boolean network was performed to find out the attractors of the biphenyl pathway which showed that the enzymes, that are involved in the biphenyl are responsible in controlling the biphenyl pathway.

The symbiotic role of insects xenobiotics is often reported but many researchers argue upon the fact that these symbionts have a greater imapct on the host ecology, nutrition, adaptation to environment and immune system. These findings may be ground for future research. Characterization and comparison of expression level of specific microbial genes involved in insecticide degradation needs to explored to further confirm the role of investigated microbiota. Investigation should be concentrated in mechanism of symbiotic acquisition and in process microbes employ to metabolize such substrate. The finding of our work provide an essential base line for future vector control and interventional studies which should focus on bacteria implicated in the detoxification process in field population.

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An Appendix

TABLE 1: Table showing microbiota extracted through literature mining in *Anopheles sp.* The table depicting that 4 major phyla of bacteria i.e Actinobacteria, Bacteroides,Firmicutes, Proteobacteria are majorly found in *Anopheles*. It also depict the source of isolation, in which developemental stage it is found and its examples with references.

Family	**Class**	**Genus**	**Anopheles Species**	**Deep Sequencing**	**Culturing**	**Non-Culturing**	**Conditions**	**Developmental Stage**	**Example**	**References**
Actinobacteria										
Microbacteriaceae	Actinobacteria	Agromyces	gambiae	✓	X	X	Semi-natural	Larval	JX186590	[218]
Brevibertericeae	Actinobacteria	Brevibateriu m	stephensi	X	X	✓	Field	Larval	FJ608062	[219]
Corynebateriaceae	Actinobacteria	Corynbacterium	funesius gambiae	✓	X	X	Field Semi-natural	Adult	GQ10973	[13, 218]
Intrasporangiaceae	Actinobacteria	Janibacter	arabiensis	X	✓	X	Field	Adult	NR_043218	[220]
Micrococcaceae	Actinobacteria	Kocuria	stephensi	X	✓	X	Field	Larval	HQ591424	[221]
Microbacteriac	Actinobact	Microbacteri	gambiae	X	✓	X	Field	Larval	HQ5914	[221]

eae	eria	um	stephensi				Laboratory		31	[222]
Propionibacteriaceae	Actinobacteria	Micrococcus	gambiae stephensi	X	✓	X	Field Laboratory	Adult	FJ608230	[187] [219]
Nocardiaceae	Actinobacteria	Propionibacterium	funestus gambiae	✓	X	X	Field Semi-natural	Adult	GQ00336	[13] [218]
Nocardiaceae	Actinobacteria	Rhodococcus	arabiensis stephensi	X	✓	X	Field	Larval Adult	AY837749	[220] [221]
Bacteroidetes										
Flavobacteriaceae	Flavobacteriia	Chryseobacterium	coustani funestus gambiae stephensi	✓	✓	✓	Field Semi-natural Laboratory	Larval Pupal Adult	HQ591432	[221, 222] [218, 219] [13]
Porphyromonadaceae	Bacteroidia	Dysgonomonas	stephensi	X	X	✓	Field	Larval	FJ608061	[219]

Flavobacteriaceae	Flavobacteriia	Elizabethkingia	gambiae stephensi	✓	✓	✓	Semi-natural Laboratory	Adult	EF426434	[52, 218] [223, 224] [187, 219]
Flavobacteriaceae	Flvobacteriia	Flavobacterium	albimanus funestus gambiae stephensi	X	✓	✓	Field Laboratory	Adult		[45, 225]
	Cytophagia	Flexibacteraceae	stephensi	X	X	✓	Field	Adult	FJ608195	[219]
Flavobacteriaceae	Flavobacteriia	Myroides	stephensi	X	✓	X	Field	Larval Adult	HQ832872	[221]
Prevotellaceae	Bacteroidia	Prevotella	gambiae	✓	X	X	Semi-natural	Adult	JN867317	[52]
Chitinophagaceae	Sphingobacteriia	Sediminibacterium	gambiae	✓	X	X	Semi-natural	Adult	FJ915158	[52]
Sphingobacteriaceae	Sphingobacteriia	Sphingobacterium	gambiae	X	✓	X	Laboratory	Pupal Adult	EF426436	[50]

Firmicutes										
Bacillaceae	Bacilli	Bacillus	arabiensis gambiae stephensi funestus	X	✓	✓	Field and Laboratory	Larval Adult	AY8377 46	[220, 222] [45, 223] [219]
Clostidiaceae	Clostridia	Clostridium	gambiae	✓	X	X	Semi-Natural	Larval	JN39157 7	[218]
Enterococcaceae	Bacilli	Enterococcus	stephensi funestus gambieae	✓	✓	X	Field	Larval Adultl	HQ5914 41	[13, 221]
Incertae Sedis Bacillales (Family XII)	Bacilli	Exiguobacterium	stephensi	X	✓	X	Field	Larval	HQ5914 39	[221, 226]
Lactobacillaceae	Bacilli	Lactobacillus	gambiae stephensi	✓	X	✓	Field and Semi-natural	Larval Adult	FJ60805 3	[218, 219]

Bacillaceae	Bacilli	Lysinibacillus	maculipennis stephensi	X	✓	X	Field	Larval	GU204964	[51]
Paenibacillaceae	Bacilli	Paenibacillus	arabiensis stephensi	X	X	✓	Field	Adult	EF426449	[219, 220]
Staphylococcaceae	Bacilli	Staphylococcuss	maculipennis gambiae funestus stephensi quadrimaculatus	✓	✓	✓	Field, Semi-Natural and Laboratory	Larval Adult	FJ608067	[227, 228] [13, 219] [229]
Strptococcaceae	Bacilli	Streptococcus	stephensi gambiae funestus	✓	X	✓	Field and Semi-natural	Larval Adult	FJ608047	[13, 52] [219]
Proteobacteria										
Acetobacteraceae	Alphaproteobacteria	Acetobacter	stephensi	X	X	✓	Laboratory	Adult		[228]
Alcaligenaceae	Betaproteo	Achromobact	stephensi	X	X	✓	Field	Adult	FJ608307	[219]

	bacteria	er							1	
Comamonadaceae	Betaproteobacteria	Acidovorax	arabiensis	X	X	✓	Field	Adult	AY837725	[220]
Moxaxellaceae	Gammaproteobacteria	Acinetobacter	albimanus gambiae stephensi funestus	✓	✓	✓	Field, Semi-natural and Laboratory	Larval Adult	FJ608267	[218, 225] [13, 52] [219, 228]
Aeromonadaceae	Gammaproteobacteria	Aeromonas	gambiae stephensi maculipennis darlingi coustani	✓	✓	✓	Field, Semi-natural and Laboratory	Larval Adult	FJ607997	[218, 225] [13, 219] [220, 221] [51, 230]
Comamonadaceae	Betaproteobacteria	Agrobacterium	stephensi	X	✓	✓	Laboratory	Larval Adult	HQ832875	[219]
Alcaligenaceae	Betaproteo	Alcaligenes	stephensi	X	✓	✓	Field	Adult	Ay8377	[45, 221]

	bacteria		funestus						39	
Anaplasmataceae	Alphaproteobacteria	Anaplasma	arabiensis	X	X	✓	Field	Adult		[220]
Burkholderiales General incertaesedis	Betaproteobacteria	Aquabacterium	gambiae	X	X	✓	Field	Adult	FN821398	[228]
Acetobacteraceae	Alphaproteobacteria	Asaia	maculipennis gambiae funestus coustani stephensi	✓	✓	✓	Field, Semi-natural and Laboratory	Adult	FJ608071	[52, 222] [13, 228] [187, 226]
Rhodocyclaceae	Betaproteobacteria	Azoarcus	stephensi	X	X	✓	Field	Larval Adult	HQ832874	[219]
Alcaligenaceae	Betaproteobacteria	Bordetella	stephensi	X	✓	X	Field	Larval	AB740924	[221]
Bradyrhizobiac	Alphaprot	Bradyrhizobi	gambiae	✓	X	X	Semi-	Adult	GU2049	[52]

eae	eobacteria	um					natural		62	
Caulobacterac eae	Alphaprot eobacteria	Brevundimon as	funestus stephensi	X	✓	✓	Field	Adult	AY3912 83	[45, 51]
Burkholderiace ae	Betaproteo bacteria	Burkholderia	stephensi gambiae	✓	X	✓	Field, Semi-natural and Laborat ory	Larval Adult		[52, 228] [223]
Enterobacteria ceae	Gammapr oteobacter ia	Buttiauxella	darlingi	X	X	✓	Field	Adult		[230]
Enterobacteria ceae	Gammapr oteobacter ia	Cedecea	funestus stephensi gambiae	✓	✓	✓	Field, Semi-natural and Laborat ory	Adult	DQ0688 69	[52, 225] [45, 231]

Enterobacteriaceae	Gammaproteobacteria	Citrobacter	stephensi darlingi	X	✓	✓	Field	Adult	EJ609234	[219, 230]
Comamonadaceae	Betaproteobacteria	Comamonas	funestus quadrimaculatus dureni stephensi	✓	✓	✓	Field and Semi-natural	Pupal Adult	EF426440	[52, 218] [50, 219] [229, 232]
Comamonadaceae	Betaproteobacteria	Delftia	gambiae	X	✓	n	Laboratory	Pupal	EF426438	[50]
Anaplamataceae	Alphaproteobacteria	Ehrlichia	arabiensis	X	X	✓	Field	Adult		[220]
Enterobacteriaceae	Gammaproteobacteria	Enterobacter	darlingi funestus stephensi gambiae albimanus	✓	✓	✓	Field, Semi-natural and Laboratory	Larval Adult	HQ832863	[227]

Enterobacteriaceae	Gammaproteobacteria	Erwinia	funestus darlingi gambiae	X	✓	✓	Field and Laborat Ory	Adult	FJ816023	[52, 223]
Enterobacteriaceae	Gammaproteobacteria	Escherichia Shigella	abaiensis funestus stephensi gambiae	✓	✓	✓	Field, Semi-natural and Laboratory	Adult	FJ608223	[45, 228]
Enterobacteriaceae	Gammaproteobacteria	Ewingella	stephensi	X	✓	✓	Laboratory	Adult		[219, 222]
Acetobacteraceae	Alphaproteobacteria	Gluconacetobacter	gambiae	✓	✓	✓	Semi-natural and Laboratory	Adult	FN814298	[45, 218] [219]

Acetobacteraceae	Alphaproteobacteria	Fluconobacter	funestus stephensi	X	✓	X	Field and Laboratory	Adult		[219]
Oxalobacteraceae	Betaproteobacteria	Herbaspirillum	stephensi gambiae	✓	✓	X	Field and Laboratory	Adult	FJ608162	[218, 221] [45, 230] [187, 219]
Comamonadaceae	Betaproteobacteria	Hydrogenophaga	stephensi gambiae	X	X	✓	Field and Semi-natural	Larval	FJ608063	[45, 225]
Xanthomonadaceae	Gammaproteobacteria	Ignatzshineria	stephensi	X	✓	✓	Field	Larval	FJ608103	[219]
Enterobacteriaceae	Gammaproteobacteria	Klebsiella	funestus farling stephensi	✓	X	✓	Field, Semi-natural	Larval Adult	HQ591433	[218, 221] [45, 230] [219, 232]

			gambiae				and Laboratory			
Enterobacteriaceae	Gammaproteobacteria	Kluyvera	gambiae funestus	X	X	✓	Field			[45, 225]
Enterobacteriaceae	Gammaproteobacteria	Leminorella	stephensi	✓	✓	✓	Field	Adult	FJ608283	[219]
Burkholderiales	Betaproteobacteria	Leptothrix	stephensi	X	✓	✓	Field	Larval	FJ608083	[219]
Enterobacteriaceae	Gammaproteobacteria	Morganella	gambiae	X	X	✓	Field	Adult		[45]
Methylobacteriacae	Alphaproteobacteria	Methylobacterium	gambiae funestus	X	X	✓	Field and Semi-natural	Adult	AB673246	[13, 52]
Methylophilace	Betaproteo	Methylophilu	gambiae	X	X	✓	Semi-	Pupal	FJ51773	[218]

ae	bacteria	s					natural		6	
Neisseriaceae	Betaproteobacteria	Neisseria	gambiae	✓	X	X	Semi-natural	Adult	JX222980	[52]
Sphingomonadaceae	Alphaproteobacteria	Novosphingobium	gambiae stephensi darlingi albimanus	✓	X	X	Semi-natural	Adult	JF690934	[218]
Enterobacteriaceae	Gammaproteobacteria	Pantoea	gambiae	✓	X	X	Field and Laboratory	Larval Adult	JF690934	[222, 225] [45, 230] [50, 219]
SAR11 cluster (not included in family)	Alphaproteobacteria	Pelagibacter	gambiae	✓	X	X	Semi-natural	Adult	GQ340243	[218]
Caulobacteraceae	Alphaproteobacteria	Phenylobacterium	gambiae	X	✓	✓	Field	Adult		[228]
Enterobacteriaceae	Gammaproteobacter	Phytobacter	gambiae	✓	X	X	Laboratory	Adult		[222]

	ia									
Erythrobacteraceae	Alphaproteobacteria	Prophyrobacter	gambiae	X	X	✓	Semi-natural	Larval	JQ923889	[218]
Pseudomonadaceae	Gammaproteobacteria	Pseudomonas	darlingi albimanus funestus gambiae stephensi maculipennis quadrimaculatus	✓	✓	✓		Larval Pupal Adult	EF426444	[218, 222] [52, 220] [228, 231] [50, 230] [219, 232]
Enterobacteriaceae	Gammaproteobacteria	Rahnella	stephensi	X	✓	X	Field, Semi-natural and Laboratory	Larval	GU204974	[51]
Burkholderiace	Betaproteo	Ralstonia	gambiae	✓	X	X	Field,	Adult	AY1918	[52]

ae	bacteria						Semi-natural and Laboratory		52	
Enterobacteriaceae	Gammaproteobacteria	Raoultella	gambiae	✓	X	X	Field	Adult	HQ811336	[218]
Rhizobiaceae	Alphaproteobacteria	Rhizobium	gambiae	✓	X	X	Semi-natural	Larval	DQ814410	[218]
Enterobacteriaceae	Gammaproteobacteria	Salmonella	gambiae funestus	X	X	✓	Semi-natural			[45]
Comamonadaceae	Betaproteobacteria	Schlegelellea	dureni gambiae maculipennis albimanus quadrimacul	✓	X	X	Semi-natural	Adult	FR774570	[52]

			atus							
Enterobacteriaceae	Gammaproteobacteria	Serratia	stephensi	✓	✓	✓	Field, Semi-natural and Laboratory	Larval Adult	FJ608101	[218]
Shewanellaceae	Betaproteobacteria	Shewanella	gambiae	X	✓	X	Field	Larval	HQ591421	[218]
Sphingomonadaceae	Gammaproteobacteria	Sphingobium	funestus stephensi gambiae	✓	X	X	Semi-natural	Adult	GU940735	[45]
Sphingomonadaceae	Gammaproteobacteria	Sphingomonas	gambiae arabiensis funestus	✓	✓	✓	Field, Semi-natural and Laboratory	Larval Adult	GU204960	[52]

Xanthomonadaceae	Alphaproteobacteria	Stenotrophomonas	gambiae stephensi	✓	✓	✓	Field and Semi-natural	Adult	EF426435	[218, 222] [52, 227] [219, 233]
Enterobacteriaceae	Gammaproteobacteria	Thorsellia	arabiensis	✓	✓	✓	Field and Semi-Natural	Larval Adult	NR_043217	[221]
Vibrio	Gammaproteobacteria	Vibrio	stephensi	X	✓	X	Field	Larval Adult	FJ608116	[218]
Enterobacteriaceae	Gammaproteobacteria	Xenorhabdus	darlingi	X	X	✓	Field	Adult	FJ608329	[52, 222] [13, 228]
Enterobacteriaceae	Gammaproteobacteria	Yersinia	gambiae funestus	X	X	✓	Field	Adult		[218, 220] [219]
Halomonadace	Gammapr	Zymobacter		✓	X	X	Field	Adult	FR8517	[219, 220]

ae	oteobacter ia								11	
Others										
		Bacillariophy ta	gambiae	✓	X	X	Semi-natural	Larval	JQ72702 9	[218]
		Chlorophyta	gambiae	✓	X	X	Semi-natural	Larval	EF11467 8	[218]
Rivulariaceae		Calothrix	stephensi	X	X	✓	Field	Larval	FJ60809 5	[219]
Deinococcaace ae	Deinococc i	Deinococcus	stephensi	X	✓	✓	Field	Larval	FJ60808 9	[219]
Mycoplasmatac eae	Mollicutes	Mycoplasma	arabiensis	X	X	✓	Field	Adult	AY8377 24	[220]
Spiroplasmatac eae	Mollicutes	Spiroplasma	funestus	X	X	✓	Field	Adult	AY8377 33	[220]
		Cyanobacteri a (Gpl)	gambiae	✓	X	X	Semi-natural	Pupal	HM5734 52	[218]
		Cyanobacteri	gambiae	✓	X	X	Semi-	Larval	JQ30508	[218]

		a (Gplla)					natural		4	
		Cynobacteria (GpV)	gambiae	✓	X	X	Semi-natural	Larval	AB2451 43	[218]
Fusobacteriaceae	Fusobacteriia	Fusobacterium	gambiae	✓	X	X	Semi-natural	Adult	548360	[52, 218]